UNIVERSITY OF SOUTHAMPTON

DESIGN AND DEVELOPMENT OF SPECIFIC ANTISENSE PROBES AGAINST THE MAJOR PROTEIN KINASE B ISOFORMS.

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ABSTRACT

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The study of cell signalling is important in elucidating the roles of many proteins in cellular functions. Signalling factors such as insulin act via receptor mediated transduction cascades to bring about a variety of effects including controlling translation, metabolic regulation and the cell cycle. Most of these effects are mediated by phosphorylation of serine, threonine or tyrosine residues of proteins and hence protein kinases have a crucial role to play in regulation.

One such important signal transduction protein is the serine/threonine kinase; protein kinase B (PKB) which is also referred to as Akt. PKB was discovered in 1990 and is a 60kDa cytosolic protein with 3 known isoforms (α, β, γ). PKB has been shown to be activated in response to a variety of factors including insulin, EGF and PDGF, and this activation has been shown to involve PI 3 kinase and the recently discovered kinase, PDK1.

To date PKB has been proposed to be involved in the regulation of many cellular processes including: apoptosis, protein synthesis and insulin stimulated glucose/glycogen metabolism. Possible roles for PKB include the translocation of GLUT4 transporters to the plasma membrane, inhibition of glycogen synthase kinase-3 (GSK-3) and activation of S6 kinase. PKB has also been proposed to act as a survival factor. Direct evidence for these and other roles for PKB is still lacking and is now urgently sought.

Towards this objective I have developed, 2 effective antisense oligonucleotides specific for either the α or β isoforms of PKB, which have been shown to deplete the levels of that isoform by over 90% in 3T3-L1 fibroblasts and adipoctyes. Optimum conditions (time/concentration) have now been laid down for these 2 probes and by using various control oligonucleotides (sense, random and mismatch) I have shown these probes to be both specific and very effective inhibitors of each target PKB isoform.

Using these probes I have already established a critical role for PKBα in the differentiation of cells (3T3-L1 fibroblasts to adipocytes). Use of these probes also suggests that PKBα and PKBβ are not involved in the growth factor induced phosphorylation and activation of S6 kinase in the cell lines tested. A suitable peptide based assay for analysing the activation of GSK-3 was developed and preliminary studies into the position of GSK-3 in signalling pathways undertaken. A specific PKBγ antisense probe has also been devised and is currently under test.

As the developed antisense probes are the first inhibitors against PKB available, it is hoped they can be used not only to establish the roles of PKB in various complex cell processes, but also aid understanding of certain diseases which this cascade may be involved in.

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ABBREVIATIONS

Arg	Arginine
BSA	Bovine serum albumin
BH3	Bcl-2 homology-3
cAMP	Cyclic adenosine monophosphate
CAMK	Calcium/calmodulin dependent protein kinase
CREB	cAMP responsive element binding
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	(Ethylenedioxy)diethyelenedianitilotetraacetic acid
Erk1/Erk2	Extracellular signal regulated kinases 1 & 2
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
FKHR	Forkhead transcription factor
Gab-1	Grb2-associated binder-1
Grb2	Growth factor receptor binding protein 2
GS	Glycogen synthase
GSK-3	Glycogen synthase kinase-3
HGF	Hepatocyte growth factor
HPLC	High performance liquid chromatography
IGF-1	Insulin-like growth factor
IgG	Immunoglobulin
IL	Interleukin
ILK	Integrin linked protein kinase
In-2	Inhibitor-2 protein
Ins	Insulin
IP_3	Inositol-D-1,4,5-tripohosphate
IR	Insulin receptor

IRS	Insulin receptor substrate
IRSK	Insulin receptor associated serine kinase
IRTK	Insulin receptor tyrosine kinase
MAPK	Mitogen activated protein kinase
MAPKAPK	Mitogen activated protein kinase activated protein kinase
MEK	MAP-kinase kinase
NOS	Nitric oxide synthase
NP-40	Nonidet NP-40 (octylphenol-ethylene oxide condensate)
ODN	Oligodeoxynucleotide
p70S6K	70kDa ribosomal S6 kinase
p85S6K	85kDa ribosomal S6 kinase
p90S6K	90kDa ribosomal S6 kinase
PBS	Phosphate buffered saline
PIF	PDK-1 interacting fragment
PDE	Phosphodiesterase
PDGF	Platelet derived growth factor
PDK	Phosphatidylionistol 3,4,5 trisphosphate-dependent protein kinase
PFK	Phosphofructokinase
PH	Pleckstrin homology
PHAS-I	Phosphorylated heat and acid stable regulated by insulin
PI3K	Phosphatidylinositol 3-kinase
PI	Phosphatidylinositol monophosphate
PIP_2	Phosphatidylinositol 3,4-bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PK	Protein kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PKG	Protein kinase G
PMSF	Phenylmethylsulphonyl fluoride
PVDF	Polyvinyldifluoride
PLC	Phospholipase C

PP	Protein phosphatase
PTEN	Phosphatase and tensin homologue deleted from chromosome 10
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
Raf-1	Ras activating factor
RAS-GAP	Ras GTPase activating protein
Rsk	Ribosomal S6 protein kinase
RTK	Receptor tyrosine kinase
S6K	Ribosomal S6 kinase (p70/p85S6K)
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Ser/Thr	Serine/threonine
SGK	Serum and glucocorticoid induced protein kinase
SH2	Src homology-2
SH3	Src homolgy-3
Shc	SH2 containing protein with homology to α-1 collagen
Sos	Son of sevenless
TBS	Tris buffered saline
TEMED	N,N,N',N',-tetramethylenediamide
TFA	Trifluoroacetic acid
TNF	Tumour necrosis factor
TOR	Target of rapamycin
TPA	12-O-Tetradecanoylphorbol 13-acetate
Tris	Tris(hydroxymethyl)aminomethane
Tween-20	Polyoxyethylenesorbitan monolaurate
UV	Ultra violet
Van	Sodium orthovanadate
VEGF	Vascular endothelial growth factor
X(Xaa)	Any amino acid in sequence motif

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CHAPTER ONE - PROTEIN KINASE B AND CELL SIGNALLING

1.1 Introduction to Cell Signalling.

1.1.1 General Introduction to Signalling

This project is an investigation into cell signalling with particular reference to the roles of insulin in signal transduction and the use of antisense techniques to investigate these functions. The research group I am working in, is or has been involved in studies into various signalling pathways particularly the proteins involved in the mitogen activated protein kinase (MAPK) cascade and how growth factors and in particular insulin affect this. Proteins investigated by the group to date include; the insulin receptor (Asamoah *et al.*1995), the insulin receptor serine kinase (Carter *et al.* 1995), MAPK (Sale *et al.* 1995), ribosomal S6 kinases, and protein tyrosine phosphatases (PTPα) (Arnott *et al.* 1999) all of which have roles in insulin signalling and/or MAP kinase pathways.

The project is mainly concerned with the signalling protein, Protein kinase B (PKB) (reviewed in Coffer *et al.* 1998, Kandel *et al.* 1999) which has been found to be insulin (and other growth factors) sensitive and so likely to be important in cellular communication (see section 1.2). In order to investigate this protein, a variety of techniques are being employed including cell culture work, antisense studies, kinase assays and Western blotting, with the goal of the research being to establish the position, roles and importance of these proteins in signal transduction in response to insulin and other factors. The initial aims were to develop isoform specific antisense probes against the major PKB isoforms and then to use these probes to investigate the roles of PKB in cell signalling. The overall aims are hence to try to further the knowledge and understanding of growth factor signalling (especially insulin) and the cellular proteins involved in mediating the desired responses.

Before discussing PKB in some detail it is firstly important to give a brief overview of cell signalling with particular reference to the roles of insulin in signalling.

1.1.2 Overview of Cell signalling

The field of cell signalling is an ever changing, growing area of research. Roles for proteins within a cascade are constantly being updated or redefined as more information on their functions and regulation is established. Also, new isoforms of these proteins or novel proteins are frequently identified as techniques used for their study and determination are improved. Signalling pathways are very important

for communication between cells and also within the cell. The signalling pathways detailed here are connected to the activation of intracellular cascades, by external factors binding to individual or specific receptors expressed at the cell surface.

Most, if not all, receptor-mediated signal transduction appears to occur via one of four basic mechanisms (reviewed in Devlin 1995, reviewed in Malarkey *et al.* 1995);

Inositol phosphate systems (e.g. Inositol 1,3,5 trisphosphate),

Ion channel systems (e.g. Na^+ channels in neurones),

Cyclic nucleotide systems (e.g. G-Protein mediated systems),

Tyrosine and serine/threonine kinase systems (e.g. MAP Kinase cascade).

In general, all these systems act to transduce the message from an external signal, for example a growth factor, via a receptor-mediated system to bring about specific effects on the cell. In these systems the external signalling factor or stimulus, binds to a membrane associated receptor via an extracellular ligand binding domain. This binding causes a specific alteration in the receptors properties usually at a conformational and/or activity level. These changes are also apparent on the intracellular face of the cell in the intracellular or cytoplasmic domain of the receptor, which acts to transduce these external factor induced changes to downstream cytoplasmic factors which ultimately result in the desired biological effects.

The inositol phosphate signalling system results in the release of intracellular calcium stores which subsequently act on a variety of downstream cellular factors including protein kinase C and calmodulin. Ion channel systems are mainly used in the nervous system for the propagation of the nerve impulse by altering membrane potential, but also have roles in changing the intracellular environment in a cell resulting in changes in specific downstream targets. Cyclic nucleotide systems result in changes in the activity or properties of downstream targets and are mainly mediated by GTP exchange-proteins, which bind to specific factors to alter their functioning. Protein kinase systems and phosphatase systems act to transduce signals through the cell, by altering the phosphorylation state and hence the properties of downstream factors resulting in a variety of biological effects (reviewed in Rhoades *et al.* 1989).

The possibility of cross-talk between these systems exists in order to bring about co-ordinated cellular responses, for example some G-protein systems and tyrosine kinase systems share similar downstream effectors and can often be activated by the same external factor and/or receptor. For example, inositol phosphate systems and kinase systems can both be activated by EGF in some cell types, with both these systems capable of activating PKC by distinct pathways. Cross talk can occur to various degrees between

systems and can be synergistic (as in PKC activation) or acting in opposition (reviewed in Malarkey *et al.* 1995).

Of these complex signalling systems, the one that has been studied in this project is the receptor tyrosine kinase systems and the downstream protein kinases and phosphatases these activate. This signalling system has been researched because this is the main method by which insulin and other factors signal and the system with which PKB is believed to associate with in its activation profile (reviewed in Malarkey *et al.* 1995). Therefore, it is first important to briefly consider the importance of phosphorylation and dephosphorylation in the control of cellular processes.

Many critical cellular processes have been shown to be tightly regulated by phosphorylation and dephosphorylation reactions (see Table 1.2) establishing an essential role for protein kinases and phosphatases in cellular regulation (reviewed in Tonks *et al.* 1995, Hunter *et al.* 1995). The phosphorylation state of a particular intracellular factor can drastically and specifically affect its properties; including the factors structure, activity, binding parameters and cellular localisation, or could result in changes in any combination of these important characteristics. Phosphorylation is also important in regulating cellular function of lipids (i.e. Phosphatidylinositol lipids - see section 1.3) and DNA/RNA emphasising the importance of this key method of cellular control. Stimuli driven phosphorylation is probably the major form of post-translational modification of key proteins.

Controlling the phosphorylation state of key cellular proteins is the major route by which upstream kinases and phosphatases exert their cellular roles and transduce the signalling messages of external factors via receptor mediated cascades. In phosphorylation-based control it is important that the phosphorylation reactions are dynamic and reversible, so that the changes phosphorylation modifications generate can be removed once the desired response has been completed. For this level of control of protein function to be effectively regulated a variety of specific kinases and phosphatases are required so only the desired targets are modified in response to the stimulus. Therefore, most cell types contain a variety of distinct kinases and phosphatases in an approximately equal balance to provide the tightly regulated control required (reviewed in Pawson 1995, Denu *et al.* 1996).

In most cells, the balance between the levels and activities of kinases and phosphatases is tightly regulated to maintain unstimulated cells in a fairly stable state. However, upon stimulation various components of these pathways are massively upregulated in a factor specific fashion to generate the desired response. The specific activation of certain kinases or phosphatases, which may have different substrate affinities and cellular localisations, combine to bring about the specific targeted response.

Table One - Summary of the Cellular Effects and Targets of Insulin:

PROCESS	EFFECT	TISSUE	SITE OF REGULATION
Glucose Transport	Increase	Fat, Muscle	GLUT4 transporter
Glycogen Synthesis	Increase	Liver, Fat, Muscle	Glycogen synthase
Glycogen Breakdown	Decrease*	Liver, Fat, Muscle	Phosphorylase kinase
Gluconeogenesis	Decrease*	Liver	Fructose 2,6 bisphosphate kinase
Glycolysis	Increase*	Liver	Pyruvate kinase / PFK-2
Triacylglyceride Breakdown	Decrease*	Fat	Triacylglycerol lipase
Pyruvate => Fatty Acids	Increase	Liver, Fat	PDH/ Acetyl CoA carboxylase
Protein Synthesis	Increase	Liver, Fat, Muscle	Translation initiation
Specific mRNA Synthesis	Increase/ Decrease	Liver, Fat, Muscle	Transcription

* Effect of insulin only apparent if another hormone elevates cAMP (Adapted from Denton 1995).

Table Two - Metabolic Proteins Which Exhibit Phosphorylation Changes in Response to Insulin

PHOSPHORYLATION	PROTEINS AFFECTED
Decrease	Glycogen synthase
	Phosphorylase kinase
	Phosphorylase
	Pyruvate dehydrogenase
	Triacylglycerol lipase *
	Pyruvate kinase (Liver)
Increase	ATP citrate lyase
	G-Subunit of protein phosphatase-1
	cAMP phosphodiesterase
	S6 ribosomal protein
	Acetyl-CoA carboxylase

* Effect of insulin only apparent if another hormone elevates cAMP (Adapted from Denton 1995).

In most cells, the majority of phosphorylation reactions are mediated by serine/threonine kinases and phosphatases, with 99% of cellular phosphorylation targeting these residues. Therefore less than 1% of cellular phosphorylation targets tyrosine residues. However protein tyrosine kinases and phosphatases are very important in cell signalling, particularly because most receptor mediated signal transduction cascades are initiated upon ligand binding, by activating the receptors intrinsic tyrosine kinase activity (reviewed in Tonks 1995, reviewed in Pawson 1995).

Having established the benefits of phosphorylation in the regulation of cellular processes and hence the importance of protein kinases and phosphatases, it is important to detail the individual pathways which utilise these essential regulator proteins. Before considering the main pathways used by insulin and other growth factors in cell signalling and also at the roles of PKB, it is first advisable to consider why the study of insulin and other growth factor signalling pathways is so important in cellular research.

1.1.3 Introduction to Insulin

Insulin is one of the most important hormones and acts via tyrosine kinase systems after binding to a specific cell membrane receptor (reviewed in Proud 1994, reviewed in White *et al.* 1994). Insulin is a 5.8kDa hormone, which primarily signals the "fed state". This hormone is manufactured in the β-cells in the islets of Langerhans, which are found scattered throughout the pancreas. Insulin is released in response to high blood levels of glucose and other factors such as amino acids, fatty acids and neural factors (reviewed in Rhoades *et al.* 1989). Insulin elicits a wide variety of cellular effects (see table 1.1 and table 1.2) including increasing glycogen synthesis, via increased glycogen synthase activity and increasing glucose transport, via the recruitment of the GLUT4 transporter to the cell membrane (reviewed in Proud 1994, reviewed in Denton *et al.* 1995).

Many of the proteins or processes affected by insulin signalling have been shown to be mediated by or regulated due to phosphorylation and dephosphorylation reactions (Cohen 1993, Marshall 1995, Krebs and Fischer 1993, Hunter 1995). For example, insulin has been shown to stimulate glycogen synthesis by a pathway which dephosphorylates and activates glycogen synthase (Proud 1994). Conversely insulin pathways act to stimulate acetyl CoA carboxylase by increased phosphorylation of this enzyme (Table 1.2). Therefore it is clear that kinase and phosphatase pathways must be very important in insulin signalling.

The study of insulin signalling is important in establishing how insulin brings about its cellular effects

and the pathways it employs to bring about these responses. In order to fully understand cell signalling it is necessary to identify the roles and importance of each of these pathways and the individual components of them (i.e. PKB). Other factors, including epidermal growth factor (EGF), have also been found to share common downstream targets and use some of the same pathways as insulin. Therefore, research into these pathways can lead to an improved picture of cell signalling in general.

This research may also have medical implications as defects in components of these pathways have been implicated in the pathology of a variety of diseases including cancers, metabolic disorders and neurological problems. A better understanding of this field may therefore lead to improved diagnosis, treatment or prevention of this and possibly other as yet unrelated conditions.

1.1.4 The Insulin Signalling Disease, Diabetes Mellitus

One of the main disorders associated with the hormone insulin is diabetes mellitus, of which there are two forms. These disorders affect more than 1% of the population of the industrial world, so are one of the most prevalent diseases and of serious concern to world health (Rhoades *et al.* 1989). The less common form of diabetes mellitus is known as juvenile onset or insulin-dependent diabetes mellitus and probably accounts for less than 20% of all cases. This form of the diabetes mellitus is caused by a defect in the production of insulin in the pancreatic β-cells and can be treated by daily insulin injections and dietary control (reviewed in Devlin 1993). The majority of diabetics, however, are classified as suffering from late onset or non-insulin dependent diabetes mellitus. In this disorder, insulin is produced normally, but the individual appears to have a resistance to the effects of insulin probably due to defects in the insulin signalling pathway. The cause of this disorder is as yet unknown, although links to obesity and possibly a genetic component have been implemented. This form of diabetes can be treated by oral hypoglycaemics and dietary control.

Both forms of diabetes if unchecked lead to hyperglycaemic episodes which can cause diabetic coma and even death. Weight problems, polyuria and fatigue are regularly seen with these disorders. Diabetes is also often associated with a number of secondary complications including artery thickening, retinopathy, neuropathy and kidney problems. It is therefore hoped that by studying insulin signalling that a better understanding of diabetes and other signalling disorders may be obtained, which will hopefully lead to improved treatments or even cures for these serious diseases (reviewed in Devlin 1993).

1.1.5 Insulin Signalling Pathway

Most, if not all, of insulin's action on cells are mediated via a receptor mediated signal transduction cascade. The insulin receptor which is present in virtually all vertebrate tissues is a 185kDa transmembrane glycoprotein and is encoded for on chromosome >9 36-37 (short arm of chromosome 19) (reviewed in White *et al.* 1994). These proteins comprise of two α and two β subunits which are covalently linked by disulphide bonds and hence has the heterotetrameric structure $\alpha_2\beta_2$. The two α subunits are located entirely outside the cell and possess the ligand binding activity of the receptor. The β subunits, conversely, traverse the cell membrane with the majority of these subunits being intracellular and possessing the insulin regulated tyrosine kinase activity, which is inhibited by the α subunits when the receptor is unoccupied (reviewed in Whitehead *et al.* 2000).

The binding of insulin to this receptor causes the receptor to become phosphorylated on specific tyrosine residues within the cytoplasmic domain. This autophosphorylation event appears to occur via a *trans*-mechanism in which the binding of insulin to the α subunit of one αβ dimer stimulates the tyrosine phosphorylation of the covalently linked β subunit of the other αβ dimer (reviewed in White *et al.* 1994). These phosphorylation events occur at tyrosine residues throughout the β subunits intracellular domain and act to enhance the receptors kinase activity about 20 fold. These phosphorylations also create target motifs for proteins known as the insulin receptor substrates (IRS), which bind to the insulin receptor and are then themselves tyrosine phosphorylated (reviewed in Yenush *et al.* 1996, 1997, 1998) and link the insulin receptor to downstream targets (see below) (reviewed in Whitehead *et al.* 2000).

The insulin receptor is also subjected to serine and threonine phosphorylation which appears to modulate the receptor's tyrosine kinase activity and/or activation time, possibly by the feedback action of downstream kinases. To date the kinases which act on this receptor and the roles they have, are yet to be determined, although the possible involvement of a novel receptor associated insulin receptor serine kinase has been postulated (Carter *et al* 1996)

To date four IRS proteins have been identified and are referred to as IRS-1, IRS-2, IRS-3 and IRS-4 respectively (White 1998, Zhou *et al.* 1999). All four isoforms possess a conserved domain structure which includes, amino terminal pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains, both of which are involved in coupling of these proteins to the insulin receptor allowing for the fast and efficient phosphorylation of key residues within the IRS proteins (Yenush *et al.* 1996 and 1997, Withers *et al.* 1999). The phosphorylation of these key tyrosine residues within suitable highly conserved motifs,

creates unique docking sites for Src-homology 2 (SH2) domain containing proteins such as Phosphatidylinositol-3 kinase (PI3K) (see section 1.3.3), Grb2 and SH2 containing phosphatase (SHP-2 or Syp) (White 1998, Zhou *et al.* 1999). These factors subsequently bind to the insulin stimulated/tyrosine phosphorylated IRS proteins. This binding modulates the activity of the SH2 domain proteins thereby eliciting the required downstream responses which bring about the actions of insulin (reviewed in White *et al.* 1994, reviewed in Whitehead *et al.* 2000, reviewed in Yenush *et al.* 1997).

IRS-1 is a 131kDa protein and has around 21 potential tyrosine phosphorylation sites. The phosphorylation of some of these sites creates the specific binding motifs for a variety of proteins which contain SH2 domains (O'Neill *et al.* 1994). The role of the IRS-1 protein is therefore to recruit SH2 proteins to the insulin signalling cascade and bring about modulation of their activity. Proteins known to bind to IRS-1 and be activated by this route include, the protein tyrosine phosphatase; SHP-2, Nck (an adapter molecule), Grb-2 (MAP kinase cascade adapter), PLCγ and the p85 subunit of PI 3-kinase (which is likely to activate PKB) (Myers *et al.* 1994, Pawson *et al.* 1993). In the presence of insulin, IRS-1 has been shown to be phosphorylated on at least 8 tyrosines and to bind and activate several of the factors previously mentioned including PI3K and Grb2 linking, IRS-1 to the PKB and MAPK cascades. IRS-1 is therefore, a key mediator of the actions of insulin within cells, acting to transduce the signals from binding of insulin to its receptor to a variety of different signalling cascades which bring about the metabolic actions of insulin (reviewed in Yenush *et al.* 1997, reviewed in Whitehead *et al.* 2000).

A necessary role for IRS-1 in insulin action is indicated with IRS-1 negative mice (homozygous IRS -/-) which show growth retardation and mild insulin resistance, which is more marked in muscle cells and adipocytes (reviewed in Ogwaa *et al.* 2000, Tamemoto *et al.* 1994). However, despite this, all the IRS-1 negative mice all survive with only minor defects. In these mice little or no effect was noticeable on insulin stimulation of the MAPK pathway indicating the essential involvement of other IRS proteins in insulin signalling. Also, in these mice, IRS-1 was found to be an essential component of insulin induced mitogenesis confirming a role for IRS-1 in this branch of signalling (Lamothe *et al.* 1998, reviewed in Yenush *et al.* 1997 reviewed in Whitehead *et al.* 2000).

The insulin responsive protein IRS-2 shows considerable structural and functional homology to IRS-1. In IRS-1 deficient mice (IRS-1 -/-) the activity of IRS-2 increased. This increase could partially rescue insulin signalling to the PI3K cascade and was sufficient to fully activate the MAPK cascade in response to insulin. This indicates that IRS-2 may be the major isoform involved in insulin signalling to these pathways. Homozygous IRS-2 negative mice (IRS-2 -/-), in contrast to the IRS-1 deficient breed, had more marked differences, with the mice being much smaller and suffering from severe diabetes which

little or no compensation from the IRS-1 isoform (Lamothe *et al.* 1998, reviewed in Yenush *et al.* 1997 reviewed in Whitehead *et al.* 2000).

It seems apparent that both IRS-1 and IRS-2 are necessary for insulin signalling to a variety of protein kinases and phosphatases and both isoforms are essential for normal/maximal insulin action. The apparent general/cross over and isoform specific roles of these two proteins in response to insulin can be shown by the potential tyrosine phosphorylation motifs each isoform contains. IRS-1 has been shown to possess 7 unique tyrosine motifs and share 13 common tyrosine phosphorylation motifs with IRS-2, which itself contains 9 unique tyrosine motifs site, setting up the opportunity for the binding of identical and different downstream components. It may be that there are isoform-specific expression differences in different cell types or individual subcellular locations between these two isoforms which may account for their occasional signalling differences/responses to insulin (reviewed in Yenush *et al.* 1997, Whitehead *et al.* 2000).

The roles of the other two known IRS proteins within insulin signalling are currently unclear, although it is believed that they play a fairly minor role compared to the two major isoforms, IRS-1 and more critically IRS-2. Although overexpression of either IRS-3 or IRS-4 in adipocytes has been shown to mimic some of the actions of insulin in these cells, IRS-4 is normally undetectable in adipocytes or muscle indicating that it does not play a role these insulin responsive cells. Insulin stimulated/tyrosine phosphorylated IRS-3 does not significantly bind or activate PI3K in these cells in response to insulin. Further evidence for a limited functioning of these minor isoforms in insulin signalling is confirmed by the fact that IRS-3 -/- and IRS-4 -/- mice exhibit normal glucose tolerance (Lamothe *et al.* 1998, reviewed in Yenush *et al.* 1997, reviewed in Whitehead et al. 2000). However, as both these IRS proteins are expressed and can be tyrosine phosphorylated in response to insulin in many cells, it is possible they have some role in insulin signalling.

The IRS signalling pathway of modulating the ligand activation of a specific receptor to suitable downstream targets may also be utilised by other signalling factors. The main factor that is likely to use this route is Insulin like growth factor (IGF). IGF mimics several of the metabolic effects of insulin, as well as being a more potent survival factor than insulin (reviewed in Yenush *et al.* 1997). This factor binds to and activates a unique receptor which shares structural and functional homology to the insulin receptor. The activation of the IGF receptor is again by autophosphorylation of key tyrosine residues in the receptors intracellular region, by the receptors intrinsic tyrosine kinase activity (reviewed in Malarkey *et al.* 1995). This phosphorylation leads to the recruitment of IRS proteins to the IGF receptor complex, where these IRS proteins are phosphorylated and activated and can therefore themselves recruit and

modulate specific downstream targets. It has also been shown that interleukin-4 (IL-4) which also signals via a unique receptor may utilise the IRS pathway as one means of propagating its signalling (reviewed in Yenush *et al.* 1997).

Receptor-mediated insulin signalling may also occur via direct insulin receptor interaction with downstream effector molecules, as is the case with other growth factor mediated signal transduction pathways (i.e. EGF, PDGF) or possibly by interactions mediated via other factors. For example, the insulin receptor has been shown to interact with and directly modulate several key proteins including the α-collagen homologue SHC and Gab1. However, it is clear from the current knowledge of insulin signalling that IRS proteins mediate the majority of insulin signalling and that downstream kinase and phosphatase pathways affected by these modulators for example PI3K and Grb2 (MAPK pathway activator) are very important in mediating the effects of insulin in cells (reviewed in Proud 1994, Whitehead *et al.* 2000).

1.1.6 Receptor Mediated Signal Transduction Cascades Mediated by Other Growth Factors

Having established how in the insulin signalling cascade, downstream factors such PI3K and Grb-2 can be recruited to IRS-1 proteins and subsequently activated, it is important to establish how these and other factors can be activated in response to other external factors which act via distinct receptors, but not IRS proteins, to modulate downstream targets. In order to detail these activation routes I shall look at the profiles for epidermal growth factor (EGF) and platelet derived growth factor (PDGF) which are fairly similar (reviewed in Malarkey *et al.* 1995).

The PDGF receptor comprises of an extracellular ligand binding domain, linked to a single transmembrane domain which traverses the cell membrane and a carboxyl terminal intracellular cytoplasmic domain which possesses an intrinsic tyrosine kinase catalytic activity (reviewed in van der Geer *et al* 1994). Upon ligand binding, the receptor oligomerizies, which results in autophosphorylation between the adjacent tyrosine kinase domains. Oligomerization and subsequent phosphorylation are essential for maximum activation of the tyrosine kinase function and also for substrate recognition and modification (reviewed in Heldin 1995). A similar dimerisation and autophosphorylation mechanism is utilised by EGF, via its unique receptor, which again possesses a receptor tyrosine kinase activity and is therefore grouped in the same receptor superfamily as PDGF, along with the fibroblast growth factor (FGF) and TrkA (nerve growth factor) (reviewed in Malarkey *et al.* 1995, Van ed Geer *et al.* 1993).

The intracellular domain tyrosine autophosphorylation sites of these RTK can be categorised into two subclasses of sites. Firstly, a subgroup of these tyrosine sites lies within the catalytic region of the intracellular subunit and are involved in the control of the kinase activity. Phosphorylation of these sites enhances the kinase activity many fold. The second subset of tyrosine residues are located outside of the kinase domain. Autophosphorylation of these residues acts as a molecular "switch" to induce the rapid recruitment of other proteins to the receptor, forming stable multi-protein complexes, which the receptors intrinsic tyrosine kinase can act on and hence modify (Pawson *et al.* 1993, reviewed in Heldin 1995).
The protein-protein interactions between the autophosphorylated RTKs and the recruited docking proteins utilise the specific interaction motif the SH2 domain. SH2 domains are regions of amino acids around 100 residues in length, based on the homology to the protein Src. They have been shown to bind specific phosphotyrosine containing sequences within proteins. The autophosphorylation sites within RTK provide specific docking motifs for SH-2 containing proteins, with the flanking residues around the phosphorylated tyrosines of the RTK, dictating which individual SH-2 containing protein will bind this site. For example, the PDGF receptor has been shown to recruit phospholipase Cγ, SHP-2, the p85 subunit of PI3K, Grb2 (growth factor receptor binding protein -2) and the GTPase activating protein Ras-GTP (reviewed in Malarkey *et al.* 1995).

There is also some evidence that different SH-2 domain containing proteins may bind to the same target motif of an RTK. This lead to the suggestion of a hierarchical binding pattern, in which the binding of a particular SH-2 domain protein to the RTK may depend on its individual affinity for this site and its relative cellular expression over a potential competing binder. For example, Grb2 and the SH-2 domain adapter protein SHC appear to compete for the same binding site on the EGF receptor. Some RTK meditated interactions with downstream SH-2 targets also appear to involve SH-2 domain adapter proteins to assist their recruitment. For example, Grb-2 binds indirectly to the EGF receptor via SHC and to the PDGFβ receptor via SHP-2, in addition to its direct binding to these receptors. In general whatever the methods involved, the net result of RTK phosphorylation/activation by their respective ligands and the subsequent recruitment and modification of SH-2 domain proteins is the propagation of a variety of downstream signals which elicit the desired biological responses.

A greater understanding of the functions of RTK pathways is very important in furthering our understanding of the roles these factors play in cellular signalling. It is also very useful in unravelling the complexity of various disease states since this may involve aberrant function of some of these RTKs or their downstream components. For example, overexpression or over-activation of EGF and/or the EGF receptor has been implicated in the progression and invasiveness of several major cancers.

1.1.7 Cellular Signalling Downstream of Growth Factor Receptors

The main advantage of signal transduction cascades is one of amplification: one growth factor can bind to and activate one receptor and then dissociate from this receptor and move to activate a second receptor and so on. These receptors then activate several copies of each of their downstream targets which then themselves acts on multiple copies of their respective downstream targets and so on until the end point targets of each cascade are reached and the desired biological responses elicited. In this way, a small quantity of ligand or a low expression of receptors can still activate a large number of downstream targets and generate significant biological responses.

From the previous information on growth factor induced activation, it is apparent that insulin and other growth factors result ultimately in the activation of a variety of protein cascades including PI3K and MAPK kinase signalling transduction pathways, via a series of downstream modulators to amplify the initial signal and result in the maximal activation of downstream targets. For example, the MAPK cascade can be amplified 10,000 fold from the initial signal (reviewed in Davis 1993).

The first of these two pathways is the MAP kinase cascade which at present is believed to manly act in the regulation of nuclear substrates (Johnson *et al.* 1994, Davis 1993). The activation of this pathway by insulin is believed to occur via the SH-2 domain containing adapter molecule Grb-2 binding to the tyrosine phosphorylated IRS-1 or IRS-2. As well as binding IRS-1/2, Grb-2 is also bound via its SH-3 domain to the polyproline motif of *sos* (son of sevenless) which is a guanine nucleotide exchange factor. The binding of *sos* to Grb-2 results in the recruitment of *sos* to the plasma membrane, where it can activate membrane associated Ras. Ras is a GTPase which is activated in a permanent complex by the guanine nucleotide exchange activity of *sos* (GTP for GDP) and then has a pivotal role in the activation of the MAPK isoforms Erk 1/2 (extracellular signal regulated kinases) and other signalling cascades, possibly including the PI3K cascade (reviewed in Davis 1993, reviewed in Denton *et al.* 1995, Rodriguez-Viciana *et al.* 1996). Ras-GTP then in turn activates the serine/threonine kinase Raf-1 (probably with a second kinase also involved) which then initiates the activation of the threonine/tyrosine dual specificity kinase: MAP kinase kinase (MEK). The activated MEK in turn, tyrosine and threonine phosphorylates and therefore activates the MAP kinase isoforms Erk-1 and Erk-2. These Erk kinases can then act to phosphorylate a variety of potential substrates leading to a modulation of their activity and ultimately various biological responses (Denton *et al.* 1995).

The activation of this cascade by other growth factors, i.e. EGF or PDGF, follows a similar downstream activation profile to that of insulin, apart from the fact that instead of binding to an IRS adapter module, in these growth factor signalling events, Grb-2 binds directly to the tyrosine phosphorylated RTK (reviewed in Malarkey *et al.* 1995). After the binding of Grb-2 to RTK, Grb-2 recruits *sos* to the complex, which results in the activation of Ras and ultimately the activation of Erk 1/2 via Raf-1 and MEK. Other factors may also feed into the activation pathway of Erk, including PKC and PI3K which enhance Erk activity, or PKA which appears to inhibit this activation (White *et al.* 1994).

Erk 1/2 are believed to be the major downstream modulators of this cascade as they have a wide range of substrate specificities *in vitro* compared to the upstream kinases Raf-1 and MEK which have more restricted potential targets. Putative substrates include other kinases i.e. MAP kinase activated protein kinase (MAPKAP Kinase 1 and 2), p90S6 kinases result in a continuation of the kinase cascade, phospholipase A2 and cytoskeletal proteins (Proud 1994, Sale *et al.* 1995). A variety of nuclear substrates also exist which are phosphorylated and activated by nuclear translocation of activated Erk. These substrates may include the transcription factors, Elk-1, Sap-1 c-fos, c-Jun and c-myc, with nuclear substrate regulation believed to be one of the major roles for the MAP kinase cascade based on current knowledge (Denton *et al.* 1995, Sale *et al.* 1999).

The Erk1/2 isoforms have also been implemented in a variety of general cellular roles in different cell types, including potential regulatory roles in transcription, translation, proliferation, differentiation and possibly apoptosis. Potential involvement of this cascade in metabolic roles including general protein synthesis, apoptosis, glycogen synthesis and glucose uptake is also suggested. However, to date many, of these putative roles are still not fully defined or the exact importance of the Erk cascade in these functions established (reviewed in Denton *et al.* 1995).

Another major insulin and growth factor sensitive protein kinase cascade is the phosphatidylinositol 3-kinase (PI3K) cascade which activates PKB and other key protein kinases including S6K (reviewed in Shepherd *et al.* 1998, reviewed in Vanhaesebroeck *et al.* 2000, reviewed in Kandel *et al.* 1999). The cascade begins with the insulin or growth factor stimulated activation of phosphatidylinositol 3-kinase (PI3K) by recruitment of the p85 regulatory subunit of PI3K, either to the activated receptor or to IRS-1/2 (reviewed in Vanhaesebroeck *et al.* 1997 & 2000, reviewed in Alessi *et al.* 1998a). This subunit binds via its SH-2 domain to phosphotyrosine motifs of the receptor or IRS protein. This interaction brings the catalytic p110 subunit of PI3K into close proximity where it becomes activated. The activated PI3K is then free to activate downstream components including PKB, S6K via its lipid or protein kinase activity. The lipid products of this kinase directly lead to the activation of PKB, which then may act on a

variety of possible substrates, including GSK-3 and S6K (reviewed in Hemmings 1997, Kandel *et al.* 1999, Vanhaesebroeck *et al.* 1999 & 2000). A full treatment of the activation of PKB by PI3K and the subsequent possible downstream consequences of PKB activation are discussed in great detail in subsequent sections (1.21 onwards)

The evidence for this pathway and its cellular function are as yet, fairly limited, which makes it an ideal target for investigation, particularly using antisense techniques to knock out components of the cascade and then analyse the effects of this on downstream proteins and hence the roles of this pathway.

1.2 Protein Kinase B - An Introduction

1.2.1 General Overview of PKB

Protein Kinase B was first discovered and sequenced in 1991 as a 57kDa cytoplasmic protein in rat brain. It was originally identified by 3 separate groups using either; i) degenerate oligonucleotide PCR (Coffer *et al.* 1991), ii) low stringency library screening using a cAMP-dependent kinase probe (Jones *et al.* 1991a and b) or iii) sequencing of human cDNA hybridising to v-Akt DNA (Bellacosa *et al.* 1991). It has been found to have 75% homology to the catalytic domain of PKC and 65% homology to the same domain in PKA, hence the name PKB i.e. between kinases' A and C. This kinase has been also been referred to as RAC Kinase which stands for related to A and C kinases, however this name is used less frequently now due to the existence of a rho-related GTPase also known as rac (Coffer *et al.* 1991). Subsequently PKB was also found to be the cellular homologue of v-Akt a protein from an acute transforming reterovirus, in the spontaneous thymoma AKT (AKT8) and so is also known as c-Akt (Bellacosa *et al.* 1991, reviewed in Coffer *et al.* 1998, reviewed in Kandel *et al.* 1999).

Homologues of PKB have been found in mammals (human, mouse, cow) birds, insects, nematodes, slime mould and yeast which contain a high degree of homology to each other (see figure 1.1) (reviewed in Kandel *et al.* 1999). For example, *Drosophilia* (Konishi *et al.* 1995a) and *C.elegans* (Waterston *et al.* 1992) PKB shows 85% identity to the previously cloned rat and human PKB.

Figure 1.1 - Protein sequence alignment for PKBα homologues. The amino acid sequences of known PKBα homologues were aligned using the ClustalW alignment program. A similarity score individual sequences and the mouse PKBα amino acid sequence is recorded as % homology. Total identity between all sequences is indicated with an *.

```
Aligning...Similarity to Mouse PKBα sequence

Sequences (3:1) Aligned. Score:  96%
Sequences (3:2) Aligned. Score:  98%
Sequences (3:4) Aligned. Score:  99%
Sequences (3:5) Aligned. Score:  95%
Sequences (3:6) Aligned. Score: 100%
Sequences (3:7) Aligned. Score:  62%
Sequences (3:8) Aligned. Score:  57%

Mouse           ----------MNDVAIVKEGWLHKRGEYIKTWRPRYFLLKNDGTFIGYK 39
vAkt            AREETLIIIPGLPLSLGATDTMNDVAIVKEGWLHKRGEYIKTWRPRYFLLKNDGTFIGYK 60
Rat             ----------MNDVAIVKEGWLHKRGEYIKTWRPRYFLLKNDGTFIGYK 39
Human           ----------MSDVAIVKEGWLHKRGEYIKTWRPRYFLLKNDGTFIGYK 39
Bovine          ----------MNDVAIVKEGWLHKRGEYIKTWRPRYFLLKNDGTFIGYK 39
Chicken         ----------MNEVAIVKEGWLHKRGEYIKTWRPRYFLLKNDGTFIGYK 39
Drosophilia     -MSINTTFDLSSPSVTSGHALTEQTQVVKEGWLMKRGEHIKNWRQRYFVLHSDGRLMGYR 59
C.              ----------MSMTSLSTKSRRQEDVVIEGWLHKKGEHIRNWRPRYFMIFNDGALLGFR 49
                                    :  :* **** *:**:*:.** ***:: .** ::*::

Mouse           ERPQDVDQR--ESPLNNFSVAQCQLMKTERPRPNTFIIRCLQWTTVIERTFHVETPEERE 97
vAkt            ERPQDVDQR--ESPLNNFSVAQCQLMKTERPRPNTFIIRCLQWTTVIERTFHVETPEERE 118
Rat             ERPQDVEQR--ESPLNNFSVAQCQLMKTERPRPNTFIIRCLQWTTVIERTFHVETPEERE 97
Human           ERPQDVDQR--EAPLNNFSVAQCQLMKTERPRPNTFIIRCLQWTTVIERTFHVETPEERE 97
Bovine          ERPQDLEQR--ESPLNNFSVAQCQLMKTERPRPNTFIIRCLQWTTVIERTFHVETPEERE 97
Chicken         ERPQDVDQR--ESPLNNFSVAQCQLMKTERPKPNTFIIRCLQWTTVIERTFHVETPEERE 97
Drosophilia     SKPADSASTPSDFLLNNFTVRGCQIMTVDRPKPFTFIIRGLQWTTVIERTFAVESELERH 119
C.              AKPKEGQPFP--EPLNDFMIKDAATMLFEKPRPNMFMVRCLQWTTVIERTFYAESAEVRQ 107
                 :* :         **:* :  .  *  ::*:*  *::* ********** .*:   *.

Mouse           EWATAIQTVADGLKRQEEETMDFR----------------------------------SGS 124
vAkt            EWATAIQTVADGLKRQEEETMDFR----------------------------------SGS 145
Rat             EWTTAIQTVADGLKRQEEETMDFR----------------------------------SGS 124
Human           EWTTAIQTVADGLKKQEEEEMDFR----------------------------------SGS 124
Bovine          EWTTAIQTVADGLKRQEEETMDFR----------------------------------SGS 124
Chicken         EWTKAIQTVADSLKKQEEEMMDFR----------------------------------SGS 124
Drosophilia     EWTEAIRNVSSRLIDVGEVAMTPSEQTDMT--------------------DVDMATIAED 159
C.              RWIHAIESISKKYKGTNANPQEELMETNQQPKIDEDSEFAGAAHAIMGQPSSGHGDNCSI 167
                .*  **..::.                                              .

Mouse           PSDNSGAEEMEVSLAKPKHRVTMNEFEYLKLLGKGTFGKVILVKEKATGRYYAMKILKKE 184
vAkt            PSDNSGAEEMEVSLAKPKHRVTMNEFEYLKLLGKGTFGKVILVKEKATGRYYAMKILKKE 205
Rat             PSDNSGAEEMEVALAKPKHRVTMNEFEYLKLLGKGTFGKVILVKEKATGRYYAMKILKKE 184
Human           PSDNSGAEEMEVSLAKPKHRVTMNEFEYLKLLGKGTFGKVILVKEKATGRYYAMKILKKE 184
Bovine          PGENSGAEEMEVSLAKPKHRVTMNEFEYVKLLGKGTFGKVILVKEKATAAYYAMKILKKE 184
Chicken         PSDNSGAEEMEVSMTKPKHKVTMNEFEYLKLLGKGTFGKVILVKEKATGRYYAMKILKKE 184
Drosophilia     ELSEQFSVQGTTCNSSGVKKVTLENFEFLKVLGKGTFGKVILCREKATAKLYAIKILKKE 219
C.              DFRASMISIADTSEAAKRDKITMEDFDFLKVLGKGTFGKVILCKEKRTQKLYAIKILKKD 227
                    .      .. :   .::*:::*:::*:*********** :** *   **:*****:

Mouse           VIVAKDEVAHTLTENRVLQNSRHPFLTALKYSFQTHDRLCFVMEYANGGELFFHLS---- 240
vAkt            VIVAKDEVAHTLTENRVLQNSRHPFLTALKYSFQTHDRLCFVMEYANGGELFFHLS---- 261
Rat             VIVAKDEVAHTLTENRVLQNSRHPFLTALKYSFQTHDRLCFVMEYANGGELFFHLS---- 240
Human           VIVAKDEVAHTLTENRVLQNSRHPFLTALKYSFQTHDRLCFVMEYANGGELFFHLS---- 240
Bovine          VIVAKDEVAHTLTENRVLQNSRHPSLTALKYSFQTHDRLCFVMEYANGGELFFHLS---- 240
Chicken         VIVAKDEVAHTLTENRVLQNSRHPFLTALKYSFQTHDRLCFVMEYANGGELFFHLS---- 240
Drosophilia     VIIQKDEVAHTLTESRVLKSTNHPFLISLKYSFQTNDRLCFVMQYVNGGELFWHLS---- 275
C.              VIIAREEVAHTLTENRVLQRCKHPFLTELKYSFQTNDRLCFVMEFAIGGDLYYHLNREVQ 287
                **: ::********.***:  .** *  *******:*******::. **:*::**.
```

```
Mouse           -RERVFSEDRARFYGAEIVSALDYLHSEKNVVYRDLKLENLMLDKDGHIKITDFGLCKEG 299
vAkt            -RERVFSEDRARFYGAEIVSALDYLHSEKNVVYRDLKLENLMLDKDGHIKITDFGLCKEG 320
Rat             -RERVFSEDRARFYGAEIVSALDYLHSEKNVVYRDLKLENLMLDKDGHIKITDFGLCKEG 299
Human           -RERVFSEDRARFYGAEIVSALDYLHSEKNVVYRDLKLENLMLDKDGHIKITDFGLCKEG 299
Bovine          -RERVFSEDRARFYGAEIVSALDYLHSEKEVVYRDLKLENLMLDKDGHIKITDFGLCKEG 299
Chicken         -RERVFSEDRARFYGAEIVSALDYLHSEKNVVYRDLKLENLMLDKDGHIKITDFGLCKEG 299
Drosophilia     -HERIFTEDRTRFYGAEIISALGYLHSQG-IIYRDLKLENLLLDKDGHIKVADFGLCKED 333
C.              MNKEGFSEPRARFYGSEIVLALGYLHANS-IVYRDLKLENLLLDKDGHIKIADFGLCKEE 346
                 .:. *:* *:****:**: **.***::  ::*********:********::*******

Mouse           IKDGATMKTFCGTPEYLAPEVLEDNDYGRAVDWWGLGVVMYEMMCGRLPFYNQDHEKLFE 359
vAkt            IKDGATMKTFCGTPEYLAPEVLEDNDYGRAVDWWGLGVVMYEMMCGRLPFYNQDHEKLFE 380
Rat             IKDGATMKTFCGTPEYLAPEVLEDNDYGRAVDWWGLGVVMYEMMCGRLPFYNQDHEKLFE 359
Human           IKDGATMKTFCGTPEYLAPEVLEDNDYGRAVDWWGLGVVMYEMMCGRLPFYNQDHEKLFE 359
Bovine          IKDGATMKTFCGTPEYLAPEVLEDNDYGRAVDWWGLGVVMYEMMCGRLPFYNQDHEKLFE 359
Chicken         IKDGATMKTFCGTPEYLAPEVLEDNDYGRAVDWWGLGVVMYEMMCGRLPFYNQDHEKLFE 359
Drosophilia     ITYGRTTKTFCGTPEYLAPEVLDDNDYGQAVDWWGTGVVMYEMICGRLPFYNRDHDVLFT 393
C.              ISFGDKTSTFCGTPEYLAPEVLDDHDYGRCVDWWGVGVVMYEMMCGRLPFYSKDHNKLFE 406
                *. * . .***************:*:***:.***** *******:*******.:**: **

Mouse           LILMEEIRFPRTLGPEAKSLLSGLLKKDPTQRLGGGSEDAKEIMQHRFFANIVWQDVYEK 419
vAkt            LILMEEIRFPRTLGPEAKSLLSGLLKKDPTQRLGGGSEDAKEIMQHRFFANIVWQDVYEK 440
Rat             LILMEEIRFPRTLGPEAKSLLSGLLKKDPTQRLGGGSEDAKEIMQHRFFANIVWQDVYEK 419
Human           LILMEEIRFPRTLGPEAKSLLSGLLKKDPKQRLGGGSEDAKEIMQHRFFAGIVWQHVYEK 419
Bovine          LILMEEIRFPRTLSPEAKSLLSGLLKKDPKQRLGGGSEDAKEIMQHRFFASIVWQDVYEK 419
Chicken         LILMEEIRFPRTLSPEAKSLLSGLLKKDPKQRLGGGPDDAKEIMQHKFFAGIVWQDVYGK 419
Drosophilia     LILVEEVKFPRNITDEAKNLLAGLLAKDPKKRLGGGKDDVKEIQAHPFFASINWTDLVLK 453
C.              LIMAGDLRFPSKLSQEARTLLTGLLVKDPTQRLGGGPEDALEICRADFFRTVDWEATYRK 466
                **:  :::** .:  **:.**:*** ***.:***** :*. **    **  : *    *

Mouse           KLSPPFKPQVTSETDTRYFDEEFTAQMITITPPDQDDSMECVDS--ERRPHFPQFSYSAS 477
vAkt            KLSPPFKPQVTSETDTRYFDEEFTAQMITITPPDQDDSMECVDS--ERRPHFPQFSYSAS 498
Rat             KLSPPFKPQVTSETDTRYFDEEFTAQMITITPPDQDDSMECVDS--ERRPHFPQFSYSAS 477
Human           KLSPPFKPQVTSETDTRYFDEEFTAQMITITPPDQDDSMECVDS--ERRPHFPQFSYSAS 477
Bovine          KLSPPFKPQVTSETDTRYFDEEFTAQMITITPPDQDDSMEGVDS--ERRPHFPQFSYSAS 477
Chicken         KLVPPFKPQVTSETDTRYFDEEFTAQMITITPPDQDDSMDCVDN--ERRPHFPQFSYSAS 477
Drosophilia     KIPPPFKPQVTSDTDTRYFDKEFTGESVELTPPDPTGPLGSIAE--EP--LFPQFSYQGD 509
C.              EIEPPYKPNVQSETDTSYFDNEFTSQPVQLTPPSRSGALATVDEQEEMQSNFTQFSFHNV 526
                :: **:**:* *:*** ***:***.: : :***.  ..:  : .  *    *.***:

Mouse           GTA---------------- 480
vAkt            GTA---------------- 501
Rat             GTA---------------- 480
Human           STA---------------- 480
Bovine          ATA---------------- 480
Chicken         GTA---------------- 480
Drosophilia     MASTLGTSSHISTSTSLASMQ 530
C.              MGSINRIHEASEDNEDYDMG- 546
                  :
```

Sequence 1: Bovine	480aa (Bovine PKBα)
Sequence 2: Human	480aa (Human PKBα)
Sequence 3: Mouse	480aa (Mouse PKBα)
Sequence 4: Rat	480aa (Rat PKBα)
Sequence 5: Chicken	480aa (Avian PKBα)
Sequence 6: vAkt	501aa (viral-Akt)
Sequence 7: Drosophilia	530aa (*Drosphilaia.* DPKB)
Sequence 8: C.	546aa (*C. elegans* PKBα)

There are certain differences between these species homologues which may affect their function. For example, the yeast homologue ypk1 does not possess the pleckstrin homology (PH) domain which is found in PKB from other species and is involved in regulation of function (Chen *et al.* 1993, Casamayor *et al.* 1999). A mammalian homologue which is very similar to ypk1 has recently been discovered. This protein, serum and glucocorticoid induced kinase (SGK) shares a high degree of homology to the classical PKB's, except for the fact that like yeast ypk's, this PKB subfamily member does not possess the regulatory PH domain (Kobayashi *et al.* 1999, Park *et al.* 1999). The finding that PKB or similar subforms exist in many different species suggests an evolutionary conservation of the protein at a basic structural level. This conservation perhaps indicates a critical role for PKB in the functioning of both simple and complex organisms (reviewed in Vanhaesebroeck *et al.* 2000, Kandel *et al.* 1999).

To date there are 3 main isoforms of PKB namely PKBα (Bellacosa *et al.* 1991, Jones *et al.* 1991a), PKBβ (Jones *et al.* 1991b, Altomare *et al.* 1995) and PKBγ (Nakatani *et al.* 1999, Brodbeck *et al* 1999, Konishi *et al.* 1995a). PKBα is encoded for on chromosome 14q32 in a region which is proximal to the immunoglobulin heavy chain locus (Staal *et al.* 1998), part of an area which is frequently affected by mutations. In humans, the mRNA sequence is 2610 base pairs with the translated region between bases 199 and 1641 encoding for a 480 amino acid protein. The gene for PKBβ is found on chromosome 19q13 (Brodbeck *et al.* 1999) and is 1741 base pairs long with the translated region falling between bases 88 and 1513 and giving rise to a 481 amino acid protein. In the gene sequences, the α and β isoforms show around 60% identity which rises to 82% identity for the protein. It was also found that in humans the β isoform has an carboxyl-terminal extended splice variant protein of 521 amino acids which to date has an unknown function or importance (Jones *et al.* 1991b).

The γ isoform is the most recently discovered PKB. In humans it contains 479 amino acids encoded for by a 1760 base pair mRNA (translated region between base 37 and 1476) and found to be located on chromosome 1q44 in humans (Brodbeck *et al.* 1999, Nakatani *et al.* 1999, Masure *et al.* 1999, Murthy *et al.* 2000). This isoform shows 83% identity to the α and 78% identity to the β forms at the protein level, with only 40% identity to these isoforms at the gene level (Masure *et al.* 1999). In rat and mouse similar homologues of PKBγ have been identified and shown to be of identical length and very similar structure/sequence at the protein level (Brodbeck *et al.* 1999, Nakatani *et al.* 1999, Murthy *et al.* 2000). In rats a truncated PKBγ subisoform is also present. In this subisoform, the PKBγ sequence is 21 amino acids shorter and the protein is only 52kDa (Konishi *et al.* 1996). This results from the truncation of the protein at the C-terminal caused by a shorter mRNA of only 1548 base pairs (translation frame 47 to 1411), with this truncation probably affecting the function and regulation of this variant (reviewed in

Coffer *et al.* 1998, Konishi *et al.* 1991). However the expression and hence importance of this subisoform has not yet been determined.

The three isoforms identified so far show a large degree of homology to each other with this identity being particularly strong in the main central catalytic domain, which confers substrate specificity and is also heavily involved in regulation. The apparent similarity in these domains coupled with key conserved residues in other sections initially suggests that the three PKB isoforms would share similar substrate specificities and be regulated in a similar fashion. Therefore it is important to establish what if any isoform differences there are in the regulation of the individual isoforms, substrate specificity, or perhaps in the cell or tissue expression and subcellular distribution of each isoform (reviewed in Coffer *et al.* 1998, reviewed in Kandel *et al.* 1999).

Of these isoforms the α isoform is by far the most predominant, expressed widely in a variety of tissues, with especially high levels found in heart, lung, brain testis and thymus (Jones *et al.* 1991a Coffer *et al.* 1991). This isoform has a low to moderate expression in the kidney, liver and spleen perhaps indicating a less important role for this isoform in these tissues. The β isoform is the second most common PKB showing a high expression level in most of the same tissues as the α form (Bellacosa *et al.* 1993) including the brain and heart. The β isoform is also very highly expressed in Purkinje cells (cerebellum), skeletal muscle and brown fat, perhaps indicating a critical role for this isoform in insulin responsive tissues (Altomare *et al.* 1998). However the PKBβ expression is lower in the kidney, spleen, testis, liver and smooth muscle. The PKBγ isoform shows a much lower general tissue distribution and expression. However, high levels of this isoform are seen in the testis and brain, with lower levels present in the spleen, heart, kidney, lungs, liver and skeletal muscle (Konishi et al. 1996, Brodbeck et al. 1999, reviewed in Kandel et al. 1999, reviewed in Chen et al. 1999)

It is, interesting to note that in rat liver although the α and β forms are present in similarly low levels, PKBα is 4 time more active (Walker *et al.* 1998) perhaps indicating some isoform specific differences in activation profiles. This situation appears to be reversed in rat adipocytes with the PKBβ form being twice as active than PKBα in these cells (Walker *et al.* 1998). The PKBβ isoform is also highly expressed and up regulated in developing embryos (Altomare *et al.* 1998) whilst the α isoform is up regulated in regenerating neurones (Owada *et al.* 1997) perhaps indicating isoform specific roles. The PKBγ isoform is also highly responsive to insulin in L6 myocytes compared to the other isoforms, again suggesting that despite the apparent identity between the isoforms there may be differences in their regulation, responsiveness and cellular roles (Walker *et al.* 1998).

Most evidence suggests PKB has a ubiquitous expression and it appears that all cells contain at least one isoform of PKB with most cells containing two or even all three forms. This suggests that individual isoforms may have distinct roles in certain cells as well as general roles which any isoform can perform (reviewed in Kandel *et al.* 1999, reviewed in Chen *et al.* 1999). Little is currently known about the regulation of the expression of PKB; however, it appears PKB translation is upregulated in cells becoming terminally differentiated. For example, PKB expression is low in the multipotent fibroblast cell line 10T1/2 but PKB protein levels are dramatically increased as these cells are induced to differentiate into myocytes by *MyoD* transformation (Altomare *et al.* 1995). Whilst the expression of PKBβ is fairly low in the 3T3-L1 fibroblast cell line, its expression appears to be markedly up regulated when this cell line is differentiated into 3T3-L1 adipocytes (Hill *et al.* 1999). The situation with the α isoform is apparently reversed in these cell lines with PKBα having a high expression in the fibroblasts but a lower expression in the differentiated adipocytes (Hill *et al.* 1999). The PKBγ isoform in contrast to the α and β isoforms does apparently not change its expression during the differentiation of the fibroblasts into the adipocytes but is instead expressed at a similar fairly low level in both 3T3-L1 cell types (Barthel *et al.* 1998).

PKB was found to be the cellular homologue of the viral oncogene v-Akt which encodes for a constitutively active protein kinase involved in transformation. This viral gene is a fusion between PKB and a truncated tripartite viral group gag (p12, p15, dp30) (Ahmed *et al.* 1993). The two domains are spliced together by a 21 amino acid region encoded for by the 5'-untranslated region of PKB plus 3 other nucleotides (Ahmed *et al.* 1993, reviewed in Coffer *et al.* 1998). The encoded viral protein homologue was found to be a myristoylated form of PKB and is therefore directly targeted to the plasma membrane and constitutively activated thereby suggesting a route for the oncogenic activation of this viral protein (Ahmed *et al.* 1993). Normal cellular PKB has a primarily cytoplasmic location with around 90% of this isoform found in the cytosol. In comparison, the constitutively active viral PKB due to the presence of the myristoylation motif is dispersed among various cellular components with 40% localised at the plasma membrane, 30% having a nuclear location and only 30% residing in the cytoplasm (Ahmed *et al.* 1993, reviewed in Coffer *et al.* 1998).

Although this indicates that under normal conditions, the vast majority of PKB is a cytoplasmic protein prior to activation, there is some evidence for isoform specific subcellular localisation. For example, whilst PKBα appears to be mainly a cytoplasmic protein, PKBβ may have a more membranous location including the possible presence of this isoform in microsomes also containing the glucose transporter, GLUT4, which PKB has been implicated in regulating (Calera *et al.* 1998). There is also some evidence

of the nuclear translocation of PKBα and PKBβ isoforms usually after their activation again indicating possible isoform specific subcellular localisation differences which may affect their biological roles (Meier *et al.* 1997 Andjelkovic *et al.* 1997 and 1998).

As the constitutively active viral PKB has a transforming or oncogenic potential, cellular PKB is classified as a proto-oncogene (Aoki *et al.* 1998). Therefore, mutations in the PKB gene sequence at a particular point could lead to the expression of a constitutively active kinase which may also have transforming properties and thus be implicated in certain cancers (reviewed in Coffer *et al.* 1998). PKBα has been shown to be overexpressed in the breast cancer epithelial cell line MCF7 (Jones *et al.* 1991a) and has been found to be have a 20 fold amplification in primary gastric adenocarcinoma. PKBα is also likely to be overexpressed in other cancers due to its chromosomal location which is in a region prone to mutation and already implicated in a variety of tumours including T-cell leukaemia/lymphoma and mixed-lineage childhood leukaemia (Bertness *et al.* 1990).

PKBβ has also been shown to be overexpressed in a number of cancers including glioblastoma and ovarian or pancreatic cancers (Jones *et al.* 1991). Large PKBβ amplifications often more than 5 fold are seen in 12.1% of ovarian and 2.8% of breast carcinomas (Bellacosa *et al.* 1995, Liu *et al.* 1998). A recent study has indicated an even more significant tumourogenic role for PKBβ by showing this isoform to be overexpressed/upregulated 3 fold in 36% of ovarian cancers studied (91 test subjects) (Yuan *et al.*2000). PKBβ is also frequently overexpressed in undifferentiated tumours indicating a possible role for this kinase in tumour aggressiveness (Cheng *et al.* 1992, 1996).

The PKB isoform, PKBγ, has recently been implicated in tumour progression and carcinogenesis. The isoform has been shown to be upregulated in several prostrate cancer types and also in certain types of oestrogen resistance breast cancers. It is apparent that PKB has roles in cellular transformation and could possibly be one major route by which cancer cells form, survive and metastasise. The possible roles of PKB in these processes will be discussed later (section 1.5)

As all isoforms of PKB are insulin responsive, it has been suggested that PKB may be linked to the insulin resistance disease, diabetes mellitus (reviewed in Kandel *et al.* 1999). To date no mutations or alterations in any of the PKB genes have been seen in patients suffering from diabetes indicating this protein is not likely to be the cause of the insulin resistance seen in this disease (Hansen *et al.* 1999). However, changes in PKB activity have been seen in some patients suffering from non-insulin dependent diabetes mellitus, possibly indicating that modification of PKB function or activity as a consequence of

the insulin resistance may contribute to the different responses and complications seen with this disorder (Krook *et al.* 1998, Rondinone *et al.* 1999). Therefore it is important to understand more about PKB's structure, activation and function, as this may be important in improving the knowledge of this signalling pathway and also may help with many potentially life threatening disorders.

1.2.2 Structure of PKB

PKB consists of three distinct domains (figure 1.2), a regulatory N-terminal 147 amino acid pleckstrin homology (PH) domain, which is includes a short glycine rich linker region at the carboxyl terminal end of this domain. This short linker is linked to the central kinase domain of around 250 amino acids which contains the main functional activity of the protein. The final subunit is the C-terminal regulatory domain which is around 80 amino acids and has a controlling role in the kinase activity and functioning (reviewed in Coffer *et al.*1998, reviewed in Kandel *et al.* 1999, reviewed in Chen *et al.*1999).

The first PKB region, the pleckstrin homology (PH) domain is so called due to its similarity to a known domain in the protein pleckstrin which is the major PKC substrate in platelets (reviewed in Downward 1995, Alessi *et al.* 1998a and b). PH domains are usually around 100 to 120 amino acids long and can be found in more than 100 other proteins, where they are believed to be involved in mediating either protein-lipid interactions, protein-protein interactions or sometimes both. Other proteins containing a pleckstrin homology domain include dynamin, phospholipase D and β-spectrin (reviewed in Fruman *et al.* 1999).

Although the sequence similarity within this domain between PH domain containing proteins is low or not conserved, the general globular peptide structure of the domain is highly conserved. The basic structure of this region consists of seven antiparallel β-strands which form 2 orthogonal β-sheets (one of 4 strands and one of 3) with an N to C terminal amphipathic α-helix (Mayer *et al.* 1993, Gibson *et al.* 1994). This structure forms a curved barrel motif, which contains a hydrophobic binding pocket. It is interesting to note that all PH domains also contain an invariant single tryptophan residue in the C-terminal α-helix, which appears to be important in ligand binding (reviewed in Chen *et al.*1999).

The hydrophobic pocket and other key residues within the PH domain have been found to generate a distinct positive electrostatic polarisation patch around the ligand binding site which has been shown to interact with both proteins and the head groups of certain phospholipids (Isakoff *et al.* 1998, reviewed in Chen *et al.* 1999). Several PH domain containing proteins have been shown to bind phospholipids with

the affinity and specificity of lipids bound varying greatly between PH domain proteins. For example, dynamin and pleckstrin bind to phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$) but not phosphatidylinositol 3,4,5-trisphosphate ($PI3,4,5P_3$), where as PKB has been shown to bind only $PI3,4P_2$ and $PI3,4,5P_3$. The residues which confer high affinity and specific phosphatidylinositol (PI) binding have recently been elucidated and found to occur at the N-terminus in a $KX_{7-13}R$/KXRHyd, where X is any amino acid and Hyd is a hydrophobic amino acid. PH domains which lack this motif or have key differences within this area bind Phosphatidylinositol (PI) lipids with a greatly reduced affinity if at all (Fruman et al.1999, Isakoff *et al.* 1998, reviewed in Chen *et al.* 1999, reviewed in Vanhaesebroeck *et al.* 2000).

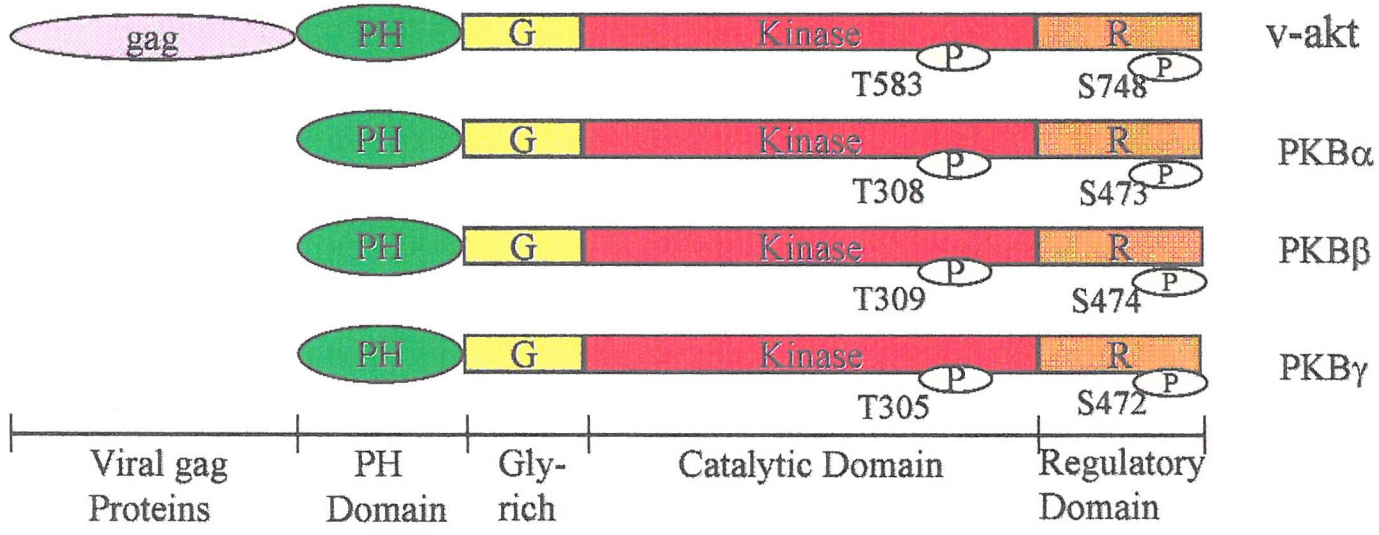
The PH domain is widely distributed amongst signalling proteins suggesting it importance in signalling networks. The lipid binding properties of this domain may be involved in membrane targeting of PH domain proteins possibly resulting in the activation and/or nuclear / mitochondrial translocation of the protein in question (reviewed in Chen *et al.* 1999). Many signalling molecules have been shown to associate with various PH domains, for example, some PKC subspecies, the βγ subunits of GTP-binding regulatory subunits and many inositol based lipids (i.e. phosphatidylinositol 3,4,5 bisphosphate and inositol 1,4,5-triphosphate) all associate with the PH domain of PKB *in vitro* (Konishi *et al.* 1995, Harlan *et al.* 1994, Frech *et al.* 1997, Feng *et al.* 1994). The importance of the PH domain in PKB activation and function is discussed in subsequent sections (see 1.3.3, 1.3.4)

The lipid binding PH domain of PKB is followed by a catalytic domain which contains the kinase activity of PKB. This domain which is around 250 amino acids contains not only the catalytic site but also an invariant ATP nucleotide binding lysine residue (K179 in PKBα), activation/phosphorylation serine/threonine sites and key substrate recognition residues.

This domain shows the maximum amount of identity between the three PKB isoforms with more than 90% identity in this region perhaps indicating similar substrate recognition and function between these isoforms. This central kinase domain is also highly conserved in many serine/threonine kinases with PKB's catalytic domain having a high degree of homology to the same domain in both PKA and PKC (reviewed in Coffer *et al.* 1998, reviewed in Peterson *et al.* 1999, reviewed in Toker 2000).

This kinase domain is followed by the last section, which is a short regulatory region. This C-terminal domain which is found in PKB and several other kinases (e.g. S6 kinase and several PKC isoforms) contains a further regulatory serine or threonine phosphorylation site involved in kinase activation and also a hydrophobic proline rich subdomain (reviewed in Peterson *et al.* 1999, Toker *et al.* 2000).

Figure 1.2 - PKB Isoforms



The basic domain structure of PKB is conserved in all the PKB isoforms and also throughout evolution with the v-Akt, *Drosophilia* and *C. elegans* homologues all consisting of similar regions. In the main the only differences between these is in the length of the domains and some of the amino acids present in each, although this is not believed to affect the functional activity of the protein (reviewed in Coffer *et al.*1998). The general structure of PKB is also very similar to many other protein kinases not only in the central catalytic domain but also within regulatory regions and sometimes the PH domain as well. Due to its particular structural similarity to the kinases; PKA, PKC, PKG, and S6K, these and other kinases have been grouped together as a kinase super-family known as the AGC Kinases (reviewed in Chen *et al.* 1999, Peterson *et al.* 1999).

1.2.3 Activation of PKB

PKB has been shown to be activated within one minute in response to a variety of growth factors in different cell types including insulin, EGF, IGF-1, PDGF and basic FGF (Alessi *et al.* 1996a/b, Franke *et al.* 1995, Burgering *et al.* 1995). PKB has been shown to be activated by vanadate treatment in adipocytes, activated in T-cells in response to treatment with interleukin-2 (Wijkander *et al.* 1997, Reif *et al.* 1997) and the treatment of epididymal fat cells with β-adrenergic agonists e.g. isoproterenol also results in PKB activation (Moule *et al.* 1997). PKB can also be activated by various cellular stresses in certain cell types including heat shock in CHO cells, hydrogen peroxide (oxidative stress) in COS7 cells and hyperosmolarity in NIH 3T3 cells (Konishi *et al,* 1996 & 1997 Bae *et al.* 1999).

Insulin has been found to lead to rapid and significant activation of PKB in many cell types. For example, insulin treatment of L6 myotubes or rat adipocytes results in around a 12 fold activation of PKB with an activation half time of about 1 minute and a sustained kinase activity lasting more than an hour (Alessi *et al.* 1996b, Cross *et al.* 1997, Hurel *et al.* 1996). Conversely, in similar cell types EGF only leads to 3 fold increase in PKB activity which returns to basal levels in about 10-20 minutes (Cross *et al.* 1997). The activation of PKB by the factors IL-2 and isoproterenol is found to lead to different levels and times for PKB activity in various cells (Moule *et al.* 1997). It therefore appears that the activation profile for PKB varies greatly between different factors and in different cell types with activation often rapid and/or transient but sometimes slower or more sustained. It may be that these factors act through several different pathways depending on the factor used and the cell type examined. Alternatively these factors may activate the same upstream activators of PKB by differing amounts resulting in differing PKB activation profiles (reviewed in Kandel *et al.* 1999). Generally, PKB activation appears to be cell and factor type specific. However, there may be differences in the activation

of specific isoforms of PKB by certain growth factors (Fujio *et al.* 1999)

In general most experiments concerning the activation profile for PKB in response to a variety of factors, study the α isoform of PKB. However, it is assumed that most of these findings also relate to the other two known PKB isoforms. Both the β and γ isoforms of PKB contain the same key residues implicated in the activation of PKBα suggesting a similar activation route for all three isoforms. There is also some experimental evidence showing that the activation of PKBβ and γ involves phosphorylation of the same residues shown to be essential for the activation of PKBα and detailed below (Walker *et al.* 1998). For the general activation profile of PKB only the route/factor activation of the PKBα isoform will be discussed.

Analysis of PKBα has revealed 4 phosphorylation sites, Serine 124, Threonine 308, Threonine 450 and Serine 473, which are all found to be phosphorylated when this protein is activated following growth factor stimulation (Alessi *et al.* 1996a/b). Two of the sites S124 and T450 were subsequently found to be phosphorylated in the basal state, as well upon growth factor stimulation, indicating that a change in the phosphorylation state of these residues does not occur with growth factor stimulation and hence is not directly responsible for altering PKB activity (Bellacosa *et al.* 1998). Despite these phosphorylation events being independent of cell stimulation there is some evidence that prior phosphorylation of these residues may be necessary/required for the correct folding/orientation of PKBα yielding maximal PKBα activation. The proposed priming role of these sites was further emphasised using the double mutant PKBα S124A/T450A which when transfected into NIH3T3 cells is activated with reduced efficiency by PDGF (Bellacosa *et al.* 1998). Both these residues which when phosphorylated serve as potential maximal activation primers have been found to be conserved in the other PKB isoforms and several PKB homologues further emphasising a likely regulatory role for these sites which are evolutionarily conserved (Bellacosa *et al.* 1998, reviewed in Chen *et al.* 1999).

When PKBα is activated by many growth factors, for example, insulin and IGF-1 it is found to be only phosphorylated over basal levels on the other two distinct residues namely; threonine 308 and serine 473, which appear to act synergistically to bring about maximal PKB activity (Alessi *et al.* 1996a). Both these key activation residues have been found to be conserved in PKBβ suggesting both the α and β isoforms are regulated in a similar fashion (Meier *et al.* 1997). Recently the human and mouse forms of PKBγ have also been found to contain the equivalent of these two phosphorylation sites suggesting a similar activation route/profile for this isoform (Brodbeck *et al.* 1999). It is also interesting to note that these two activation site residues are conserved in evolution (i.e. present in *Drosophilia* and *C.elegans*)

and so the pathways involved in the activation of PKB and its homologues are also likely to be similar (reviewed in Coffer *et al.* 1998, reviewed in Chen *et al.* 1999).

However, in the truncated variant of rat PKBγ only the equivalent of the threonine 308 is present in this subisoform. This subisoform lacks the final 23 C-terminal residues and so the regulatory serine 473 is missing (Konishi *et al.* 1996). As yet it is unknown as to how important this deletion variant is but it has been suggested that in rats this form of PKBγ may have a different method of regulation or only use some of the pathways the other two isoforms use (Alessi *et al.* 1996a/b, Walker *et al.* 1998). Alternatively the serine 473 (or equivalent) site may act as a "turbo charger" to obtain a higher activation level in the isoforms/species it is present in (reviewed in Coffer *et al.* 1998). Further study of activation and possible roles of this truncated variant and its corresponding full length PKBγ in a variety of cells and/or species will hopefully establish the importance of this subisoform of PKB. The other splice variant of PKB, the human PKBβ2 subisoform, which has an additional 40 amino acids at its C-terminus contains all the serine and threonine regulatory phosphorylation sites and appears to follow normal PKB activation (Jones *et al* 1991b). However as with the species specific rat PKBγ subisoform, any unique roles for this species specific PKBβ subisoform are currently unknown and need further study.

Mutation analysis of PKBα has revealed that phosphorylation of both the serine 473 and threonine 308 is required for maximal activation of the kinase (Ahmed *et al.* 1997). Mutation of either of these residues to alanine greatly reduced the ability of IGF-1 or insulin to stimulate PKB which an 85% reduction in insulin stimulated activity seen in the T308A mutant (Alessi *et al.* 1996b). However, mutation of either residue to alanine does not prevent the other residue from becoming phosphorylated in response to growth factor stimulation suggesting that phosphorylation of each site occurs independently, with phosphorylation at both sites acting synergistically to bring about maximal activation (Alessi *et al.* 1996a, Walker *et al.* 1998). Mutation of either of the residues to aspartate which mimics the negative charge supplied by phosphorylation results in the partial activation of PKB with mutation of both serine 473 and threonine 308 to aspartate resulting in an even more active form of the enzyme. In fact the D308T/D473S double mutant could not be further activated by growth factor treatment indicating the importance of both these residues in PKB activation (Alessi *et al.* 1996a/b, Ahmed *et al.* 1997).

In vitro phosphorylation of serine 473 by MAPKAP kinase-2 also results in partial PKB activation with subsequent growth factor stimulation resulting in threonine 308 phosphorylation and maximal PKB activation. This indicates how these two residues act synergistically to generate a high level of PKBα activity (Alessi *et al.* 1996b, Walker *et al.* 1998). There is some recent evidence that with PDGF

stimulation whilst phosphorylation of S473 contributes to PKB activation, phosphorylation on this site is not essential. However, all research to date still indicates both these residues play important roles in maximal PKB activation. Phosphorylation of the T308 site has therefore been proposed as the key event in PKB activation, a fact that has been confirmed in all PKB studies performed (reviewed in Kandel *et al.* 1999).

The threonine 308 is located in the subdomain VIII of the catalytic domain of this kinase, nine residues upstream of a conserved Ala-Pro-Glu motif. This site is also found as an activating phosphorylation site in many other kinases, particularly members of the AGC family (reviewed in Peterson *et al* 1999, reviewed in Toker 2000). Serine 473 is located in the C-terminal of the catalytic domain and has the motif FPQFSY. This site therefore lies in the consensus sequence F-X-X-F/Y-S/T-F/Y which is found in several other growth factor cascade protein kinases such as PKC, $p90^{rsk}$ and S6 kinases (reviewed in Cohen 1997, reviewed in Belham *et al.* 1999). In PKB and other AGC kinases both these sites are evolutionary conserved and present in most AGC family members/isoforms, they are found to always be located around 170 amino acids apart which suggests similar conserved activation mechanisms (reviewed in Cohen 1997 & 1998, Peterson *et al.* 1999). It is therefore possible that common kinases may act on these two motifs resulting in the activation of a variety of signalling proteins. This has yet to be confirmed although the recently discovered 3-Phosphoinositide-dependent kinase 1 (PDK1) has emerged as a possible candidate for such a role as it acts on threonine 308 motif of PKB (Alessi *et al.* 1997a/b reviewed in Vanhaesebroeck *et al.* 2000) and has also been shown to phosphorylate S6 kinase *in vivo* (Alessi *et al.* 1997b, Pullen *et al.* 1998)

For maximal activation of PKB, the PH domain of PKB is an essential requirement for the pathways which phosphorylate PKB and generate the active protein (Klippel *et al.* 1997). Mutation of a PH domain arginine residue essential for PI binding (PKB R25C) resulted in the generation of an inactive PKB which could not bind any PI lipids or become phosphorylated and activated *in vitro* (Franke *et al.* 1997, Bellacosa *et al.* 1998). Similar point mutations which reduce PI binding have also been found to abrogate the activation of PKB by a variety of growth factors (James *et al.* 1996, Stokoe *et al.* 1997). In agreement with these findings, mutations in the PH domain which increase PKB's affinity for PI lipids (i.e. PKB E40K) have been shown to greatly enhance the growth factor induced phosphorylation and activation of PKB further emphasising a critical role for the PH domain in activation (Franke *et al.* 1997, Stokoe *et al.* 1997, Alessi *et al.* 1997b)

In the absence of PH binding PI lipids a full length PKB could not be activated by PDK1. However when the PH domain of PKB is removed, generating a deletion PH domain mutant, phosphorylation and

activation using the PKB kinase PDK1 (see section 1.41) could be achieved without the addition of 3'PIs (Alessi *et al.* 1997a/b, Datta *et al.* 1995). It appears that the PH domain of PKB when unoccupied by PI lipids masks the phosphorylation sites of PKB, thereby preventing phosphorylation and activation of PKB. However, in the presence of bound 3'PI lipids (probably generated by PI3K - see section 1.41) the PH domains conformation is likely to altered, revealing the previously hidden phosphorylation sites which can then be acted on by specific kinases (i.e. PDK1) resulting in PKB activation. It seems that the PH domain of PKB plays a negatively regulatory role which is removed in the presence of specific 3'PI lipids generated after growth factor stimulation via the PI3K cascade (reviewed in Coffer *et al.* 1998, reviewed in Chen *et al.* 1999, reviewed in Vanhaesebroeck *et al.* 2000).

The PH domain of PKB is also likely to be involved in targeting PKB to the membrane which also aids maximal phosphorylation and activation of PKB (Andjelkovic *et al.* 1997, Datta *et al.* 1995). When bound by 3'PI lipids to its PH domain PKB is rapidly translocated to the plasma membrane (Andjelkovic *et al.* 1997, Franke *et al.* 1997, Kohn *et al.* 1996). The requirement of membrane targeting as a mechanism for PKB activation is emphasised by the fact that the membrane targeted v-Akt is constitutively active and that the addition of a membrane localisation or myristoylation signal to PKB leads to the generation of a constitutively active mutant (Coffer *et al.* 1991, Andjelkovic *et al.* 1997 Meier *et al.* 1997). The apparent requirement of membrane targeting for maximal PKB activation is likely to be due to a membranous location for the PKB activating kinases. It appears that the role of the PH domain in PKB activation is several fold. The binding of specific 3'PI lipids to this domain results in the membrane targeting of PKB and the unmasking of the activation phosphorylation sites. Both of these mechanisms are likely to act together to generate the maximally active PKB (Frech *et al.* 1997, Stephens *et al.* 1998, Datta *et al.* 1995, reviewed in Coffer *et al.* 1998, Chen *et al.* 1999).

The PH domain linked model for PKB activation proposed has recently been given a further level of complexity. It has been suggested that PKB exists as a multimer in cells with the PH domains of each PKB mediating this interaction. Experiments using the yeast-2 hybrid system or mutant PKB studies show that PKB-PKB interactions can occur but only between the same PKB isoform i.e. α-α interactions. In this hypothesis it is believed that in resting cells the PKB multimer is held in an inactive conformation via intermolecular interactions between the PH domains of individual PKB molecules (Lin *et al.* 1999, reviewed in Cheng *et al.* 1999). When the activating serine and threonine sites of an individual PKB are phosphorylated a conformation change occurs in this molecule which relieves the inhibitory effect it has on its partnering PKB molecule (i.e. the one it is interacting with). Phosphorylation of one PKB molecule does not lead to its activation but rather a "deinhibition" of its neighbour. The phosphorylated PKB does not therefore require an intrinsic kinase activity to deregulate

its partner and this partner does not require phosphorylation of its own sites to obtain activation. In this model, phosphorylation can be said to cause intermolecular derepression of the PKB complex. Whilst this model is still somewhat controversial, evidence for this or a similar activation system is mounting and so further investigation is required to establish the exact activation profile for PKB (Datta *et al.* 1995, reviewed in Coffer *et al.* 1998).

Therefore, the activation of PKB is an extremely complex process that we still do not fully understand. At a structural level certain key features of PKB have been identified to be involved in or necessary for maximal activation. For example, the PH domain of PKB has been proposed to be involved in PKB oligomerization, 3'PI lipid binding, PKB translocation and regulation of access to critical phosphorylation sites. These critical phosphorylation sites have been identified as T308 and S473 which when phosphorylated act synergistically to activate PKB (reviewed in Chen *et al.* 1999, Coffer *et al.* 1998, Kandel *et al.* 1999). Phosphorylation of these two residues has been found to be both necessary and sufficient for maximal PKB activation (Alessi *et al.* 1996a/b, reviewed in Vanhaesebroeck *et al.* 2000). Having now established the key features of PKB which act to bring about its activation it is important to detail the upstream pathways which act on these features and bring about alterations in their functions/state to activate PKB. Therefore, it is appropriate to look at the upstream PKB activation pathway(s) which act to mediate growth factor and other stimuli signalling resulting in PKB activation.

1.2.4 The PKB Pathway

1.2.4.1 The PI 3-Kinase Family

The presence and importance of the PH domain in the phosphorylation and activation of PKB led to a great deal of research into the importance of PH domain binding PI lipids in this activation pathway. This meant that a great deal of research targeted the likely generators of these lipids, particularly the protein responsible for the generation of 3'-Phosphatidylinositol lipids namely phosphatidylinositol 3'-Kinase (PI3K) (reviewed in Vanhaesebroeck *et al.* 1999, reviewed in Toker 2000).

The PI3K family were first discovered in the late 1980s as unique proteins which phosphorylate the D-3 hydroxyl group of the inositol head groups of various phospholipids via a distinct lipid kinase activity (reviewed in Vanhaesebroeck *et al.* 1997 & 1999, Fruman *et al.* 1998). As well as this lipid kinase activity these proteins were also shown to possess a separate protein kinase activity, with both activities

likely to be involved in PI3Ks downstream roles. PI3K has been found widely expressed in cells and across species indicating a critical evolutionarily conserved role for this kinase. The phospholipid 3'-kinase activity of PI3K has shown to undergo activation in response to many growth factors (i.e. insulin, PDGF, EGF) and also mitogenic stimulation (reviewed in Alessi *et al.* 1998, reviewed in Vanhaesebroeck *et al.* 1999). PI 3-Kinase activity has also been found to be increased in transformed cells indicating a critical cell cycle regulatory role for this kinase (Klippel *et al.* 1998, reviewed in Alessi *et al.* 1998, reviewed in Vanhaesebroeck *et al.* 1999).

PI 3-kinase and its products have been proposed to mediate many intracellular events including PKB and S6 Kinase activation (King *et al.* 1997, Sable *et al.* 1998), Rac phosphatase regulation and regulating processes such as glucose transport, protein trafficking and cytoskeletal functions (Malarkey *et al.* 1995). PI3K may also be involved with the Ras/MAP kinase pathway since certain Ras isoforms i.e. Ras and R-Ras shown to interact with and activate PI3K and also the PI3K PIP_3 products are believed to regulate Raf-Ras interactions and hence MAP kinase activity (Marte *et al.* 1996). Thus understanding PI3Ks roles in cell signalling is a critical step in unravelling the complex signalling story.

Inositol containing phospholipids (PIs) have an inositol phosphate group at position 3 of their glycerol backbone with fatty acids at positions 1 and 2, and comprise of approximately 10% of the total cellular lipid. If the PI lipids contain no other additional phosphate it is called phosphatidylinositol (PI), with this being the major PI present in cells. However in cells all free hydroxyl groups of the inositol ring of PI lipids apart from the 2' and 6' position can be phosphorylated in a variety of combinations to yield many different PI lipids. The other major PI lipids present in cells are PI 4 Phosphate and PI 4,5 bisphosphate which each make up around 5% of cellular PI lipids. The D3 phosphorylated inositol lipids generated by the PI3K family comprise of less than 0.2% of the total cellular PI, but are subject to by far the highest external stimuli induced variation in their levels and are potentially very important in cell regulation. In cells PI3K proteins generate PI 3 Phosphate (PI3P), PI 3,4 bisphosphate ($PI3,4P_2$) and PI 3,4,5 trisphosphate (PI $3,4,5P_3$) with the later two products believed to be involved in PKB activation (reviewed in Vanhaesebroeck *et al.* 1999, reviewed in Fruman *et al.* 1998, reviewed in Chen *et al.* 1999).

There has been found to be at least 9 multiple isoforms of PI3K based on gene splicing/rearrangement which can be broadly divided into 3 distinct classes, namely, class I, II and III. These classes are separated based on the substrate recognition, functioning and mechanisms of regulation.

i) Class I

Class I PI3K are heterodimers of around 200kDa comprising of a 110-120 kDa catalytic subunit linked to a 50-100 kDa adapter or regulatory subunit. These phosphorylate all classes of PI but have an major order of preference for $PI3,4P_2$ > PI4P > PI. Class I PI3Ks are the major class of PI3K and have been shown to regulate a host of cellular functions including, protein synthesis, lipolyis, glucose metabolism (i.e. glucose uptake and glycogen synthesis) and apoptosis. There are two subclasses, I_A and I_B which share similar lipid substrate specificities but vary in their mechanism of regulation (Stephens *et al.* 1993, reviewed in Vanhaesebroeck *et al.* 1999).

The Class I_A PI3K are a group of kinases which phosphorylate phosphatidylinositol lipids usually phosphatidylinositol 4,5 bisphosphate (its major cellular substrate) at the D3 position of the Inositol ring (Woscholski *et al.* 1997). PI3K is composed of two subunits an approximately 110kDa catalytic (p110) subunit and an 85kDa regulatory (p85) subunit (Pons *et al.* 1995, reviewed in Vanhaesebroeck *et al.* 1997). There are 3 classes (α, β, γ) of the p110 catalytic subunit which range in molecular weight from 110-113kDa and are multi-domain proteins. These catalytic subunits form heterodimeric complexes with the 85kDa regulatory subunits (reviewed in Alessi *et al.* 1998, Vanhaesebroeck *et al.* 1997 & 1999).

The regulatory subunits are encoded for by 3 mammalian genes p85 α, β, γ, with alternative splicing sites giving rise to at least 7 polypeptides all of which contain 2 SH2 domains (Inukai *et al.* 1996 & 1997). The major form of the class I PI3K involved in insulin response has recently shown to be the p110β/p85α complex, which was shown to have roles in the stimulation of glucose transport via GLUT4 translocation to the plasma membrane in 3T3-L1 adipocytes (Ozanne *et al.* 1997).

Class I_A PI3Ks have been found to be regulated by receptor and non-receptor tyrosine kinase by at least 2 mechanisms (reviewed in Shepherd *et al.* 1998). In the first mechanism, activation occurs via the tandem SH2 domains of the p85 subunit binding to phosphotyrosine residues found in a Y-X-X-M motif (Cuevas *et al.* 1999). For example, with insulin stimulation the insulin receptor tyrosine kinase is activated and this results in phosphorylation of IRS proteins. These adapter proteins (i.e. IRS-1) contain several Y-X-X-M motifs which the p85 subunit can bind. This binding results in the membrane recruitment of the p110 catalytic subunit, its tyrosine phosphorylation and hence its activation (reviewed in Vanhaesebroeck *et al.* 1999, reviewed in Chen *et al.* 1999, reviewed in Alessi *et al.* 1998a/b)

In the second activation mechanism for class I PI3K, GTP-ras interacts with the p110 subunit via an effector site located within this catalytic domain with this association leading to PI3K activation (Rodriguez-Viciana *et al.* 1996). It is most likely that both mechanisms are activated in response to

growth factors and act in tandem to bring about maximal activation of PI3K. However, it is unclear, as to whether ras activation is required for insulin induced PI 3-Kinase activity or whether the IRS binding route is sufficient. The membrane localisation and activation of PI3K causes the phosphorylation of inositol phospholipids at the D3 position generating $P3,4,5P_3$ which is the rapidly converted to PI $3,4P_2$ by the action of a specific 5'-phosphatase (reviewed in Alessi *et al.* 1998b, Shepherd *et al.* 1998).

The only class 1_B identified to date is the p110γ catalytic subunit which complexes with a unique 101kDa regulatory subunit p101. P110γ/p101 heterodimers have been found to be activated by the Gβγ subunits of heterotrimeric G-proteins with the p101 subunit found to be essential for this activation. The Class 1_B PI3K shows a much more restricted tissue distribution than the ubiquitously class 1_A PI3K, being only abundant in white blood cells. Therefore the likely importance of class 1_B PI3K in PKB activation is limited to a few cell types.

ii) Class II

Class II PI3K family members are between 170kDa and 210kDa in size and consist solely of a large catalytic domain and are therefore, monomeric. These proteins are not regulated by an additional regulatory subunit but instead posses a C-2 lipid binding domain which appears to be involved in regulation of this PI3K class. They act mainly on PI and PI4P however, there is some variability between subisoforms of this group. Also certain sub-isoforms of this group i.e. PI3KC-2α show a marked resistance to the inhibitory effects of the PI3K inhibitors wortmannin and LY294002 which are known to successfully inhibit other PI3K isoforms including the related PI3KC-2β. As this class of PI3K can generate $PI3,4P_2$ which is implicated in PKB activation, it is possible this class may have some role in the PKB pathway discussed later (reviewed in Shepherd *et al.* 1998, Alessi *et al.* 1998b).

iii) Class III

The final mammalian class of the PI3K family, the class III PI3Ks, are homologues of the *S. cerevisiae* Vp534p which are they only PI3K class present in yeast. This group to date has had little research and so questions about its expression, functioning and roles need to be addressed. This family has been shown to only act on the lipid PI, so only generate PI3P and are therefore not likely to be involved in PKB activation. Also within the PI3K super family, several structurally related kinases have been identified and are sometimes grouped together collectively as class IV PI3Ks. These proteins which include, DNA dependent protein kinase, TOR (target of rapamycin) and ATM (ataxia telangiectesia mutated) all possess homology to PI3K in their catalytic domain. However, as yet no lipid substrates for these proteins have been identified indicating that they exert their effects solely via the serine/threonine kinase activity (review in Vanhaesebroeck *et al.* 1999).

1.2.4.2 PI3K Involvement in PKB Activation

PI3K and its lipid products were proposed as the first known upstream regulators of PKB based on several key experimental findings. Firstly, the growth factor stimulated (i.e. insulin, PDGF, EGF) activation of PKB in many cell types has been found to be prevented by the addition of the fungal inhibitor wortmannin to the cultures (Franke *et al.* 1995, Kohn *et al.* 1996). Wortmannin even at low nM concentrations is known to irreversibly inhibit class 1_A PI3K by forming a schiff base with a lysine residue in the catalytic domain of PI3K, preventing the generation of PI 3'lipids. Therefore these experiments using wortmannin to inhibit not only PI3K but also PKB, indicated PI3K as an upstream activator of PKB (Cross *et al.* 1995, Datta *et al.* 1996).

Secondarily, inactivation of PI3K activity by preventing its binding to and activation by PDGF bound PDGFβ receptors also inhibits PKB activity (Franke *et al.* 1995). PDGF activation of PKB was found to be dependent on the key PDGF receptor residues, Y240 and Y251, which are binding sites for the p85 regulatory subunit of PI3K and hence involved in the activation of PI3K (Franke *et al.* 1995). It is apparent from these findings that PI3K is essential in the growth factor inducted activation of PKB.

A second PI3K inhibitor LY294002, which acts as a competitive inhibitor of ATP binding to PI3K, has not only been found to inhibit the growth factor induced activation of PI3K, but also the subsequent activation of PKB further emphasising a role for PI3K upstream of PKB (Cross *et al.* 1995, Datta *et al.* 1996). Overexpression of dominant negative mutants of the p85 subunit of class 1_A PI3K also have been found to prevent the activation of PKB whilst constitutively active p110 PI3K activates PKB in the absence of external stimuli (Klippel *et al.* 1996). In many studies, PH domain mutants of PKB which do not bind the PI 3'lipid products of PI3K, were found not to be activated by PDGF whereas immunoprecipitated PKB could be activated by the addition of the PI3K products $PI3,4P_2$ and $PI3,4,5P_3$ (Cross *et al.* 1995, Alessi *et al.* 1996, Hemmings 1997).

It has been found that the cellular levels of $PI\ 3,4,5P_3$ rise by between 10-100 times (depending on cell type / species) within 30 seconds of insulin stimulation with a similar rise of $P3,4P_2$ following very rapidly (Vanhaesebroeck *et al.* 1997, Woscholki *et al.* 1997). The rapid activation profile for PKB in response to insulin also indicates an upstream position for PI3K and lipid products. These lipid products of PI3K activation have a critical role to play in insulin and other growth factor pathways. Therefore, based on the evidence using PI3K inhibitors and class 1_A PI3K mutants, PI3K is critically involved in PKB activation (Backer *et al.* 1992, Lam *et al.* 1994).

The 3'-Phosphoinositol products of PI3K have been shown to bind to the PH domain of PKB with this binding likely to result in the recruitment or anchoring of PKB to the cell membrane with the major PKB binding lipids being PI3,4 P_2 and PI3,4,5P_3 (Kohn *et al.* 1996, Franke *et al.* 1997). Interestingly, the other 3'PI-lipids PI3P and PI3,5P_2 have been shown not to bind to or activate PKB indicating that only specific 3'PIs activate PKB (Franke *et al.* 1995).

The binding of the PI3,4P_2 and/or PI3,4,5P_3 to PKB's PH domain has been shown to lead to PKB activation and to be a critical step in this event (Stokoe *et al.* 1997, Stephens *et al.* 1998, reviewed in Kandel *et al.* 1999). Which of these two lipids is the most important in activating PKB is somewhat controversial, with current evidence based on binding affinities and activation profiles favouring PI3,4,5P_3 as the major activating lipid (James *et al.* 1996, Stokoe *et al.* 1997, reviewed in Kandel *et al.* 1999). However in, platelets the activation of PKB is biphasic, with PI3,4,5P_3 binding initially and leading to rapid and transient activation of PKB followed by the subsequent secondary binding of PI3,4P_2 resulting in a more sustained activation (Banfic *et al.* 1998).

Despite the requirement of PI3K lipids in the activation of full length PKB, the presence of these lipids is not sufficient to activate PKB without the concomitant phosphorylation of the T308 and S473 (PKBα) residues and therefore the presence of protein kinase activity is also required (reviewed in Kandel *et al.* 1999, Chen *et al.* 1999). This was shown by the fact that PI 3-lipids could not activate purified/recombinant PKB *in vitro* (James *et al.* 1996). Inhibition of PI3K activity by chemical inhibitors or dominant negative mutants also prevents phosphorylation of PKB, indicating that PI3K and its lipid products have a critical role to play in these phosphorylation events (reviewed in Shepherd *et al.* 1998). There is no evidence that PKB is directly phosphorylated by PI3K and so the role of this kinase and its lipid production must be to facilitate PKB phosphorylation by other kinases (reviewed in Alessi *et al.* 1998). The requirement of these PKB kinases is further shown by the fact that PKB mutants with their PH domain removed can still be phosphorylated and activated *in vitro* in the absence of PI3K lipids (Kohn *et al.* 1996).

The current evidence for the direct role of PI 3,4,5P_3 (and to a lesser extent PI 3,4P_2) interactions with PKB is that they target PKB to the plasma membrane where it is phosphorylated and activated by distinct kinases. These lipids may also facilitate PKB activation by these kinases causing a conformational change or dimerisation of PKB or possibly directly activating a PKB kinase (Hemmings 1997, Franke *et al.* 1997, Alessi *et al.* 1997). Therefore, it is now important to look at the potential kinases which may be involved in phosphorylating and activating PKB and whether PI3K has a role in their actions on PKB.

1.2.4.3 PDK1

Recently a PKB kinase (PDK1) was identified and isolated in rabbit skeletal muscle. This kinase is a ubiquitously expressed 556 amino acid/63kDa monomeric kinase (Alessi *et al.* 1997a/b/c). This kinase was found to contain a PI3,4,5P_3 binding PH domain similar to PKB but at a position C-terminal to the catalytic domain (Stephens *et al.* 1998). The amino terminal kinase or catalytic domain was also found to be similar to that of PKB, PKA and PKC and hence PDK1 is another member of the A,G,C family of serine/threonine protein kinases (Alessi *et al.* 1997a/b/c, Stephens *et al.* 1998). PDK1 was found to be ubiquitously expressed in all mammalian cell types studied and homologues of this kinase were found in many other species including yeasts (*S. pombe* and *S. cerevisiae*) and *Drosophilia* suggesting evolutionary conservation of this kinase (Niederberger *et al.* 1999, Alessi *et al.* 1997a, reviewed in Vanhaesebroeck *et al.* 2000).

Analysis of this kinase *in vitro* and *in vivo* has shown that it directly phosphorylates PKBα on threonine 308 leading to a 30-fold increase in PKB activity (Alessi *et al.* 1998a/b). Subsequently PDK1 was also shown to phosphorylate the β and γ isoforms of PKB on their respective kinase domain threonine, with similar affinities/kinetics, indicating that PDK1 shows no PKB isoform specific targeting (Walker *et al.* 1998). Mutation of PDK1 to a kinase dead or inactive form was found to completely abrogate the growth factor-induced activation of PKB, indicating that PDK1 is a key regulator of PKB phosphorylation and activation (Alessi *et al.* 1997b/c, Stephens *et al.* 1998).

This kinase was originally found to be maximally activated by low micromolar concentrations of the D enantiomers of PI 3,4P_2 and PI 3,4,5P_3. PI 3,4,5P_3 is the most important PDK1 mediator, being involved in the membrane recruitment and activation of PDK1 (Stokoe *et al.* 1997, Currie *et al.* 1999). No other inositol lipids have any effect on PDK1 activity, indicating a possible direct role for only PI3K generated lipids in PDK1 recruitment/activation. This kinase was therefore given the name Phosphatidylinositol 3,4,5-trisphosphate - dependent protein kinase-1 (PDK1) since PI 3,4,5P_3 is the strongest activator (Alessi *et al.* 1997b/c).

This kinase has also been shown to be insensitive to wortmannin and so is not a member of the PI3K family but is activated by their lipid products. Hence it is likely that this kinase is one of the kinases which mediate PKB activation by insulin and other growth factors via PI3K. This kinase may also play roles in regulating other actions of the PI3K derived second messengers PI 3,4,5P_3 and PI 3,4P_2 and is therefore likely to be the subject of further investigations (Alessi *et al.* 1997a/b/c, reviewed in Cohen *et al.* 1997, reviewed in Vanhaesebroeck *et al.* 2000).

There is now evidence to suggest that PDK1 may in fact be constitutively active in cells due to the continual presence of small concentrations of the activating PI 3'lipids at the plasma membrane which may be sufficient to activate PDK1 (Anderson *et al.* 1998). For example, PI3K agonists have been shown not to alter the phosphorylation or activity of PDK1. The activity of PDK1 was not affected by mitogenic stimuli (Andjelkovic *et al.* 1999, Currie *et al.* 1999), indicating that PDK1 may be already active. However, it is unclear whether the $PI3,4,5P_3$ quantities present in unstimulated cells are sufficient to maximally activate PDK1 and hence further activation of PDK1 by growth factor stimulated PI3K activity is likely to be required (Pullen *et al.* 1998, Casamayor *et al.* 1999, Currie *et al.* 1999).

Whatever the precise mechanism of $PI3,4,5P_3$ in PDK1 activation it is clear that this lipid binds to PDK1 with a high affinity and is involved in regulating its activity. This is likely to be via binding to PDK1's PH domain which may aid PDK1 localisation to the plasma membrane and thus modulate its activity (reviewed in Vanhaesebroeck *et al.* 2000). PDK1 translocation to the plasma membrane is increased upon PDGF stimulation, which is likely to be via increasing binding of PI3K lipids products to the PH domain of PDK1 (Anderson *et al.* 1998). The importance of PDK1's PH domain in PDK1 activity was also shown by the fact that deletion of this domain resulted in a PDK1 mutant which only phosphorylates PKB at 5% of the rate of full length PDK1 (Alessi *et al.* 1997b/c). The rate of phosphorylation of PKBα on T308 by PDK1 was increased about 1000 fold in the presence of $PI3,4,5P_3$/$PI3,4P_2$ again indicating roles for these lipids in facilitating maximal PDK1 activity towards PKB (Alessi *et al.* 1997a/b/c).

The proposed mechanism for the T308 phosphorylation and activation of PKB is as follows: The PI3K lipid products $PI3,4,5P_3$ and $PI,3,4P_2$ bind to the PH domains of PKB and PDK1 resulting in the localisation of both of these proteins to a proximal location on the plasma membrane. These lipids appear to enhance the activity of PDK1 towards PKB and facilitate its phosphorylation of the T308 (PKBα) site of PKB. This is likely to involve removing the inhibitory/masking effect of PKB's unoccupied PH domain by inducing a conformation change in PKB which exposes the T-loop threonine site for phosphorylation by PDK1 (reviewed in Kandel *et al.* 1999, reviewed in Chen *et al.* 1997, reviewed in Vanhaesebroeck *et al.* 2000).

Many questions about the regulation of PDK1 still need to be answered. The precise role of PI3K lipid products in PDK1 activation still remains controversial; and needs more investigation. The cellular location of PDK still need to be addressed, some evidence suggests that PDK1 is translocated to the plasma membrane when activated and so is predominantly found in this region. However, evidence also points to a large proportion of PDK1 remaining in a cytosolic location even after stimulation (reviewed

in Chen *et al.* 1999, reviewed in Vanhaesebroeck *et al.* 2000).

PDK1 itself also has several phosphorylation sites at S24, S241, S393, S396 and S410 which appear to be important in PDK1 regulation (Casamayor *et al.* 1999). Of these sites, S241 appears to be a key residue in PDK1 activation with mutation of this residue to alanine abolishing PDK1 activity whilst mutation of the other sites did not affect PDK1 activity (Casamayor *et al.* 1999). This phosphorylation site was found to resistant to dephosphorylation by PP2A whereas the other sites were all dephosphorylated by this phosphatase (Casamayor *et al.* 1999). S241 lies in the activation loop of PDK1 between subdomains VII and VIII in the equivalent position to the residue PDK1 phosphorylates on its protein kinase substrates possibly indicating an autophosphorylation mechanism (Casamayor *et al.* 1999). However, the phosphorylation of PDK1 at these sites does not seem to be affected by growth factor stimulation and no PDK1 kinases have as yet been proposed and so the importance of these sites is unclear.

It is apparent therefore that the activation profile of PDK1 needs to be clarified to establish not only the cellular location and activity of this kinase in unstimulated cells, but also how these parameters change in response to cellular stimulation. Evidence suggests a role for PI3K and its lipids products in maximal activation of PDK1 so the importance of these and other factors in regulating PDK1 need to be addressed (reviewed in Vanhaesebroeck *et al.* 2000).

The threonine 308 of PKB phosphorylated by PDK1 has been found to lie in the T-loop of the protein between domains VII and VIII in the sequence TFCGTPEYLAPE which is also identical in PKBβ and γ. The PDK1 phosphorylation site including the downstream amino acids have been found to be a conserved motif in other A,C,G kinases with the general sequence requirement established as TF/LCGTXXYXAPE/D (Walker *et al.* 1998, reviewed in Vanhaesebroeck *et al.* 2000). Many different proteins apart from PKB have been found to contain this PDK1 phosphorylation motif including PKA, S6K and PKC. This raises the possibility that PDK1 could also act on and phosphorylate the homologous site in other kinases and thus be an important branch point in signalling, especially as the equivalent PKB T308 activating phosphorylation is likely to have similar regulatory roles in other A,G,C kinases (reviewed in Peterson *et al.* 1999).

Of the kinases which share the conserved PKB site, PDK1 has already been shown to phosphorylate S6 kinase, PKA, p90RSK and several PKC isoforms including the atypical subforms PKCλ/PKCζ, the novel PKCδ and conventional PKC isoform PKCβII at their homologous sites (Belham *et al.* 1999, Good *et al.* 1998, Alessi *et al.* 1998, Pullen *et al.* 1998). As with the phosphorylation of PKB, the phosphorylation

of several PKC isoforms (i.e. PKCλ/PKCζ) requires the presence of lipid co-factors to assist PDK1s in their activation (Bandyopadhyav *et al.*1999, Chou *et al.* 1998). The phosphorylation and activation of the novel PKCs; PKCε and PKCδ, and the atypical PKCζ has been shown to be stimulated by PI3K lipid products and conversely inhibited by the PI3K inhibitor LY294002, which again points towards the PI3K/PDK1 pathways involvement in these activations (Standaert *et al.* 1999, Chou *et al.* 1998, reviewed in Peterson *et al.* 1999, Vanhaesebroeck et al. 2000)

A second group of PDK1 substrates including S6K and SGK require prior phosphorylation at a second site to enhance PDK1 directed phosphorylation and activation (Alessi *et al.* 1998, Kobayashi *et al.* 1999). For example, a catalytically inactive mutant of PDK1 has also been shown to block growth factor stimulated activation of S6 Kinase, emphasising a likely role for PDK1 in the phosphorylation and activation of this and other kinases of the same group (Pullen *et al.* 1998, Balendran *et al.* 1999, reviewed in Cohen 1999). This priming phosphorylation event will be investigated in chapter 5 with reference to the activation of S6K where this phosphorylation increases the activity of this kinase.

A third subset of possible PDK1 substrates require an interaction with Rho-GTP in order for PDK1 to access and phosphorylate the T-loop site. This group which includes the PKC related kinases (PRK 1 and 2) possess an N-terminal Rho-binding domain which when occupied by Rho induces a conformational change in these proteins allowing PDK1 to interact with these kinases and phosphorylate the T-loop site (Flynn *et al.* 2000). The final group of possible PDK1 substrate have been found to be constitutively phosphorylated at the T-loop site, with this phosphorylation event not influenced by PI3K or other known inputs. This group of substrates which includes p90RSK and PKA may therefore be phosphorylated by the constitutively active PDK1 as soon as they are synthesised and then regulated by other post-PDK1 phosphorylation events in response to upstream inputs (Cheng *et al.* 1998, Jensen *et al.* 1999, Frodin *et al.* 2000).

Therefore, it appears, that not only has PDK1 been found to be an essential kinase in the T-loop phosphorylation and activation of PKB but has also been shown to be a key factor in regulating several other important cellular proteins. It is apparent that PDK1 can act as a direct downstream modulator of PI3K function to not only PKB but also possibly S6K and several PKC isoforms. Cellular roles for PDK1 in glucose transport (via PKB and/or PKCζ), protein synthesis (via S6K) and cell survival in response to a variety of growth factors including insulin, EGF and PDGF have already been proposed. It is clear that PDK1 is likely to be important in the control/regulation of key events and thus needs further investigation to establish its precise cellular roles (reviewed in Vanhaesebroeck *et al.* 2000, reviewed in Chen *et al.* 1999, reviewed in Peterson *et al.* 1999).

1.2.4.4 PDK2

For maximal PKB activation, phosphorylation of the activating serine of PKB (S473 in PKBα) is also required. Mutation studies on this residue revealed that if it is mutated to alanine maximum activation of PKB could not be achieved, whereas mutation to aspartate partially activated PKB (Alessi *et al.* 1996). In both cases, phosphorylation of T308 still occurred irrespectively with a subsequent increase in activation. A similar situation was observed with T308A and T308E mutations indicating that these two phosphorylation events are independent and act synergistically to maximally activate PKB (Alessi *et al.* 1996, Bellacosa *et al.* 1998). Incubation of partially active (T308 phosphorylated) PKB with PI 3,4,5P_3 did not result in further phosphorylation or activation of PKB *in vitro*, indicating that the serine 473 phosphorylation is unlikely to be a PI3'lipid induced autophosphorylation event and therefore another upstream kinase is likely to be involved. (Alessi *et al.* 1997c).

Mutation studies have shown that the phosphorylation of this serine site is also sensitive to the Phosphatidylinositol lipids and so this unknown kinase has been termed phosphatidylinositide dependent kinase -2 (PDK2). The insulin-induced phosphorylation of this residue is also prevented by inhibitors of PI3K, indicating the involvement of the PI lipids in its activation/regulation (Alessi *et al.* 1996).

The serine 473 site in PKB has been shown to lie within the regulatory C-terminal domain in a region which is highly conserved within the A,C,G kinase family (reviewed in Peterson *et al.* 1999). In this family, this site has been found to lie within the consensus motif P-X-X-P-S/T-F/W with the phosphorylated serine or threonine always lying 160-165 amino acids downstream of the conserved serine/threonine site phosphorylated by PDK1. Kinases shown to contain both the PDK1 site and this potential PKD2 site include not only all the PKB isoforms, but also S6K, many PKC isoforms and the rho-dependent protein kinase (reviewed in Peterson *et al.* 1999, reviewed in Vanhaesebroeck *et al.* 2000).

The search is on for the identity of this kinase, which along with PDK,1 is likely to be involved in the phosphorylation and activation of a wide variety of kinases and so have a critical role to play in cell signalling. Although MAPKAP Kinase-2 phosphorylates this residue *in vitro* this kinase is not stimulated to any extent by IGF-1 or insulin in many cell lines and inhibition of the MAPKAP-2 activating pathway had no effect on insulin's activation of PKB (Alessi *et al.* 1996). MAPKAP-2 is therefore unlikely to be PDK2.

Another more promising candidate for PDK2 is the integrin linked protein kinase (ILK) (reviewed in

Dedhar 2000). This kinase has been found to link with the cytoplasmic domains of integrins β1, 2 and 3 and so is usually located in the proximity of the plasma membrane (Radava *et al.* 1997). ILK also has been found to suppress anoikis (an anchorage dependent form of apoptosis) and stimulate progression, processes which PKB has also been implemented in (Radava *et al.* 1997, Assoian *et al.* 1997, reviewed in Dedhar *et al.* 2000, Datta *et al.* 1999). ILK is stimulated by PI3K and its activation has been shown to be prevented by inhibitors of PI3K with these findings placing ILK downstream of PI3K (Delcommenne *et al.* 1998). This is further evidenced by the facts that ILK has also been shown to require the PI3K lipid product, $PI3,4,5P_3$ for maximal activity with ILK activity negatively regulated by the 3`phosphatase, PTEN (see section 1.3.4) (Delcommenne *et al.* 1998, Morimoto *et al.* 2000).

Most importantly in this pathway, ILK has also been found to bring about phosphorylation of PKBα at the serine 473 site with a kinase deficient ILK preventing this phosphorylation and activation of PKB (Delcommenne *et al.* 1998, Lynch *et al.* 1999). Activation of ILK has also been shown to lead to the phosphorylation and inhibition of GSK-3 a likely downstream, substrate of PKB, again linking these two kinases (Wu 1999, Troussard *et al.* 1999, Delcommenne *et al.* 1998, Persad *et al.* 2000). *In vitro* ILK has previously been shown to directly phosphorylate S473 of PKBα raising the possibility that ILK may be the elusive PDK2 (Delcommenne *et al.* 1998, reviewed in Dedhar 2000). Recently ILK has been shown not to contain a kinase activity suitable for a direct PKB phosphorylation event and has been proposed to act via a second as yet unknown kinase, to mediate phosphorylation and activation of PKB (Balendran *et al.* 1999, Lynch *et al.* 1999).

Use of dominant negative mutants of ILK in several cell lines also been shown to suppress PKBα S473 phosphorylation and lead to increased apoptosis and G1 to S phase cell cycle arrest, linking ILK to PKB S473 phosphorylation (Wu 1999, Troussard *et al.* 1999, Persad *et al.* 2000). These studies indicate that in certain cell lines, particularly anchorage dependent ones, ILK is a likely upstream regulator of S473 phosphorylation and PKB activation (Hannigan *et al.* 1996, Delcommenne *et al.* 1998, Persad *et al.* 2000). However, whether these studies make ILK an attractive direct candidate as PDK2 or whether ILK acts between PI3K and an unknown PDK2 at least in some cell types is unclear and therefore one which more than merits further investigation (reviewed in Kandel *et al.* 1999, reviewed in Dedhar 2000).

Alternatively recent evidence has shown that there is the possibility that PDK1 could phosphorylate the serine 473 site leading to maximal PKB kinase activity (reviewed in Vanhaesebroeck *et al.* 2000). Earlier *in vitro* studies showed that PDK1 could not directly phosphorylate this site on PKB (Alessi *et al.* 1997a&b) however the new data shows this may not be the case and PDK1 may be able to acquire PDK2 activity (Balendran *et al.* 1999a). It has been shown that the kinase domain of PDK1 interacts with a

fragment of the carboxyl domain of PRK2 a rho-dependent and lipid dependent kinase which has been subsequently termed the PDK1-interacting fragment (PIF) (Balendran *et al.* 1999a). The interaction of PIF with PDK1 occurs via binding of the modified PDK2 consensus motif in the PRK2 fragment to a hydrophobic motif in the small lobe of PDK1 (Balendran *et al.* 1999a, Biondi *et al.* 2000). In PIF the phosphorylated serine or threonine residue, usually within this motif, is replaced by an aspartate, thus mimicking phosphorylation of this site, with this modification shown to be essential for interaction. This fragment has been shown to comprise the final 78 C-terminal amino acids including a putative PDK2 site and may be generated by alternative splicing of the PRK2 gene or possibly after a post-translational cleavage event (Balendran *et al.* 1999a, Biondi *et al.* 2000, reviewed in Vanhaesebroeck *et al.* 2000).

Upon this interaction PDK1 was found to obtain activity towards the PDK2 site. Thus this converted form of PDK1 was able to phosphorylate PKBα on both the threonine 308 site and the serine 473 site in a PI3,4P_2 and PI3,4,5P_3 dependent manner (Biondi *et al.* 2000, Balendran *et al.* 1999a&b). Interestingly in the presence of PIF, PDK1 is converted from a form that may not be activated directly by PI3,4,5P_3 to a form that is activated more than 3 fold by this lipid (Balendran *et al.* 1999a&b, Biondi *et al.* 2000). Also, in brain extracts the major PDK2/S473 activity which phosphorylates PKB in a PI3,4,5P_3 dependent manner could be immunoprecipitated by a PDK1 antibody perhaps further indicating a likely role for PDK1 as a PDK2 kinase (Balendran *et al.* 1999a).

These results appear to show that PDK1 could in fact also be the unknown kinase PDK2 gaining this second activity upon its interaction with the PIF region of PRK2 (Balendran *et al.* 1999). It will be interesting to see if this situation is apparent in other cell lines and how such a interaction may affect the other possible roles of PDK1, particularly as most other A, G, C kinases also contain this PDK2 hydrophobic motif (reviewed in Chen *et al.* 1999, reviewed in Toker *et al.* 2000). The role of PDK1 in phosphorylating the T389 site of S6K as well as the T229 site has also been investigated (Balendran *et al.* 1999b). Some data suggests that PDK1 is capable of phosphorylating both the PDK1 and PDK2 sites under normal unoccupied conditions however in the presence of the PIF fragment it appears that PDK1 does not phosphorylate the PDK2 site and cannot phosphorylate the PDK1 either (see chapter 5) (Balendran *et al.* 1999b).

Recent evidence has again raised the possibility of the phosphorylation of PKB at the S473 site is in fact an autophosphorylation event (Toker *et al.* 2000). This evidence is based on the findings that the phosphorylation of this hydrophobic site motif in PKB requires an intact intrinsic kinase activity. In these studies, kinase inactive PKB mutants (K179M) could still be phosphorylated on the T308 site in response to IGF-1 but could not be phosphorylated on the S473 site (Toker *et al.* 2000). However in the

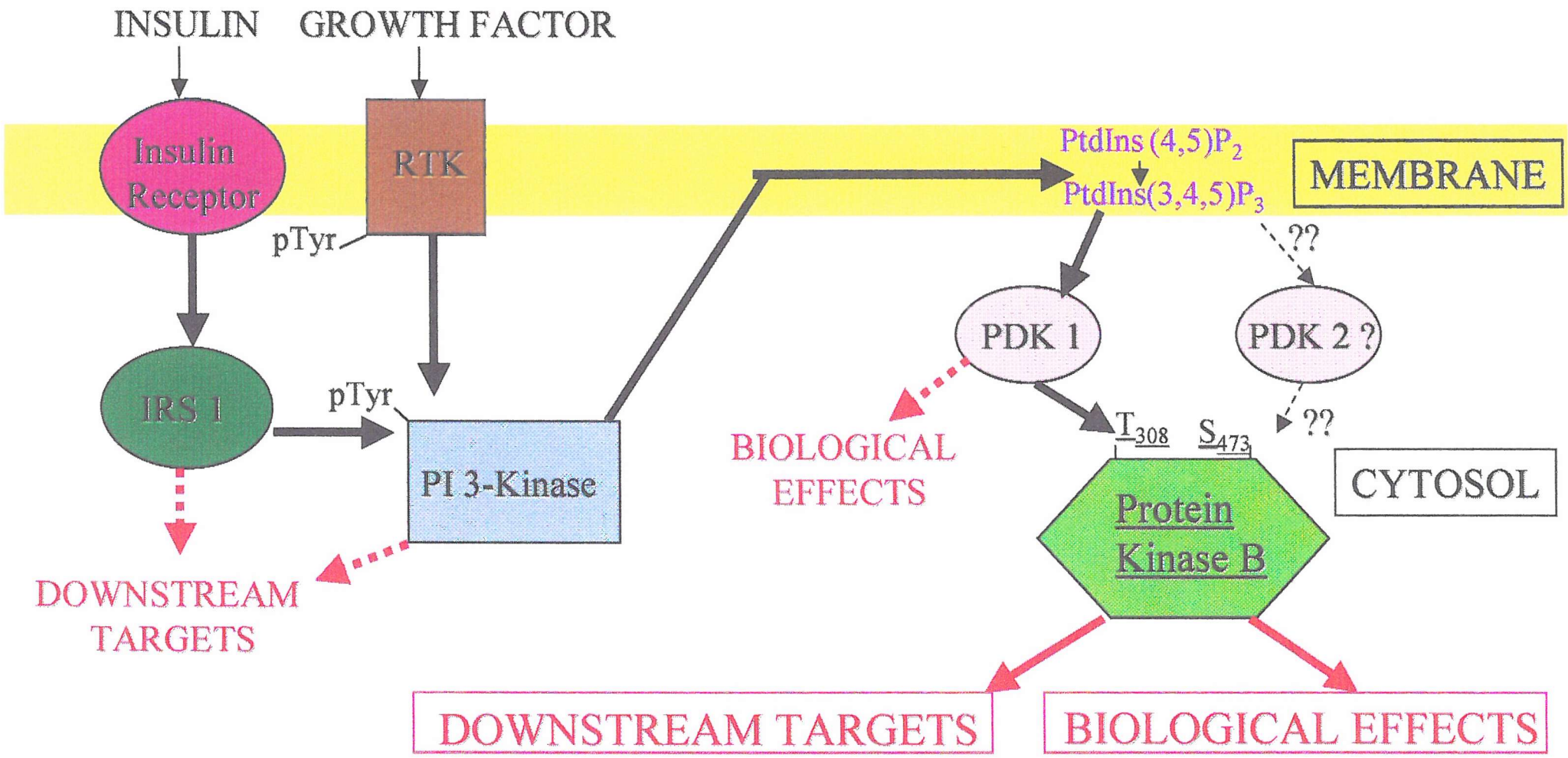
wild type enzyme, both sites could be phosphorylated in response to IGF-1 stimulation indicating the importance of PKB's intrinsic kinase activity in the hydrophobic motif serine phosphorylation. The phosphorylation of S473 was also found to be dependent on prior phosphorylation of the T308 site as shown by T038A and T308E mutants. This event was found to be sufficient to stimulate PKB autophosphorylation on S473 *in vitro* (Toker *et al.* 2000).

There are therefore several possible candidates for the identity of PDK2, including PDK1 or PKB itself. From experimental studies it appears that some of these candidates show more promise than others as the PDK2 kinase, but the overall situation is unclear. Also, there may be more as yet unidentified candidates for the role of this crucial signalling kinase especially as this area of research is still fairly new. Hence it is apparent that more work is required to elucidate the exact mechanism by which insulin and other growth factors bring about PKB activation and how PI3K, PDK1 and PKB itself contribute to this complex activation process (reviewed in Cohen *et al.* 1997, Coffer *et al.* 1998). A simplified diagram of the receptor linked activation of PKB via the PI3K/PDK1 pathway is shown in figure 1.3

1.2.4.5 Alternative Routes for PKB Activation

Recent evidence has also shown that cytokines such as interleukin-2 activate PKB via receptor mediated activation of PI 3-Kinase (Reif *et al.* 1997). However, it is also becoming apparent that PKB cannot only be activated by PI 3-Kinase but also by other as yet undefined mechanisms. For example, the β-adrenergic agonist, isoproterenol, has been shown to activate PKB in a wortmannin-insensitive mechanism and hence not using class 1_A PI3K (Moule *et al.* 1997). This factor has been shown to act on PKB via the β_3-adrenoreceptors using a mechanism which has been found not to involve changes in cAMP levels (Liao *et al.* 1998). Also the activation of PKB by this agonist has been shown not to lead to the characteristic SDS-PAGE mobility shift for activated PKB seen after growth factor stimulation (Moule *et al.* 1997).

Figure 1.3 - Simplified PKB Activation Pathway



It is possible that such an activation still involves the proposed constitutively active PDK1 since PDK1 is ubiquitously expressed and so follows a similar activation route downstream of PI 3-kinase. It may be that this mechanism is via a wortmannin insensitive class II PI3K or via other possible PKB kinases. Other possible mechanisms for this activation include acting via an as yet unknown wortmannin insensitive, G protein βγ subunit activated PI 3-Kinase or by direct interaction of PKB with $G_{\beta\gamma}$ subunits (Murga *et al.* 1998, reviewed in Vanhaesebroeck *et al.* 1999). In fact, it has recently been shown that the PH domain of PKB can interact with $G_{\beta\gamma}$ subunits and with PKCα and δ subtypes *in vitro* (Konishi *et al.* 1995). Another possibility is that the proposed wortmannin insensitive route is confined only to the cell types used in this study (rat adipocytes) and so is not a major PKB activation route. This is evidenced but the fact that other groups have failed to repeat this wortmannin insensitive activation. Hence, further work is required to establish how β-Adrenergic agonists stimulate PKB

PKB has also been shown to be activated by cellular stress, for example heat shock (i.e. temperature elevation of COS-7 cells from 37°C to > 44°C) and osmolarity stress (Meirer *et al.* 1998, Konishi *et al.* 1996). In heat shock treatment, this activation of PKB has been found to be related to and greatly enhanced by its association with heat shock protein 27 (Hsp 27) (Konishi *et al.* 1997). As with β-adrenergic agonist stimulation, this activation of PKB has been found to occur by an unknown wortmannin insensitive mechanisms, so a role for PI3K in this is unlikely. In this activation, the PH domain of PKB was shown to be indispensable and in heat shock cells PKC-δ was shown to interact with this domain and be phosphorylated *in vitro* by PKB indicating a possible role of PKB's PH domain in responding to cellular stress (Konishi *et al.* 1996). However, there is some evidence that these routes may indeed follow the documented wortmannin-sensitive PI3Kroute, with previous differences due to a less specific mixed histone PKB assay used in initial experiments (Shaw *et al.* 1998).

PKB activity has also been shown to be increased by agents which elevate cellular cAMP levels with this possibly via PKA (Sable *et al.* 1997, Filippa *et al.* 1999). PKB has also been shown to be phosphorylated by PKA with this phosphorylation leading to PKB activation. To date the significance of this possible route is unclear, as the sites on PKB phosphorylated by this kinase have yet to be resolved and these findings have not been shown to occur *in vivo*. It has however been shown that activation of PKB by this route is a lot weaker than the common PI3K/PDK1 route with questions about the requirements of the PH domain and S473 site also raised (Filippa *et al.* 1999).

Agents which elevate intracellular calcium levels have also been reported to activate PKB in a PI3K independent manner through the calcium/calmodulin dependent protein kinase kinase (CAMKK) (Yano *et al.* 1998). PKB has also been shown to be phosphorylated by CAMKK on T308 in the presence of

$PI3,4,5P_3$. Calcium antagonists have also been shown to prevent $PI3,4,5P_3$ formation and PKB activation which may indicate a link to PI3K via this route (Wang *et al.* 2000). However, the relative importance and role of this has been questioned as other groups have been unable to show that CAMKK is capable of directly phosphorylating PKB and also have been unable to find any activation of PKB by increased cellular calcium levels in kidney, fibroblast and neuronal cells (Pullen *et al.* 1998, Conus *et al.* 1998, reviewed in Vanhaesebroeck *et al.* 2000)

PKB has also been shown to interact with, be phosphorylated and negatively regulated by the protein kinase C isoform, PKCδ although again the physiological significance if any has yet to be determined. Therefore, it is apparent that the evidence concerning the PI3K independent activation of PKB is very patchy and inconclusive. It is, however, apparent that regulation of PKB activity is a fairly diverse and complex area which is likely to be cell/tissue type and stimulator specific and is therefore a rich source of study for a long while to come. One alternative route for these PI3K independent mechanisms of PKB activation is that these routes act to inhibit phosphatases involved in the negative regulation of PKB.

1.2.5 Negative Regulation of PKB

It is appears that the major event in the activation of PKB is the phosphorylation of the T-loop threonine residue (i.e. T308 in PKBα) and the C-terminal hydrophobic domain serine (i.e. S473 in PKBα). However this activation profile, in response to most growth factors/stimuli, has been found to be rapid and transient and therefore methods of regulating this event are required. In unstimulated cells and in order to inactivate PKB these residues are dephosphorylated, indicating the action of specific phosphatases is required. The negative regulation of PKB has been found to not only involve protein phosphatases which act directly on the threonine/serine activation sites, but also lipid phosphatases which act to remove the PI3K generated, PKB activating lipids $PI3,4P_2$ and more importantly $PI3,4,5P_3$ (reviewed in Chen *et al.* 1999, reviewed in Kandel *et al.* 1999).

When activated PKB is treated *in vitro* with protein phosphatase 2A (PP2A) it is found to be dephosphorylated on T308/S473 and inactivated indicating that PKB is a substrate for this phosphatase. (Andjelkovic *et al.* 1996, Meier *et al.* 1997). Also, hyperosmotic shock rapidly inactivates PKB and this is preceded by the dephosphorylation of PKB (Meier *et al.* 1998). Evidence for a role of PP2A in the *in vivo* dephosphorylation and inactivation of PKB has also recently been identified. The agents vanadate, peroxyvanadate and okadaic acid all act to inhibit cellular phosphatase activity particularly PP2A and

have all been shown to activate PKB again indicating a role for PP2A in the negative regulation of PKB (Meier *et al.* 1998). Prolonged activation of PKB is also seen in the presence of calyculin A, another PP2A inhibitor, emphasising a potential role for PP2A in the inactivation of PKB (Meier *et al.* 1998). Therefore, it is likely that PP2A and possibly other protein phosphatases act to remove the activating phosphorylations on PKB and down regulate PKB back to basal levels once the PKB activating stimulus is finished (reviewed in Kandel *et al.* 1999).

The second major mechanism of down regulating or negatively effecting PKB activity appears to via specific 3' and 5' lipid phosphatase activity directed towards the PI products of PI3K previously shown to activate PKB. The first lipid phosphatases proposed to have a role in regulating PKB activity are the SH-2 domain containing inositol 5'phosphatases SHIPs which have been shown to hydrolyse $PI3,4,5P_3$ to $PI3,4P_2$ (Habib *et al.* 1998). Overexpression of this phosphatase in cells has been shown to inhibit PKB activity and promote apoptosis in a variety cell lines by the inhibition of the PI3K/PKB survival pathways (Aman *et al.* 1998, Liu *et al.* 1998). SHIP null mutant mice have been shown to have increased levels of $PI3,4,5P_3$ and prolonged PKB activation, coupled with decreased levels of $PI3,4P_2$ which indicates the relative importance of $PI3,4,5P_3$ over $PI3,4P_2$ in the activation and functioning of PKB (Aman *et al.* 1998, Liu *et al.* 1997 & 1998). These SHIP-/- mice demonstrate defects in triggering apoptosis and have excessive cellular survival in myeloid cells indicating a role for PI3K/PKB in cell survival and for SHIP in negatively regulating these events (Aman *et al.* 1998, Liu *et al.* 1997 & 1998).

The second major lipid phosphatase activity involved in PKB regulation, recently found to be present in many cell types, was an inositol polyphosphate 5'phosphatase which has subsequently been referred to as PTEN/MMAC1 (reviewed in Di Cristofano *et al.* 2000). PTEN (phosphatase and tensin homologue deleted from chromosome 10), MMAC1 (mutated in multiple advanced cancers) or TEP1 (TGFβ-regulated epithelial cell enriched phosphatase) was found to be located on chromosome 10q23 within a genomic region which suffers loss of heterozygosity in many human cancers (Myers *et al.* 1998, Cantley *et al.* 1999). In fact, mutations or deletions in this gene have been identified in a large number of different tumours including prostate cancers, glioblastomas and endometrial derived carcinomas (Li *et al.* 1997, Suzuki *et al.* 1998).

Mutations in the PTEN gene have been found to be present in more than 80% of patients suffering from one of three autosomal dominant disorders: Cowdens disease, Bannayan-Zonana syndrome and Lhermitte-Duclos disease, which share similar features including multiple benign tumours and an increased susceptibility to thyroid and breast malignancies (Liaw *et al.* 1997). Therefore, inactivation of PTEN has been implicated in the progression of a large number of tumours with loss of function also

linked to a predisposition towards cancer, demonstrating a critical role for PTEN as a tumour suppressor gene. The fact PTEN is one of the most commonly mutated genes in human cancers (second only to p53) further emphasises the role PTEN plays in tumour suppression (reviewed in Di Cristofano *et al.* 2000, reviewed in Datta *et al.* 1999).

PTEN is a 55kDa protein which has been found to contain a specialised tyrosine phosphatase domain which is capable of dephosphorylating not only tyrosine residues but also serine/threonine residues (Davis *et al.* 1998). This domain correlates with the region where most gene mutations in tumours occur, underpinning the importance of this region/activity in the tumour suppression function of PTEN. However PTEN was found to be an inefficient protein phosphatase suggesting this activity was not the main functioning of PTEN. Subsequently, PTEN was found to be very active towards highly acidic substrates suggesting it does not act primarily on proteins. The major substrate for PTEN was then found to be the PKB activating lipid, $PI3,4,5P_3$, which PTEN cleaves at the D3 phosphate group generating an inactive $PI4,5P_2$ product (Maehama *et al.* 1998, Myers *et al.* 1998).

The crystallographic structure has revealed the catalytic site of PTEN to consist of an extra wide hydrophobic pocket which can accommodate the head group of $PI3,4,5P_3$. This site is surrounded by three positively charged residues (K125, K128, H93) which account for PTENs preference for highly acidic substrates such as $PI3,4,5,P_3$ and help generate its lipid phosphatase activity. The catalytic site of PTEN is also very deep and so could possibly bind a phosphotyrosine or a phosphoserine/threonine residue and still sometimes act as a protein phosphatase (Lee *et al.* 1999).

The fact that PTENs main substrate is $PI3,4,5P_3$, placed this protein as a negative regulator of the PI3K pathway which generates this lipid (Stambolic *et al.* 1998, Haas-Kogan *et al.* 1998). In quiescent cells, the levels of $PI3,4,5P_3$ are very low but increase rapidly via the action of PI3K in response to growth factor stimulation (reviewed in Chen *et al.* 1999). Thus it appears that PTEN's cellular roles are two fold, firstly to keep the levels of $PI3,4,5P_3$ low in quiescent cells to prevent inappropriate activation of the targets of this lipid i.e. PKB, and secondly to return the high post stimulation levels of $PI3,4,5P_3$ back to the low normal levels and thus inactivate/negatively regulate the various downstream targets of this lipid including PKB (Wu *et al.* 1998, Wang *et al.* 1999, reviewed in Datta *et al.* 1999).

Therefore, PTEN has been shown to directly reduce both the levels of $PI3,4,5P_3$ and the activity of PKB in a variety of cell types (reviewed in Datta *et al.* 1999, reviewed in Kandel *et al.* 1999). Overexpression of PTEN has also been shown to induce apoptosis in many cell types with this suppression of cellular survival linked to the PI3K/PKB pathway (Wang *et al* 2000, Tolkacheva *et al.* 2000, Liu *et al.* 1999,

reviewed in Datta *et al.* 1999). Thus fact was confirmed by experiments where similar apoptotic responses to those with PTEN were induced by PI3K and PKB mutants and PI3K inhibitors (Wang *et al* 2000, Tolkacheva *et al.* 2000). PTEN overexpression has also been linked to G1 growth arrest and to the inhibition of other PI3K lipid activated proteins including integrin linked kinase and phospholipase C (Zhundel *et al.* 2000, Morimoto *et al.* 2000). In fact, the endogenous levels of PTEN have been found to be increased in mid-late G1 phase where it may act as a control point negative regulator in cell cycle progression possibly via interaction with retinoblastoma (Rb) (Persad *et al.* 2000, Paramio *et al.* 1999).

In contrast to these overexpression studies, loss of PTEN function has also been found to have significant affects on cellular function. For example, loss of PTEN function has been shown to cause an accumulation in $PI3,4,5P_3$ which results in PKB hyper-activation leading to protection against various apoptotic stimuli (Cantley *et al.* 1999, Nakashima *et al.* 2000). PKB activity and cellular survival have also been shown to occur in PTEN -/- glioblastoma cells (Haas-Kogan *et al.* 1998, Li *et al.* 1998). It is likely that this increase in PI3K lipid products and therefore PKB activity, is one of the mechanisms by which several PTEN -/- cancers exhibit their apoptotic resistance and hence tumour potency and progression (Lu *et al.* 1999, Di Cristofano *et al.* 1999, Wu *et al.* 1998, reviewed in Datta *et al.* 1999).

Further evidence linking PTEN lipid phosphatase activity to negative regulation of the PI3K/PKB pathway, is seen with the fact that overexpression of PTEN in 3T3-L1 cells inhibits glucose uptake and GLUT4 transporter translocation, events which have been previously attributed to the PI3K pathway (Nakashima *et al.* 2000, reviewed in Alessi *et al.* 1998). This overexpression of PTEN also prevents membrane ruffling which may link PTEN activity to the regulation of other proteins (Nakashima *et al.* 2000). In fact, the protein phosphatase activity of PTEN may also be linked in part to tumour invasiveness since PTEN has also been shown to dephosphorylate and inactivate focal adhesion kinase (FAK) and reduce membrane ruffling and tumour spreading via this activity (Maier *et al.* 1999, Morimoto *et al.* 2000).

Therefore, it is apparent that the PTEN phosphatase is a very important regulator of PKB activity and that this phosphatase appears to be mutated in a variety of cancers and cancer related syndromes which therefore places not only PTEN but also PKB as key mediates of cancer cell functioning. PTEN is an important protein in cells as it appears to be critical in modulating a range of factors downstream of PI3K and in the balance of key cellular processes including cell growth verses growth arrest and cell survival verses apoptosis. It is clear that the discovery of PTEN is a key event in cell signalling and along with other potential negative regulators of PI3K/PKB needs urgent study (reviewed in Datta *et al.* 1999).

1.2.6 PKB Movement Post Activation

Once PKB has been activated by the PI3K/PDK1 and membrane localisation, most of it dissociates from the plasma membrane to activate its mainly cytosolic substrates (Mitsuuchi *et al.* 1998). There is some evidence, in fact, that there are differences in the substrate specificities of membrane-localised and cytosolic-activated PKB. This is shown by the fact a membrane targeted constitutively active PKB has been shown to activate p70S6K but a double aspartate (T308D/S473D) constitutively active mutant does not activate p70S6K. As well as functioning to activate cytosolic and possibly membrane localised substrates, PKB has also been proposed to act on several nuclear substrate and hence may itself also have a nuclear location. For example, the constitutively active v-Akt has been shown to be distributed in the cytosol, nucleus and at the plasma membrane (Ahmed *et al.* 1993)

A detectable proportion of PKB has been proposed to translocate to the nucleus rapidly following growth factor induced activation. For example, following insulin stimulation and PKB plasma membrane translocation, both PKBα and PKBβ have been shown to rapidly translocate to the nucleus in 293 cells (Andjelkovic *et al.* 1997, Meier *et al.* 1997). The phosphatase activator ceramide has also been shown to prevent both activation and nuclear translocation of PKB in PC12 cells, via the dephosphorylation of PKB using a specific ceramide activated phosphatase (Salinas *et al.* 200).

Recently, the 14kDa protein product of the Tcl1 oncogene has been shown to interact with the with the PH domain of PKB and promote the nuclear localisation of PKB (Pekarsky *et al.* 2000). In Tcl1 negative cells stimulated with 10% FBS more than 90% of the activated PKB resides in the cytoplasm however with expression of Tcl1 the localisation ratio is approximately 50% cytosolic and 50% nuclear (Pekarsky *et al.* 2000). The association of Tcl1 with PKB has been found to not only lead an increased nuclear localisation of PKB, but also to increase the activity of PKB around 5 fold acting in synergism with the growth factor activated PI3K/PDK1 pathway to maximise PKB activity (Pekarsky *et al.* 2000). Therefore, PKB may have roles to play in both cytoplasmic and nuclear control and hence it is important to define clear substrates for PKB.

1.27 Possible Cellular Roles of PKB

Because of the fairly recent discovery of PKB and its associated pathway little is yet known about most of its cellular roles. It is believed that most of PKB's actions are on cytoplasmic processes, although evidence points to possible nuclear translocation of PKB and hence PKB may also target nuclear substrates. There is also the possibility of some mitochondrial interaction between PKB and mitochondrial membrane proteins particularly in relationship to cell survival (see later). This is likely to be a oversimplification of what is rapidly becoming a very complex and diverse signalling pathway which we are only just starting to explore.

Likely cellular roles for PKB can be broadly split into 2 groups namely i) metabolic roles (usually linked to insulin signalling pathways) including glycolysis, glucose transport, glycogen /lipid/ protein synthesis and transcription/translation and ii) cell cycle roles including cell survival / death, cellular differentiation and cell proliferation. However, within these umbrella headings there is likely to be a great deal of cross over with shared downstream substrates or cellular functions which could come under either heading. We can also divide PKB's functioning into inferred direct PKB substrates, proteins believed to be effected indirectly by PKB and cellular processes proposed to be effected by PKB via an as yet undefined route. Also it is clear that many of the proposed roles for PKB which are detailed below, may be cell / tissue or species specific and depend on the PKB levels/isoforms expressed and what other signalling factors they contain and/or respond to.

Most experiments to date proposing cellular roles for PKB are based on overexpression studies involving dominant negative or constitutively active mutants of PKB. Therefore caution is needed in interpreting potential roles for PKB based on these studies since overexpressed PKB can act in an non-physiological fashion. Overexpressed PKB may act on non-physiological substrates or interfere with the normal functioning of upstream or downstream endogenous proteins. For example, PKB mutants may interact with PDK1 and prevent it from acting on its other downstream targets (i.e. AGC kinases). Alternatively PKB mutants could bind to substrates preventing other AGC kinases from phosphorylating them (reviewed in Vanhaesebroeck *et al.* 2000).

Many other PKB functionality studies are based on the *in vitro* actions of PKB so again may not relate to physiological conditions. The recently discovered SGK kinase has also raised questions about the exact cellular roles of PKB (Kobayashi *et al.* 1999). This kinase has been shown to be regulated in a similar PI3K dependent fashion to PKB and to phosphorylate the same target sites on proteins which PKB can act on (Kobayashi *et al.* 1999, Park *et al.* 2000). Therefore it is important to confirm genuine cellular

roles for PKB and not attribute to PKB some of SGK's actions.

1.2.7.1 Potential Direct PKB Substrates

1.2.7.1.1 Glycogen Synthase Kinase-3

The first potential PKB direct substrate to be discovered was glycogen synthase kinase-3 (GSK-3), which PKB can directly phosphorylate and inactivate *in vitro* (Cross *et al.* 1995). Overexpression of PKB in a variety of cells has also been shown to lead to the phosphorylation and inactivation of GSK-3 (Cross *et al.* 1995, Alessi *et al.* 1996).

GSK-3 was originally identified as a serine/threonine kinase that phosphorylates and inactivates glycogen synthase (Cohen *et al.* 1982/1985) but is itself serine phosphorylated and inhibited in response to insulin stimulation. Insulin activates GS by promoting dephosphorylation of this at a cluster of serine residues (3a, 3b, 3c and 4) which are those specifically phosphorylated by GSK-3 (Paulter *et al.*1988). Therefore, it was proposed that insulin acts to inhibit GSK-3 whilst stimulating a specific protein phosphatase to bring about GS dephosphorylation and activation and hence the conversion of glucose to glycogen (Cohen 1993).

GSK-3 has also been found to be phosphorylated on serine residues notable serine 9 in β and 21 in α, with phosphorylation at these sites leading to a rapid and significant decrease in activity (Wang *et al.* 1994, Stambolic *et al.* 1994). *In vitro* studies and when overexpressed *in vivo* PKB has been shown to inactivate GSK isoforms by phosphorylating the inactivating serine residues, phosphorylated in response to growth factor treatment (Cross *et al.* 1995). The time course for PKB activation by growth factors i.e. insulin of 1 minute also fits in with the growth factor induced inactivation time for GSK-3 of around 2 minutes (Hurel *et al.* 1996, Alessi *et al.* 1996, Shaw *et al.* 1997, Van Weeren *et al.* 1998).

Growth factor mediated inhibition of GSK-3 in many cell types has been shown to be removed by the PI 3-kinase inhibitor wortmannin, which also inhibits growth factor stimulated PKB activation (Welsh *et al.* 1994). These experiments suggest PKB may have a role in regulating the activity of GSK-3 by direct phosphorylation and inhibition. Therefore PKB may be involved in modifying many of the cellular processes GSK-3 has been implemented in, especially the regulation of glycogen synthesis via GSK-3 inhibition of Glycogen Synthase (Cross *et al.* 1997, Hurel *et al.* 1996, reviewed in Coffer *et al.* 1998).

GSK-3 has been shown to phosphorylate many substrates which can be split into two distinct groups. The first group of substrates has been found to require prior phosphorylation by a different kinase before GSK-3 can phosphorylate them. Substrates within this group usually require phosphorylation on serine or threonine residues 4 places C-terminal of the site GSK-3 acts on. Substrates in this group include glycogen synthase, which is previously phosphorylated by casein kinase 2, and the G-subunit of PP1 after prior phosphorylation by PKA. The substrates within this group appear to have a conserved structural motif of S-X-X-X-S(P)- for GSK-3 to be active towards them, with other members of this group including CREB protein and ATP citrate lyase (Plyte *et al.* 1992). The other group of substrate classification are those which do not have a requirement for a prior phosphorylation, with members of this group including the transcription factors c-Jun and L-myc and the microtubule associated protein tau with GSK3β hyperphosphorylation of this neuronal protein being linked to the development and progression of Alzheimer's disease (Imahori *et al,* 1997, Takasmama *et al.* 1993). The other notable GSK3 substrate is the eukaryotic initiation factor 2 protein (eIF2) which has been shown to be phosphorylated and inactivated by GSK3's action at a type I GSK3 phosphorylation motif. Therefore if GSK-3 is a direct target of PKB this suggests a pivotal role for PKB in regulating a variety of processes which GSK-3 has been implemented in.

1.2.7.1.2 PKB target motif

The discovery of GSK-3 as an *in vitro* direct substrate which PKB can phosphorylate lead to the search for and elucidation of a PKB substrate consensus sequence. By using synthetic peptides based on the amino acid sequence around the serine site of GSK-3 which PKB targets, Alessi *et al* 1996c), were able to defined the minimum sequence requirements for a possible PKB substrate. It was found that this minimum PKB substrate sequence motif based on the GSK-3 site was Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd; where Xaa is any amino acid, Yaa and Zaa are small residues (not glycine) and Hyd is a bulky hydrophobic residue (Phe, Leu).

It has been found that the three PKB isoforms, α, β, γ show indistinguishable specificity towards a range of synthetic peptides based on the established PKB target motif. This suggests that these three isoforms may share very similar cellular substrates with any found differences in the functioning between these isoforms likely to be due to their individual expression levels, sub-cellular distribution and activation profiles by upstream factors. This motif has already led to the discovery of other possible PKB substrates by database searches and subsequent overexpression studies and likely to enable more potential PKB substrates to be identified.

That said caution should be used in assigning proteins containing this motif as potential PKB substrates as this motif site can also be phosphorylated by other protein kinases including p70S6K and MAPKAPK-2. The PKB related protein, SGK, shares an identical target motif to PKB and has been shown to phosphorylate many of the same downstream targets *in vivo* (Park *et al.* 1999, Kobayshi *et al.* 1999). Therefore SGK could act on the same group of potential substrates as PKB. This kinase has also been shown to reside on the PI3K/PDK1 pathway and to be activated in response to a variety of growth factors in a similar activation profile to PKB (Park *et al.* 1999, Kobayshi *et al.* 1999). Two additional SGK isoforms have subsequently been identified and found to phosphorylate the same target motif (Park *et al.* 1999, Kobayshi *et al.* 1999). Therefore it is important to devise direct and specific inhibitors of endogenous levels of PKB and SGK to elucidate the individual roles of these two proteins.

Database analysis of proteins containing the PKB target site has revealed that well over 1000 human proteins contain this site and so could in theory be acted on by PKB or SGK, although it is unlikely that PKB targets this many downstream proteins. PKB has also been shown *in vitro* and when overexpressed *in vivo* to phosphorylate some proteins on sites outside this devised consensus motif suggesting that this motif is not the only site of PKB action. This points towards other factors being critically involved in establishing PKB substrates, including the structure and sequence surrounding the proposed PKB target motif which act to provide the necessary environment and accessibility for PKB.

The discovery of the PKB target motif which PKB phosphorylates lead to a great deal of research into potential downstream targets of PKB. This research lead to the discovery of a series of proteins which have been shown to be phosphorylated by PKB *in vitro* and also to be possible *in vivo* substrates based on overexpression and dominant negative mutant studies. Therefore, this evidence may point towards these proteins being direct cellular targets for PKBs kinase activity and hence that they may act downstream of PKB to bring about the cellular roles PKB has been proposed to control. These proteins and the potential action of PKB on them are detailed below.

1.2.7.1.3 Heart 6-Phosphofructo 2-Kinase

One protein which contained an amino acid sequence which falls within the desired motif is the heart muscle, glycolytic enzyme 6-phospofructo 2-kinase (PFK2). This protein is involved in the stimulation of glycolysis by increasing the activity of the related protein 6- phosphofructo 1-kinase (PFK1), a glycolytic enzyme, which converts Fructose 6-phosphate into Fructose 1,6 bisphosphate and is one of the key control points of glycolysis (Deprez *et al.* 1997, Lefebvre *et al.* 1996).

The regulation of PFK1 is the major control point by which the rate of glycolytic flux i.e. the pace of glycolysis is controlled. PKF1 activity is inhibited and hence the breakdown of glucose slowed by high levels of ATP and by increased amounts of citrate which enhances ATP's inhibition of PFK1 This inhibitory effect of ATP is reversed by a high AMP level which is indicative of a low energy state within the cell. Hence when the cell requires energy (i.e. low ATP/ high AMP) or building blocks (i.e. low citrate) the PFK1 activity and hence glycolysis increases. Conversely, high levels of these products mean the cell has an abundance of biosynthetic precursors and energy and so PFK1 activity is switched off and glycolysis stopped (Stryer 1988).

PFK2 catalysis the formation of fructose 2,6 bisphosphate a potent activator of PFK1 from fructose 6 phosphate. Fructose 2,6 bisphosphate was found to activate PFK1 by converting this tetrameric enzyme from the "tense" to the "relaxed" state thus increasing PFK1 affinity for its substrate fructose 6 phosphate and diminishing the inhibitory effects of ATP. PFK2 is a 53kDa peptide which has also been shown to contain a fructose bisphosphatase2 activity which acts to hydrolyse the fructose 2,6 bisphosphate back to fructose 6 phosphate, thus inactivating PFK1. Hence PFK2 is described as a tandem enzyme since in contains these two opposing enzymatic activities (Stryer 1988).

In heart muscle, the kinase activity of PFK2 has been found to be greatly enhanced by phosphorylation. Subsequent analysis relieved that this enzyme contained two PKB phosphorylation sites S466 (R-M-R-R-N-S-F) and S483 (R-P-R-N-Y-S-V) which are both phosphorylated by overexpressed PKB resulting in PFK2 activation. This phosphorylation and activation was found to be sensitive to wortmannin but not rapamycin or PD98059 placing PFK2 downstream of PI3K. Insulin was also found to increase the Vmax of the enzyme again via of PI3K pathway. These studies led to the proposal that PKB maybe the direct regulator of heart muscle PFK2 activity and therefore be involved in glycolysis (Deprez *et al.* 1997, Lefebvre *et al.* 1996).

Recent evidence, however, has pointed towards other PI3K activated kinases being involved in the regulation of PFK-2. For example, studies using PI3K inhibitors and kinase dead mutants of PDK1 and PKB suggest a role for PI3K/PDK1 but not for PKB in this phosphorylation (Bertrand *et al.* 1999). Also, very recently a 57kDa wortmannin-sensitive and insulin stimulated kinase which phosphorylates PFK-2 has been partially purified. This novel kinase was found to act downstream of PI3K/PDK1 but not to be any of the known PDK1 targets, PKB, SGK or PKCζ and hence represents a unique PDK1 activated kinase which may have other cellular role and hence, requires further investigation (Deprez *et al.*2000). Therefore, the role of PKB in the PFK-2 pathway is unclear with other kinases perhaps more

physiologically important in this phosphorylation event hence more study is need to establish what if any role PKB has in controlling glycolytic flux. These experiments also highlight the danger of assigning direct substrates for PKB based solely on *in vitro* and *in vivo* overexpression studies and emphasise the need for direct inhibitors of endogenous PKB.

1.2.7.1.4 Endothelial Nitric Oxide Synthase (eNOS)

Another protein with the PKB target motif and hence a putative substrate for PKB is nitric oxide synthase. The endothelial form of this protein was found to contain 2 potential PKB phosphorylation sites within its amino acid sequence at positions S653 and S1179 with the neurone form of NOS having the same site at S1415 (Michell *et al.* 1999). Studies revealed that PKB can phosphorylate eNOS at the 1179 site with this phosphorylation leading to the production of Nitric oxide (NO) which has been shown to be involved in gene regulation and angiogenesis (Dimmeler *et al.* 1999). Conversion of the S1179 to an aspartate residue to mimic phosphorylation generated an active eNOS further showing the importance of this site in eNOS activation and NO generation (Michell *et al.* 1999). However, phosphorylation at serine 653 in the endothelial form or any phosphorylation of the neuronal NOS was found not to be important for the generation of NO (Fulton *et al.* 1999, Dimmeler *et al.* 1999)

The use of wortmannin or LY294002 and a dominant negative mutant of PKB blocked the release of NO from endothelial cells indicating the involvement of the PI3K/PKB pathway in eNOS activation and hence NO generation (Gallis *et al.* 1999, Fisslthaler *et al.* 2000). Also, PKB activation was found to be sufficient to enhance NO production two fold even at resting calcium levels (the major NOS activator). This calcium independent activation was subsequently explained in experiments showing that PKB phosphorylation of S1179 imposes a negative charge on eNOS which increases eNOS activity by increasing electron flux at the reductase domain and by reducing calmodulin dissociation from the activated eNOS when calcium levels are low (McCabe *et al.* 2000). It is also interesting to note that shear stress (viscous drag) which is a major source of NOS activation physiologically not only caused phosphorylation of eNOS but also was shown to cause the phosphorylation and activation of PKB (Fulton *et al.* 1999).

It appears that PKB may have a direct role in regulating endothelial NO production via direct phosphorylation and activation of NOS and thus is likely to be involved in regulating blood pressure, vascular remodelling and angiogenesis as these are the major roles of the released NO (Fulton *et al.* 1999, Dimmeler *et al.* 1999a). NO has also been reported to be involved in vascular endothelial growth

factor (VEGF) induced cell survival cell migration and increased blood flow/angiogenesis in tumour cells (Snyder *et al.* 1999). VEGF has also been found to activate PKB and constitutively activate PKB mutants bypass the need for VEGF in signalling to eNOS, therefore PKBs involvement in eNOS activation may be linked to its roles as a survival factor and in tumourogenesis (Dimmeler *et al.* 1999b, MoralesRuiz *et al.* 2000)

1.2.7.1.5 Forkhead transcription Factors

The transcription factors FKHR, FKHRL and AFX are members of the Forkhead family of transcription factors and are the human/mammalian homologues of the DAF16 transcription factor in *C.elegans* (Anderson *et al.* 1998). All these transcription factors posses a 100 amino acid Forkhead domain and a C-terminal transactivation domain which are involved in DNA binding and transcriptional activation. These transcription factors are involved in regulating gene expression and particularly in the control of cell proliferation and differentiation. In humans, FKHR binds to the insulin responsive sequence DNA sequence and is involved in the upregulation of a variety of genes including IGF-binding protein, glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (PECK) and the apoptosis inducer FasL (Guo *et al.* 1999). The upregulation of these genes and the overall transcriptional activity of FKHR was found to be inhibited by an insulin stimulated kinase cascade which results in phosphorylation of FKHR on S253 and therefore cytoplasmic localisation of the transcription factor (Nakae *et al.* 1999, Kops *et al.* 1999, reviewed in Datta *et al.* 1999)

FKHR was found to contain 3 PKB phosphorylation sites within its amino acid sequence; T24, S253 and S316, which were all shown to be phosphorylated in 293 cells co-transfected with either PKB or its upstream activator PDK1 (Biggs *et al.* 1999, Rena *et al.* 1999, Tang *et al.* 1999) The other Forkhead family transcription factors FKHRL, AFX and the *C.elegans* homologue Daf16 have also subsequently be shown to be phosphorylated by PKB at these sites, in overexpression studies or *in vitro* (Takaishi *et al.* 1999). These 3 potential sites for PKB phosphorylation are conserved in all the Forkhead transcription factors identified so far and also similar sites have been identified in a variety of other transcription factors perhaps indicating a more general role in PKB in regulating transcription (reviewed in Datta *et al.* 1999).

Phosphorylation of FKHR was shown to occur in cells treated with IGF-1 an event which was abolished by wortmannin treatment (inhibitor of PI3K) but not by PD98059 (MEK inhibitor) treatment indicating this phosphorylation event is downstream of the PI3K pathway (Brunet *et al.* 1999, Biggs *et al.* 1999,

Rena *et al.* 1999, Tang *et al.* 1999). The *in vivo* phosphorylation of FKHR was found to occur in a hierarchical fashion in which overexpressed PKBs' phosphorylation of S253 in the DNA binding domain is a prerequisite of phosphorylation of the T24 and S316 sites (Nakae *et al.* 2000). Insulin has been shown to cause the phosphorylation of FKHR on all three potential PKB sites and cause inhibition of FKHR transcriptional activity, IGF-1 fails to bring about phosphorylation of FKHR on the T24 and does not inhibit FKHR transcriptional activity (Nakae *et al.* 2000). This indicates that the T24 site is essential for the inhibition of FKHR transcriptional activity. There is some evidence from PKB overexpression studies, that in response to insulin the T24 site is phosphorylated by a PI3K activated kinase distinct from PKB, but this occurs only after prior phosphorylation of the PKB target site S253 by overexpressed PKB (Tomizawa *et al.* 2000).

Phosphorylation at these residues was shown to decrease FKHR transcriptional activity, to promote nuclear export of the factor and to increase binding of FKHR to the cytosolic protein 14-3-3. It is presently unclear whether the phosphorylation of the FKHR actually creates a nuclear export signal within FKHR causing its removal from the nucleus or whether the phosphorylation on of FKHR in some way masks a nuclear localisation signal causing FKHR to be retained within the cytoplasm (Delpeso *et al.* 1999, reviewed in Kops *et al* 1999). Mutation of these residues to alanine, rendered FKHR resistant to phosphorylation, nuclear export and to the suppression of its transcriptional activity. These mutants were also found to cause 293T cells to undergo DNA binding dependent apoptosis. (Brunet *et al.* 1999, Biggs *et al.* 1999, Rena *et al.* 1999, Tang *et al.* 1999). The PKB overexpression studies suggest a possible role for PKB in regulating the expression of genes involved in both metabolic and cell survival pathways via an inhibition of FKHR transcription factors and further indicates critical roles for PKB in a variety of cellular events particularly apoptosis (reviewed in Datta *et al.* 1999).

1.2.7.1.6 cAMP Responsive Element Binding Protein (CREB)

Another transcription factor proposed to be directly regulated by PKB phosphorylation is cAMP responsive binding protein, CREB. CREB is shown to activated in response to a variety of stimuli including growth factors, peptide hormones and neuronal activity and itself activates transcription of a variety of target genes including the bcl-2 anti-apoptotic protein. The transcriptional activity of CREB has been found to dependent on the phosphorylation of S133 within CREBs kinase inducible domain, with phosphorylation at this site sufficient for maximal transcriptional activity (reviewed in Shaywitz *et al.* 1999).

This critical S133 has been shown to be phosphorylated *in vitro* by PKA, MAPK, CAMK and more recently PKB, with phosphorylation at this site acting to increase the binding of the CREB co-activator CBP (Du *et al.* 1998). Phosphorylation of this site has subsequently shown to be sensitive to wortmannin, placing it on a PI3K directed pathway (Pugazhenthi *et al.* 2000). Surprisingly, this putative PKB phosphorylation site has been found not to lie within the PKB target motif perhaps indicating that other sequences/factors influence PKB targets. Links between PKB and CREB have recently been suggested from PKB overexpression experiments, showing a direct route for PKB through CREB to the induction of anti-apoptotic bcl-2 expression and linking PKBs phosphorylation of S133 on CREB to cellular survival (Walton *et al.* 2000, Pugazhenthi *et al.* 2000). Therefore, evidence is mounting linking PKBs possible activation of the CREB transcription factor to its roles as a survival factor (reviewed in Datta *et al.* 1999)

1.2.7.1.7 Human Telemerase Reverse Transcriptase Subunit (hTERT)

Another protein found to contain a PKB phosphorylation motif and shown to be phosphorylated by overexpressed PKB is the human Telemerase Reverse Transcriptase subunit (hTERT). This protein was found to contain 2 potential PKB target motifs, at S227 (GARRRGGSAS) and S824 (AVRIRGKSYV). Treatment of human melanoma cell lysate with recombinant PKB resulted in the phosphorylation of both of these sites leading to an enhancement of telemerase activity. This up-regulation of hTERT was found to be involved in maintaining the length of telomeres which is linked to cells survival since short telomeres cause cells to enter senescence. The PKB induced phosphorylation of S824 on hTERT has also been linked to survival and proliferation of cells during development, ageing, neoplasia and tumour progression (Kang *et al.* 1999, reviewed in Liu *et al* 1999)

The use of the growth factors also caused the phosphorylation of hTERT and increased telemerase activity with the PI3K inhibitor wortmannin found to prevent this phosphorylation and the upregulation of the telemerase. Therefore, a possible role for PKB in the enhancement of telemerase activity and so cell survival / longevity is indicated by these results (Kang *et al.* 1999).

1.2.7.1.8 Raf-1 kinase

Raf-1 kinase is a serine and threonine protein kinase which is an essential component of the MAPK cascade. It is activated by recruitment to the plasma membrane by the action of the GTPase Ras, and

subsequently once activated dissociates from the membrane and phosphorylates and activates MEK which is the upstream kinase activator of MAPK. Recombinant PKB has been found to phosphorylate Raf-1 *in vitro* on S225 which lies within the PKB target motif and is found in the regulatory domain of Raf-1. This phosphorylation has been shown to cause the inactivation of Raf-1 kinase activity and its sequestration by 14-3-3 proteins which results in the inhibition of the MAPK cascade (Rommel *et al.* 1999).

This potential PKB phosphorylation event has been shown *in vivo* by the use of dominant negative (K179A) and constitutively active PKB mutants. Also, transfected PKB has been shown to interact directly with raf-1 kinase and phosphorylate it in breast cancer cells with this causing inactivation of the MAPK cascade and a shift for growth arrest to cell proliferation (Zimmermann *et al* 1999). Interestingly, PKB phosphorylation of Raf-1 and inhibition of the MAPK cascade has only been found to occur in terminally differentiated skeletal muscle myotubes and not their myoblast precursors indicating a possible cell type/differentiation state specific role for this action of PKB (Rommel *et al.* 1999). This finding may also fit in with conflicting reports as to the role of PI3K in the MAPK cascade which show that in certain cases PI3K inhibitors also partially inhibit the MAPK cascade whereas in other they act to activate this pathway (Rommel *et al.* 1999, Zimmermann *et al* 1999). Therefore, it is likely that PKB may be involved in mediating cross talk between different cell signalling pathways and that this is a potential role which needs further characterisation.

1.2.7.1.9 Phosphodiesterase 3β (PDE3β)

A PKB target site has also been found in the metabolic protein phosphodiesterase 3β (PDE3β)which acts to regulate the cellular levels of cAMP. PDE3β activity has been shown to be stimulated in response to insulin and act to hydrolyse cAMP, decreasing the activity of PKA and inhibiting a variety of cellular effects including PKA lipase activated lipolysis. The PDE3β activity has also been shown to be stimulated by leptin and it is believed that the actions of this protein is one way in which insulin and leptin antagonise the hormone glucagons effects on cells.

The activation of PDE3β has subsequently be found to be directly related to phosphorylation on S302. This phosphorylation and activation is also inhibited by wortmannin indicating a role for the PI3K pathway. PKB has been shown to directly phosphorylate this protein *in vitro* and overexpression studies using dominant negative and constitutively active proteins have shown that PKB interacts with, phosphorylates and activates PDE3β *in vivo*. A possible direct role for PKB phosphorylation and

activation of PDE3β has also been shown in leptin stimulated cells, suggesting the PKB route pathways has a key metabolic role in reducing the levels of cAMP and the consequences of this. A role for the PKB/PDE3β pathway in thymidine incorporation and pro-apoptotic BAD phosphorylation/inhibition in FDCP2 cells has also recently been proposed, indicating the complex roles PKB may play in mediating cell cycle and survival events. PKB possible phosphorylation and activation of PDE3β has also been implicated in *Xenopus oocyte* germinal vesicle breakdown, suggesting a role for this pathway in meiosis/development.

Recently questions over the involvement of PKB in PDE3β phosphorylation/activation have been raised based on experiments using the non-specific kinase inhibitor ML-9 which not only inhibits PKB but other downstream kinases including PKA and p90S6K. This inhibitor which does not inhibit PI3K activity was not found to affect PDE3β phosphorylation/activation in response to insulin whereas the direct PI3K wortmannin did. This suggests a role for distinct PI3K activated protein kinases, other than PKB in the phosphorylation of PDE3β. However due to the non-specific nature of this inhibitor and the fact these experiments were performed in only one cell type (rat adipocytes) it is unclear as to their importance, or PKBs physiological roles in PDE3β activation (Kitamura *et al.* 1998/1999).

1.2.7.2.0 Mammalian Target of Rapamycin (mTOR)

mTOR or FRAP/RAFT is a novel PI3K related kinase which is involved in cell cycle G1 progression, translation and possibly cellular survival. It is the direct target of the inhibitor compound rapamycin and studies using this compound and the PI3K inhibitor wortmannin have placed mTOR downstream of PI3K but upstream of p70S6K and 4EBP1 which are key mediates of a variety of cellular events including translation (reviewed in Thomas *et al.* 1997, Dufner *et al.* 1999)

A PKB target motif was identified within the amino acid sequence of mTOR and PKB was subsequently found to phosphorylate this site *in vitro* (Dennis *et al.* 1996, Scott *et al.* 1998). The growth factor induced phosphorylation of S2448 was subsequently found to occur *in vivo* and be blocked by inhibitors of PI3K or dominant negative mutants of PKB (Nave *et al.* 1999, Scott *et al.* 1998). A constitutively active PKB mutant was also shown to phosphorylate this site on mTOR *in vivo* (Nave *et al.* 1999). The involvement of amino acids, which themselves are strong activators of protein synthesis, has also been found to be necessary for this phosphorylation event and the activation of mTOR (Nave *et al.* 1999). The activation of mTOR by growth factor stimulation, has not only been shown to be dependent on the

phosphorylation of S2448, but also a wortmannin sensitive auto-phosphorylation event on S2481 (reviewed in Thomas *et al.* 1997).

Therefore the PKB overexpression studies points towards a possible role for PKB in the activation of mTOR and may help to explain the possible roles of PKB in protein synthesis perhaps by mTORs actions on p70S6K or 4EBP1 (PHAS-I) (reviewed in Dufner *et al.* 1999, Kandel *et al.* 1999). (see section 1.28 and Chapter 5)

1.2.7.2.1 Insulin Receptor Substrate (IRS)

The insulin receptor substrate 1 (IRS-1) protein acts as a modulator between the insulin receptor (and IGF-1 receptor) and key downstream signalling proteins including PI3K and Grb-2. IRS-1 has been found to not only be phosphorylated on key tyrosine residues, which are involved in substrate recognition, but also to be phosphorylated on serine and threonine sites which regulator the activity of this protein. Of these potential sites of S/T phosphorylation, 12 have shown to negatively regulate IRS-1 activity and downstream functions by reducing IRS-1 signalling to the downstream components. These phosphorylation events may not only reduce IRS-1 direct activity towards downstream substrate but also act to promote dephosphorylation of the IRS-1 tyrosine residues key to substrate recognition. To date the kinases which negatively regulate IRS-1 have yet to be identified although one possible candidate is GSK-3. Since GSK-3 can potentially be negatively regulated by PKB this may indicate a role for PKB in maintaining or prolonging IRS-1 signalling (Eldar *et al.* 1997, reviewed in Whitehead *et al.* 2000).

A more direct role for PKB in maintaining IRS-1 function has recently been suggested. Within the structure of IRS-1, 4 potential phosphorylation sites have been identified with the PKB target motif RXRXXS and have subsequently been shown to be phosphorylated by PKB *in vitro* (Li *et al.* 1999). The phosphorylation of these 4 sites *in vivo* has been shown to be dependent on PI3K, suggesting a possible role for PKB, and have been shown to positively regulate IRS-1 function and prolong its activation time. The extension of IRS-1 activation time afforded by phosphorylation of these sites has been found to be due to the protection against tyrosine phosphatase attack conferred to IRS-1 by phosphorylation at 4 key serine residues (Paz *et al.* 1999). Therefore, these findings indicate a possible role for PKB in maintaining the tyrosine phosphorylation state and hence function/activity of IRS-1 which is itself an upstream activator of PKB. This possibly suggests PKB may function as a positive feedback regulator of its own activation pathway and thus may act to prolong its own activation in response to insulin and IGF-1 (reviewed in Vanhaesebroeck *et al.* 2000).

1.2.7.2.2 Breast Cancer Susceptibility Gene Product (BRCA1)

The breast cancer susceptibility gene (BRAC1) encodes for a 220kDa nuclear phosphoprotein which acts as a transcription factor. The protein has been found to be mutated and inactivated in a variety of cancers particularly breast cancer and has been found to act as a tumour suppressor, with roles in DNA repair and transcriptional regulation (Bertwistle *et al.* 1998). This protein has been found to contain a potential PKB target motif which has been shown to be phosphorylated by PKB *in vitro*. The use of PI3K inhibitors or a dominant negative K179A PKB mutant has been shown to prevent this phosphorylation of BRAC1 *in vivo* indicating that PKB could be the cellular protein that targets this site (Altiok *et al.* 1999). This phosphorylation site on the BRAC1 protein lies within its nuclear location sequence and whilst to date an exact modulatory role for this phosphorylation event has yet to be identified it may involve changes in the nuclear targeting of this protein. Therefore, this suggests that PKB may have a role to play in modulating the functioning of a key tumour suppressor and gives further evidence to the likely roles PKB plays in cellular survival and carcinogenesis (reviewed in Vanhaesebroeck *et al.* 2000).

1.2.7.2.3 Kappa B Kinase (IκKα)

I Kappa B kinases (IκKs) are a series of protein kinases which are involved in the activation of the transcription factor NF-κB which is involved in controlling cell growth and oncogenesis and is further discussed in section 1.2.7.3 (May et al. 1997, Mayo *et al.* 2000). IκKs act to enhance NF-κB activation by phosphorylating a series of inhibitory proteins known as IκB and preventing these proteins from interacting with NF-κβ and retaining NF-κβ within the cytoplasm. The phosphorylation of these IκB inhibitory proteins targets them for degradation and thus removes them from effecting NF-κB subcellular location. Therefore, the function of IκKs are to prevent IκB from inhibiting NF-κB translocation to the nucleus and thus allow NF-κB to function as a transcription factor inducing genes that control such functions as proliferation and apoptosis suppression (anti-apoptotic IAP proteins) (Baumann *et al.* 200, Foo *et al.* 1999, Burrow *et al.* 2000, reviewed in Datta *et al.* 1999).

It has been found that in order for IκK to exert these stimulator effects on NF–κB via IκB phosphorylation/degradation IκK itself needs to be phosphorylated (Kane *et al.* 1999, Khwaja *et al.* 1999). The phosphorylation site which is important for this activity has been found to be T23 and to lie within a PKB phosphorylation site (Romashkova *et al.* 1999). This site has been shown to be not quite

the optimal PKB target motif since it contains a glycine residue at the C-terminal of the region rather than a hydrophobic residue. However, despite this, overexpressed PKB has been shown to directly phosphorylate IκKα leading to increased phosphorylation and degradation of IκB and increased translocation of NF-κB to the nucleus (Zho *et al.* 2000, Ozes *et al.* 1999). This indicates another possible direct role for PKB in pathways which regulate key cellular processes including proliferation and apoptosis/survival (Jones *et al.* 2000, Madrid *et al.* 2000, reviewed in. Datta *et al.*1999).

1.2.7.2.4 Human Caspase 9

Caspase 9 is a protease that is critical in the initiation and progression of apoptosis. It is activated by the release of cytochrome C from the mitochondria and the complexing of cytochrome C with Apaf-1 (Budihardjo *et al.* 1999). This complex then brings about the cleavage of pro-caspase 9 to its active form which then triggers the activation of other caspases by proteolytic cleavage (reviewed in Wolf *et al.* 1999, 2000)

Overexpressed PKB phosphorylates human caspase 9 at S196 with this phosphorylation resulting in inactivation of this kinase (Cardone *et al.*1998). It is not clear exactly how this activation occurs although it may be via inactivation of the intrinsic catalytic activity of this caspase. This phosphorylation has functional consequences, since extracts of cells overexpressing activate PKB block cytochrome C mediated caspase 9 activation *in vitro*. The phosphorylation and inhibition of caspase 9 activity by overexpressed PKB has been shown to be specific for this caspase since PKB does not appear to act on other caspases including caspase 3 or caspase 8 (Fujita *et al.* 1990). PKB mediated phosphorylation and inactivation of caspase-9 may also be species specific, since mouse and rat capsae-9 proteases are not phosphorylated or inactivated by PKB (Cardone *et al.*1998).

A role for PKB in mediating some of its anti-apoptotic effects via modification of caspase activity was originally proposed when it was found that a constitutively active PKB inhibited the general function of the caspase cascade perhaps by regulating one of the initial/upstream caspases possibly caspase 9 (Kennedy *et al.* 1997, Ahmed *et al.* 1997). A possible role for PKB was also shown in cells induced to undergo apoptosis by the action of TNFα, which could be rescued by an insulin induced PKB activity acting to phosphorylate and inhibit human caspase 9, but not another potential pro-apoptotic protein BAD (see 1.2.7.2.5) (Hermann *et al.* 2000). Therefore it is possible that one way PKB may act as a survival factor, at least in human cells, is via the phosphorylation and inactivation of the pro-apoptotic protein caspase 9.

1.2.7.2.5 Pro-Apoptotic BAD Protein

The final widely studied protein possessing a potential PKB target motif is the Bcl-2 family protein BAD. BAD is a pro-apoptotic protein which is 23kDa and is only a distant member of the Bcl-2 family since its only homology to this group is the BH3 domain it contains (reviewed in Downward 1999). BAD has a restricted tissue distribution so its overall importance in apoptosis signalling is unclear. BAD has been shown to form heterodimers with the pro-survival proteins Bcl-2 and Bcl-xl via this BH3 domain. This heterodimerisation prevents the pro-survival proteins from having their anti-apoptotic effects and so drives the cell towards apoptosis (Zha *et al.* 1996, Gajewski *et al.* 1996). Phosphorylation of BAD was found to prevent the heterodimerisation of BAD to the pro-survival Bcl-2 factors and thus promote cell survival (Yang *et al.* 1995). When phosphorylated BAD was shown to be bound by the ubiquitously expressed 14-3-3 proteins and was therefore sequestered away in the cytosol unable to interact with the pro-survival machinery. It was subsequently found that in order to be bound by 14-3-3 protein BAD had to be phosphorylated at two sites, S112 and S136 within a 14-3-3 binding motif (Zundel *et al.* 1998, Zha *et al.* 1996). Recently, evidence for a third phosphorylation motif in the BH3 domain of BAD has been proposed with studies showing that phosphorylation of S155 being necessary and sufficient for inhibition of BAD activity by binding to 14-3-3 proteins and hence cellular survival (Tan *et al.* 2000, Zhou *et al.* 2000).

BAD was found to be hyperphosphorylated and thus its death promoter role abolished in cells treated with serum, IGF-1 or IL-3 as well as other survival factor treatment. This phosphorylation and the anti-apoptotic properties it gives were also found to be prevented in many cell types by inhibitors of PI3K activity including wortmannin and stress-induced ceramide. These results place BAD as a potential downstream target of the PI3K pathway which is known to be involved in cell survival (Datta *et al.* 1997, Del Peso *et al.* 1997, Blume-Jensen *et al.* 1998).

BAD contains a PKB target motif around the S136 site (R-G-R-S-R-S-A). Analysis of this site using active/ inactive PKB mutants showed that overexpressed PKB could phosphorylate this site in many cells (Datta et al. 1997, Fang et al. 1999). This suggests that one route by which PKB may confer cell survival is by the phosphorylation of BAD which results in binding to 14-3-3 proteins and thus frees the pro-survival Bcl-2 members to carry out their anti- apoptotic functions (Davies *et al.* 1998, Zundel *et al.* 1998, reviewed in Coffer *et al.* 1998). Evidence based on overexpression studies indicates that the PI3K/PKB/BAD pathway could act as a major cell survival pathway in a variety of cell types which express BAD (Jiang *et al.* 2000, Lu *et al.* 1999, Konishi *et al.* 1999, Neshat *et al.* 2000, Santos *et al.* 2000, reviewed in Datta *et al.* 1999)

PKB has been shown not to phosphorylate BAD at either of the other two sites which may be important for BAD hyperphosphorylation, binding to 14-3-3 and hence cellular survival. A role of the MAPK kinase cascade in the phosphorylation of the S112 site and hence cellular survival has recently been suggested (Scheid *et al.* 1999, Peruzzi *et al.* 1999). When phosphorylated at this site in cells BAD loses the ability to heterodimerise to Bcl-2/Bcl-xl and cannot prevent their anti-apoptotic action. The Ras/Raf/MEK/MAPK signalling pathway has been shown to act via MAPKAP (RSK) to phosphorylate S112 of BAD and promote cell survival with this anti-apoptotic function inhibited by the upstream MEK inhibitor PD98059 (Fang *et al.* 1999, Bonni *et al.* 1999, Shimamura *et al.* 1999). There are several lines of evidence therefore that growth factors may use the PI3K and MAPK pathways to deliver anti-apoptotic effects with these two kinase cascades converging at the point of BAD phosphorylation and inhibition to induce cellular survival (Fang *et al.* 1999, Neshat *et al.* 2000, Tan *et al.* 1999). Another potential PI3K activated BAD kinase has recently been proposed with the finding that overexpressed p21 activated protein kinase (PAK) can directly phosphorylate S112 and S136 of BAD and thus confer an anti-apoptotic effect in FL5.12 cells (Schurmann *et al.* 2000). In the context of PAK phosphorylation and activation of BAD, it has been shown that PKB may function as an upstream activator of PAK and so may signal to inhibit BAD and promote cell survival via the PAK kinase (Tang *et al.* 2000)

The phosphorylation of BAD at the novel S155 site has also recently been shown to occur via direct action of PKA and to be sufficient to inactivate BAD's apoptotic function perhaps indicating the importance of this site in anti-apoptotic signalling via BAD inactivation (Harada *et al.* 1999, Tan *et al.* 2000). It is also worth noting that in MC/9 cells PKB has been found to be activated by IL-4 and confer cell survival by a route which does not involve BAD phosphorylation (Scheid *et al* 1998). Therefore, it is apparent that PKB may act to rescue cells from apoptosis by routes other than the phosphorylation and inactivation of pro-apoptotic BAD (Hinton *et al.* 1999, Craddock *et al.* 1999, Scheid *et al.* 1998, Hermann et al. 2000, reviewed in Datta et al. 1999).

It is clear that the phosphorylation of BAD and hence inhibition of its pro-apoptotic signal is one route by which PKB could function as a survival factor. However, the importance of PKB in inhibiting BAD signals in the cell is unclear, since other kinases and phosphorylation sites appear to also be involved. The general picture appears to be one in which a variety of different signals and kinase pathways can converge at the level of BAD phosphorylation to bring about inhibition of apoptosis. The relative importance of each pathway depends on the expression levels of the individual proteins, the cell type studied and the anti-apoptotic stimuli applied. Therefore, in order to understand the functioning of PKB in the regulation of apoptosis via BAD a great deal of further study is required.

1.2.7.3 Possible Roles of PKB in Cell Survival

Evidence above indicates that PKB may be linked to cell survival and apoptosis regulation by its possible direct phosphorylation and modulation of a variety of key survival/apoptosis factors. These include possible direct phosphorylation and inactivation of the pro-apoptotic BAD and GSK3 proteins or inhibition of the expression of pro-apoptotic proteins (i.e. Fas ligand) via inactivation of the forkhead transcription factor. Possible positive effects for PKB on cell survival may include the activation of IκK thus increasing NF-κB transcriptional activity (i.e. increased expression of IAPs), enhancement of the anti-apoptotic human telomerase activity and activation of CREB and eNOS. As evidence for PKBs direct roles against these agents is based on PKB overexpression studies and so may not relate to physiological conditions, there are many questions as to the physiological importance of PKB in modulating these effects in cells and the relative significance of these factors in apoptotic control.

There are several other possible routes by which PKB could act as an anti-apoptotic factor. For example, overexpression of PKB in various cell lines and primary cultures has been found to prevent apoptosis that has been induced by survival factor (i.e. serum) withdrawal or UV-light exposure (Kauffmann *et al.* 1997). IGF-1 induced survival has been shown to involve the PI 3-Kinase pathway and possibly PKB activation, with the use of the PI 3-Kinase inhibitors or dominant negative PKB mutants preventing survival (Gooch *et al.* 1999, Kulik *et al.* 1999, Dudek *et al.* 1997, reviewed in Datta *et al.* 1999). Inhibition of PI 3-Kinase by these factors however does not prevent the inhibition of apoptosis in cells transfected with constitutively active PKB (Gap *et al* 1998, Dudek *et al.* 1997, Franke *et al.* 1997, Hemmings 1997).

Overexpressed PKB has been shown to prevent apoptosis in Rat1a fibroblasts by inhibiting the Ced3/ICE (Interleukin-1 -converting enzyme)-like proteases or Caspases. These proteases which form part of a cleavage directed cascade finally resulting in the cleavage of major cellular proteins including, DNA repair proteins (i.e. the poly(ADP-ribose) polymerase), RNA splicing proteins (U1) and cell cycle proteins (Rb protein), resulting in cell death. IGF-1 stimulated activation of overexpressed PKB can protect rat retinal ganglion cells via phosphorylation and inactivation of caspase-3 (Kermer *et al.* 2000). These findings suggest that one route by which PKB may promote survival is via the inactivation of the caspase cascade which is a key pathway in the degradation of cellular proteins and promotion of apoptosis (Kennedy *et al.* 1997, reviewed in Datta *et al.* 1999, reviewed in Budihardjo *et al.* 1999).

PKB has been found to be one of the first proteins cleaved and degraded by the action of caspase

cascades when a cell is committed to programmed cell death by apoptosis. For example, in human neuroblastoma and rat PC12 neural cells, the activation of caspase-3 by the mitochondrial release of the apoptotic stimuli cytochrome c and ATP, directly cleaves PKB and results ultimately in DNA fragmentation and apoptosis (Francois *et al.* 1999). Caspase-dependent cleavage of PKB also occurs in jurkat cells stimulated to undergo apoptosis by Fas ligation, indicating a major function of caspases is to turn off survival pathways which could otherwise interfere with the apoptotic response (Widman *et al.* 1998). Subsequently, the cleavage of PKB by caspase action has been shown to occur at three distinct sites; firstly two sites between the N-terminal PH domain and the kinase domain (TVAD108G and EEMD119F) and secondly in the carboxyl terminal regulatory domain (SETD434T). The cleavage at these sites generates 40 and 44kDa fragments and occurs fairly early in the apoptotic response resulting in acceleration of apoptotic cell death (Rokudai *et al.* 2000)

PKB may also prevent apoptosis by upregulation of the levels or activities of anti-apoptotic factors. For example, a constitutively active PKB mutant increased the expression of the survival factor Bcl-2 in BAF/3 cells, resulting in inhibition of apoptosis induced by growth factor withdrawal (Ahmed *et al.* 1997, Reif *et al.* 1997). Hepatocyte growth factor and VEGF have been shown to increase Bcl-xL expression and cellular survival via a PI3K/PKB sensitive pathway in renal epithelial and vascular endothelial cells respectively again suggesting a possible role for PKB in enhancing the expression of anti-apoptotic proteins (Liu 1999, Tran *et al.*1999). PKB may also regulate T-lymphocyte and hemopoietic progenitor cell survival via increased Bcl-2 expression (Jones *et al.* 2000,Tang *et al.* 2000). The upregulation of Bcl-2 protein expression could occur via PKBs potential direct activation of transcription factors. For example, enhanced CREB activity induced by overexpressed PKB signalling leads to an increase Bcl-2 promoter activity and cell survival (Pugazhenthi *et al.* 2000)

Possible roles for PKB in inducing the expression of anti-apoptotic proteins are also seen with the evidence that PKB may enhance the transcriptional activity of NF-κB. This activation may be via direct phosphorylation and activation of IκKα by PKB, with the consequences of this being activation of NF-κB and cell survival. Evidence also suggests PKB could signal to NF-κB via a route distinct from that involving IκKα (reviewed in Mayo *et al.* 2000). For example, overexpressed PKB induces the expression of the IAP anti-apoptotic proteins; survivin and XIAP, via NF-κB activation in VEGF stimulated cells (Tran *et al.* 1999). Overexpressed PKB activates the transcriptional activity of NF-κB by stimulating the transactivation domain 1 of the p65 subunit of NF-κB (Madrid *et al.* 2000). However, evidence also suggests that the PKB and NF-κB survival routes lie on distinct and separate pathways. For example, IL-1 or TNFα activates PKB and prevents apoptosis without any activation of NF-κB (Madge *et al.* 2000).

Conversely, the activation of NF-κB and cell survival has been found to occur independently of PI3K/PKB activation possibly via the MEK/ERK or p38 MAPK cascades (reviewed in Downward 1998, reviewed in Mayo *et al.* 2000, Madrid *et al.* 2000, Baumann *et al.* 2000, RemacleBonnet *et al.* 2000).

Recently, data has emerged suggesting PKB may act at the mitochondrial level to protect cells from apoptosis. Use of a constitutively active PKB mutant in UV treated Rat1a cells prevents the mitochondrial membrane potential changes and cytochrome C release normally associated with caspase activation and apoptosis (Kennedy *et al.* 1999). The mechanism by which PKB could act on the mitochondria is unclear. However, it is likely to be evolutionarily conserved since overexpressed PKB exhibits similar anti-apoptotic effects in *Drosophilia*. Growth factor deprivation can induce apoptosis by decreasing the accessibility of the mitochondrial matrix to ADP which leads to hyper-polarisation of the inner mitochondrial membrane and matrix swelling. Overexpressed PKB can prevent initial swelling of mitochondria and the hyperpolarisation of the membrane so it possible that PKB helps to maintain mitochondrial accessibility to ADP thus preventing the initiation of apoptosis (Kennedy *et al.* 1997)

PKB has been proposed to be involved in protecting cells from another form of programmed cell death known as anoikis (Bachelder *et al.* 1999). Upon detachment from the extracellular matrix, epithelial cells enter into this form of apoptosis which usually functions as a safeguard to prevent detached cells from surviving in an inappropriate location. Expression of an active PKB mutant in MDCK cells rescues these cells from this cell death trigger, induced when cells were detached and placed in suspension (Khwaya *et al.* 1997). Inhibitors of PI3K also caused detachment and apoptosis of MDCK cells grown on plastic dishes (Tang *et al.* 1999). Therefore, a role for PI3K/PKB in preventing cells from undergoing anoikis and possibly in the regulation of cell anchorage is implied. The possibility of active PKB protecting detached cells from apoptosis also indicates one possible route it by which PKB could be involved in cell metastasis and function as an oncogene (Khwaya *et al.* 1997).

In summary, PKB may act by a variety of mechanisms to deliver an anti-apoptotic signal with the methods used depending on the cell type used and the apoptotic stimuli employed. It may be that PKB utilises more than one pathway to generate survival signals and the mechanisms used may be related to the expression of the various downstream effectors. It is also interesting to note that inhibition of directed or programmed cell death is one possible mechanism by which constitutively active or mutated PKB may act as an oncogene (Kennedy *et al.* 1997, Ahmed *et al.* 1997).

In some cells, PKB activity still correlates with the activation of potential anti-apoptotic downstream factors, but this event does not promote cell survival indicating that PKB and these downstream factors

may have non-survival roles in cells, perhaps in cell cycle regulation (reviewed in Coffer *et al.* 1998, reviewed in Kandel *et al.* 1999). It is clear that in many cells PKB may have a critical role to play in cell survival pathways. However, recent evidence suggests that in certain cell types, activation of PKB may not contribute to cell survival and therefore other possible mechanisms of promoting cell survival need to be investigated.

1.2.7.4 Possible Metabolic Roles

1.2.4.1 Glucose Uptake and Lipid/Glycogen Synthesis

PKB is proposed to be involved in many key metabolic processes including glucose transport, glycolysis and the synthesis glycogen, proteins and lipids. These potential roles for PKB could occur by the possible direct actions of PKB on key metabolic proteins such as GSK-3 or via presently unidentified routes. These and other possible roles have been studied in a variety of cell types mainly by the use of PKB mutants and as yet the direct targets for PKB in these process are unclear (reviewed in Coffer *et al.* 1998, reviewed in Kandel *et al.* 1999).

PKB has been proposed to be involved in glucose uptake in response to growth factors possibly via the expression and/or membrane translocation of the main glucose transporters GLUT1 and GLUT4 (Ueki *et al.* 1998). For example, in mouse 3T3-L1 adipocytes and rat adipocyte cells, activated PKB enhances glucose uptake and causes an increase in the translocation of the insulin responsive GLUT4 glucose transporter to the plasma membrane (Kohn *et al.* 1996, Cong *et al.* 1997, Tanti *et al.* 1997). In L6 muscle cells, activated PKB increases glucose uptake via the GLUT4 transportor route and stimulates glycogen synthesis via glycogen synthase activation. In these cells, a dominant negative mutant of PKB inhibits insulin-induced translocation of GLUT4 to the plasma membrane (Hajduch *et al.* 1998). Insulin, and, to a lesser extent, exercise increased GLUT4 translocation in human skeletal muscle via the PI3K/PKB pathway again suggesting a role for PKB in glucose uptake in many cells (Thorell *et al.* 1999).

In primary rat adipocytes stimulated with insulin, GLUT4 vesicles contained a PKBβ activity which could phosphorylate the vesicle components including GLUT4 (Kupriyanova *et al.* 1999). A similar possible isoform specific role for PKBβ is suggested in 3T3-L1 adipocytes, where insulin increased the association of PKBβ with GLUT4 containing vesicles and increased the translocation of this glucose

transporter to the plasma membrane (Calera *et al.* 1998). In 3T3-L1 adipocytes which may preferentially express the β isoform, a PKBβ directed increase in GLUT4 translocation and glucose uptake has been suggested to occur in response to insulin but not PDGF stimulation (Hill *et al.* 1999). Further experiments in 3T3-L1 adipocytes suggest, that insulin stimulated translocation of GLUT4 can occur via two routes, one involving the vesicle associated adapters, SNAP-23 and synaptobrevin-2/cellubrevin which are mobilised by constitutively active PKB and the other a PKB independent route which may involve recycling endosomes (Foran *et al.* 1999).

A constitutive active PKB mutant also increased the synthesis of the ubiquitously expressed GLUT1 transporter in 3T3-L1 adipocytes resulting in an increased glucose flux directed towards lipid synthesis (Kohn *et al.* 1996). A role for PKB in increasing GLUT1 expression in 3T3-L1 adipocytes undergoing a prolonged exposure to insulin has also been suggested (Barthel *et al.* 1999, Taha *et al.* 1999). Other experiments using a constitutively active PKB mutant in 3T3-L1 cells showed no role for GLUT1 transporters in glucose uptake mediated by PKB with GLUT4 being the sole transporter utilised by a constitutively active PKB pathway (Foran *et al.* 1999).

Based on overexpression studies, a role for PKB in glucose uptake via GLUT4 and to a lesser extent GLUT1 seems likely. It is therefore apparent that PKB may have a role in controlling glucose influx and metabolism in a variety of cell types (Kohn *et al.* 1996, Ueki *et al.* 1999, Wang *et al.* 1999). Recently, the roles of PKB in glucose transport have been questioned, with the findings that in rat adipocytes insulin increases GLUT4 translocation via PDK1 activation of PKCζ, without any significant involvement for PKB in this increased glucose uptake (Kotani *et al.* 1998, Bandyopadhyay *et al.* 1999, Grillo *et al.* 1999). A potential role for PKC isoforms in glucose uptake is also suggested since insulin can activate the PKCλ/ζ isoforms and stimulates their translocation to GLUT4 containing vesicles, thereby increasing glucose uptake (Standaert *et al.* 1997 & 1999). There is also conflicting evidence from PKB mutant studies concerning the roles of PKB in glucose uptake. The T308A/S473A and K179A inactive PKB mutants had little effect on insulin stimulated GLUT4 translocation, where as the K179A/T308A/S473A inactive mutant was found to inhibit this event by around 50%. Therefore, it is apparent that PDK1 may be a key branch point in glucose uptake with the PKB and/or PKC pathways possibly contributing to this uptake in response to different stimuli or in different cells.

The expression of a constitutively active PKB in 3T3-L1 adipocyte cells increased glucose uptake/flux and directed the increased cellular glucose towards lipid synthesis instead of glycogen synthesis which may be a reflection on functioning of GSK-3 in these cells (Kohn *et al.* 1996). Increased triacylglycerol synthesis in response to insulin in hepatocytes and adipocytes may also be dependent on the PI3K/PKB

and Erk pathways suggesting PKB's involvement in regulating lipid synthesis (Carlsen *et al.* 1999, Valverde *et al.* 1999). However, ML-9 a non-specific PKB inhibitor which also inhibits PKA and RSKs, did not prevent the PI3K sensitive antilipolytic effect of insulin in rat adipocytes suggesting PKB does not have roles in regulating lipid synthesis in these cells (Smith *et al.* 2000). A possible long-term role for PKB in regulating lipid synthesis is postulated because insulin stimulation of the fatty acid synthase promoter and hence fatty acid synthase expression can be mediated by the PI3K/PKB pathway (Wang *et al.* 1998, 1999 and 2000).

A potential role for PKB in stimulating glycogen synthesis, possibly via the inhibition of GSK-3 and enhancement of glycogen synthase activity is proposed in a variety of cells and in response to a many different growth factors. Use of constitutively active and dominant negative PKB mutants, suggest a role for PKB in enhancing glycogen synthesis in insulin stimulated L6 muscle cells (Ueki *et al.* 1998), insulin and EGF activated hepatocytes (Peak *et al.* 1998) and insulin stimulated rat adipocytes (Cross *et al.* 1995). The potential role of PKB and possibly other PDK1/PKB activated proteins in the regulation of GSK-3 and so glycogen synthesis is discussed in Chapter 6. It should also be noted that glycogen synthesis can also be activated via the growth factor induced activation of a protein phosphatase (PP1) which dephosphorylates the inhibitory GSK-3 targeted phosphorylation sites on glycogen synthase and so stimulates glycogen synthesis via this route (Lavoie *et al.* 1999, reviewed in Lawrence *et al.* 1997).

1.2.7.4.2 Protein Synthesis

In many cell types, including L6 muscle cell and 3T3-L1 adipocytes, the use of PKB mutants suggest that PKB may have a role in growth factor induced protein synthesis although the exact functioning of PKB is not known (Ueki *et al.* 1998). A constitutively active PKB mutant increased the cellular levels of the adipostatic hormone leptin, by acting at the level of translation in 3T3-L1 adipocytes (Barthel *et al.* 1997). A PI3K/PKB activated increase in the translation of the cell cycle regulator cyclin D is seen in colon carcinoma and breast cancer cells stimulated with serum again suggesting a general role for PKB in modulating protein synthesis (Kitamura *et al.* 1998, Muise-Helmericks *et al.* 1998).

Constitutively active PKB causes the phosphorylation of the 4E-Binding Protein (PHAS-I/4E-BP1) which leads to 4E-BP1 dissociation from the initiation factor 4E leading to mRNA translation (Ueki *et al.* 1998, Gingras *et al.* 1998, Takata *et al.* 1999). 4E-BP1 has been identified as an inhibitor of translation which acts by binding to the translation initiator 4E, preventing this factor from forming an initiation complex and thereby activating translation (Gingras *et al.* 1999). 4E-BP1 has been shown to

be phosphorylated on multiple sites in response to a variety of stimulators with these phosphorylation events causing 4E-BP1 to dissociate from and stop inhibiting, the eukaryotic initiation factor 4E resulting in initiation of translation (MotheSatney *et al.* 2000).

It is unclear whether there is any direct phosphorylation of 4E-BP1 by PKB since no PKB target motif has been found in 4EBP1 (reviewed in Vanhaesebroeck *et al.* 2000). However, one possible route for the inactivation of 4E-BPI by PKB is via mTOR, which overexpressed PKB can activate (possibly by direct phosphorylation) and which once activated can directly phosphorylate and inactivate 4E-BP1 leading to translation initiation (Scott *et al.* 1998, Brun *et al.* 1997, Nave *et al.* 1999). The phosphorylation of 4E-BP1 is also sensitive to wortmannin and rapamycin (an inhibitor of mTOR) suggesting the involvement of PI3K and mTOR and thereby raising the possibility that PKB is a key mediator in signalling between these pathways resulting in the inactivation of 4E-BP1 (Barash 1999).

Insulin can stimulate the PI3K/PKB/mTOR pathway resulting in inactivation of 4E-BP1 and stimulation of GLUT1 expression which suggests a possible physiological role for PKB in regulating translation via the mTOR/4EBP1 route (Taha *et al.* 1999, reviewed in Pullen *et al.* 1997). Evidence exists that PKB-independent inactivation of 4E-BP1 may occur in response to certain stimuli. For example, TPA or phenylephrine inactivate 4E-BP1 independently of PKB resulting in the stimulation of translation in human embryonic kidney and Rat1a fibroblasts respectively (Rybkin *et al.*2000, Herbert *et al* 2000). Therefore, it is apparent that the role PKB may play in initiation of translation via 4E-BP1 inactivation is a complex process involving other factors depending on the cell type or stimuli used.

Another possible role for PKB in the initiation of translation and possibly other downstream cellular events is its potential actions on p70S6K, a pathway which will be discussed in Chapter 5. *In vitro* PKB phosphorylates and activate p70 S6 kinase, a 70kDa ribosomal kinase which acts on the 40S (S6) ribosomal subunit to bring about initiation of protein translation (Burgering *et al.* 1995, Kuroda *et al.* 1998). There is some evidence, based on PKB overexpression studies, that direct phosphorylation and activation of p70S6K by PKB can occur *in vivo* in response to certain factors including heat shock and PDGF. Alternatively PKB could signal via mTOR to bring about p70S6K phosphorylation and activation. However, in other cells the role of PKB in p70S6K activation is unclear, since it is apparent that PDK1 can directly phosphorylate and activate p70S6K *in vivo* (Alessi *et al.* 1997, Pullen *et al.* 1998). In certain cell types it appears PKB may be necessary but not sufficient for p70S6K activation with this route perhaps contributing to the potential role of PKB in translation (reviewed in Proud *et al.* 1997, reviewed in Dufner *et al.* 1999).

The possible contribution of PKB in protein synthesis could occur via its phosphorylation and inhibition of GSK3, which when active can phosphorylate and inactivate the protein synthesis initiation factor eIF2. By inhibiting GSK3, PKB could indirectly bring about the activation of eIF2 and hence stimulate protein synthesis (Welsh *et al.* 1997). Therefore, studies suggest that PKB could be involved in regulating protein translation and therefore implicated in a large number of differing cellular processes (Burgering *et al.* 1995, reviewed in Kandel *et al.* 1999, reviewed in Vanhaesebroeck *et al.* 1999).

1.2.7.5 Other Possible Roles

Overexpression studies indicate PKB may have roles in cellular differentiation and proliferation. For example, transfection of a constitutively active PKB into 3T3-L1 fibroblasts causes them to spontaneously differentiate into the adipocyte form (Kohn *et al.* 1996, Gagnon *et al.* 1997). Induction of PKBβ activity correlates with the differentiation of Sol8 muscle myoblasts into myotubes (Calera *et al.* 1998). Possible roles for PKB in cellular differentiation are discussed in Chapter 5. PKB may also act as a key cycle regulator in certain cells For example, PKB activity is induced by cell cycle withdrawal and myogenesis with this activity possibly contributing to differentiation of these cells and perhaps conferring apoptosis resistance (Fujio *et al.* 1999). PKB may also affect cell proliferation in a variety of cell lines which may be one possible mechanism by which PKB can function in cancer cells to induce not only survival but also proliferation or transformation of these cells (reviewed in Kandel *et al.* 1999, Datta *et al.* 1999).

Studies suggest PKB may act on E2F, a cell cycle regulator which is involved in proliferation and c-myc expression (Brennan *et al.* 1997/1999). Activated PKB increases the expression of cyclin D, another cell cycle regulator at the level of mRNA translation and may increase cyclin D stability further indicating possible roles for PKB in the cell cycle (Muise-Helmericks *et al.* 1998, Diehl *et al.* 1998). PKB directed upregulation of c-myc or down regulation of the cyclin kinase inhibitor p27 may also be possible ways in which PKB could act to regulate the cell cycle (Ahmed *et al.* 1997, Sun *et al.* 1999, Busse *et al.* 2000). The negative PTEN regulator can induce cell cycle arrest and direct cells towards apoptosis in a mechanism which could involve inhibition of the possible functioning of PKB as a cell cycle regulator (Wang *et al.* 1999, Lu *et al.* 1999). Possible mechanisms by which PTEN could induce growth arrest act via the inhibition of PKB, include inactivation of retinoblastoma (Rb) or p27 (a CDK inhibitor) activation (Paramio *et al.* 1999, Lu *et al.* 1999)

Roles for PKB in negatively and positively regulating transcription are also suggested by its possible actions on the transcription factors FKHR and CREB. As well as these potential roles for PKB in affecting transcription, overexpressed PKB has been shown to regulate the gene expression of IGFBP-1 and IGFBP-5 by affecting promoter activity (Cichy *et al.* 1998, Duan *et al.* 1999). PKB may also increase the gene expression of the atrial natriuretic factor or ANF1, a β-adrenergic receptor agonist possibily via the inhibition of GSK-3 (Morisco *et al.* 2000).

Other possible cellular functions for PKB include regulating the proliferation, migration and actin organisation of endothelial cells in response to VEGF, perhaps mediated via PKB activation of eNOS (MoralesRuiz *et al.* 2000). PKB may also function in T-cell and neutrophil activation, migration, motility and the respiratory burst possibly in response to activation by PI3Kγ (Sasaki *et al.* 2000, Hirsch *et al.* 2000). Recently, PKB has also been proposed to inhibit Rac1-GTP binding by directly phosphorylating Rac1 on serine 71 which lies within a PKB target motif, however as yet the physiological consequence of this has not been established (Kwon *et al.* 2000).

Evidence indicates that PKB may have many diverse and important cellular roles. However, these potential roles need clarification due to the problems associated with the use of PKB mutants in assigning the functioning of PKB. Therefore direct and specific inhibitors of PKB function are urgently required to establish the precise cellular roles of PKB. The use of antisense inhibitors to directly target the individual isoforms of PKB therefore provides an attractive possibility. With these specific inhibitors it should be possible to directly affect the endogenous levels of PKB and hence ascertain its cellular functioning in physiological conditions.

1.5 Introduction to Antisense Theory

1.51 Methods of Altering Protein Function

In order to establish the roles a particular protein (i.e. PKB) has in a signalling pathway, it is important to find a way of altering the function of this particular protein and seeing how this change affects its role in the pathway. There are a number of methods such an alteration can be achieved by, either up regulating or down regulating the activity or levels of the protein under study.

Many of the techniques employed to bring about these changes, involve the use of over-expression systems in which the activity of an endogenous protein is altered by transfecting in foreign DNA encoding for a form of the test protein. This DNA can encode for either wild type protein, constitutively active protein or dominant negative protein. The effects of these mutants on the pathway under test can then be studied in the hopes of clarifying the roles of the targeted protein. Whilst these techniques have yielded a great deal of information as to the likely role of many proteins including PKB in cell signalling, the obtained data should still be viewed with some caution. The main drawback with such a system is that any changes to the function of the protein within the cell, is a direct result of the added non-endogenous protein mutants and so does not involve the native cellular level or activity of the protein. This could mean that obtained results do not relate to the cells normal function or to physiological conditions.

The other way to analyse the roles of the protein in question, is to alter the cellular levels or activity of the protein directly, in other words to target the endogenous protein. Such alterations can be attempted at either the DNA, mRNA or protein level to bring about the desired changes.

Targeting at the protein level involves the use of chemical inhibitors, which interact with the test protein and prevent its action. They usually either prevent the activation of the protein by targeting its activation sites, or prevent its downstream effects by interfering with the active site preventing the binding or regulation of its substrates. Whilst such chemical inhibitors have proved to be very effective in the study of cell signalling, with examples including wortmannin (PI-3' Kinase), PD98059 (MEK1) and rapamycin (mTOR/p70 S6 Kinase), there are very few in circulation (reviewed in Toker *et al.* 2000). This is because they are difficult to design and often do not give complete inhibition of their target protein.

Another technique is to target the DNA of the test protein and prevent transcription and hence ultimately stop protein production. One such method involves binding a drug to the DNA, thus preventing transcription factor binding and hence inhibiting transcription initiation. However, as with chemicals designed to work at the protein level, such compounds have proved difficult to design and often act non-specifically. Alternatively, a specifically designed synthetic oligonucleotide can be used to hybridise to a particular region of the target DNA forming a DNA triplex, which prevents DNA unwinding and hence inhibits transcription. The main drawbacks with the triplex method is that there are difficulties in getting the oligonucleotide into the nucleus of the cells, difficulties in producing a strongly hybridising probe and the optimum conditions (low pH and temperature) are non-physiological.

The final method of altering endogenous protein function is by targeting at the level of translation, either by binding the mRNA with a drug, or using complementary synthetic oligonucleotides, known as antisense probes. As with other methods, knocking out translation of a single protein by binding a drug to the mRNA has proved to be very difficult, with problems of specificity being the main stumbling block. In antisense techniques, the designed oligonucleotide hybridises to a complementary region of the mRNA and prevents translation of the mRNA, either by preventing ribosome binding or causing digestion of the mRNA. Antisense has proved to be a very successful technique in the study of many cellular proteins, as it is possible to eliminate most potential pitfalls. It is also the method this group has previously used, to study signalling proteins such as MAP Kinase (Sale *et al.* 1995) and protein tyrosine phosphatase α (Arnott *et al.* 1999). Antisense techniques are therefore, my main strategy for studying the possible cellular roles of PKB.

1.52 Overview of Antisense

Antisense is an emerging method for altering protein levels and hence affecting the activity and therefore roles, a particular protein has in the cell. It has already proved to be a very useful tool in cell biology with successful antisense based strategies already directed against PKC and the EGF and PDGFreceptors and used to identify their particular functions in cell signalling (Chaikofi *et al.* 1995, Liu *et al.* 1992, reviewed in Stein *et al.* 1993). Hence it is becoming an ever more popular tool for the cell biologist, due to the fact that it is directed against endogenous protein levels and can be used to target specific isoforms of a particular protein (reviewed in Rou 1997).

The use of antisense as a possible therapeutic agent has also been investigated due to its potential to act as a so-called "magic bullet" drug, directed specifically against an aberrantly functioning protein in a disease state. Although the initial hopes for antisense as a new "wonder drug" or miracle cure has yet to be fully achieved. However, the technique is starting to show promise in a pharmacological role with the first antisense drug; Vitrulene™, which targets a virus causing Cytomegaloviral retinitus, recently being granted a medical license by the US government (reviewed in Stein 1999). Therefore, there may be a bright future for antisense drugs in combating many diseases, especially in targeting aberrantly functioning proteins in a variety of cancers (reviewed in Stein *et al.* 1993, 1997, 1999).

The first use of antisense as any sort of cellular tool was in 1978, when Zanernik and Stephenson successfully directed antisense oligonucleotides against the Rous Sarcoma Virus (reviewed in Coleman 1990). Since this breakthrough for antisense techniques, oligonucleotide probes have been used to target

many signalling proteins and hence elucidate some of their roles. Within this group, successful oligonucleotide antisense strategies have been used to deplete Erk1, Erk2 and PTPα and investigate their cellular roles (Sale *et al.* 1995, Arnott *et al.* 1999).

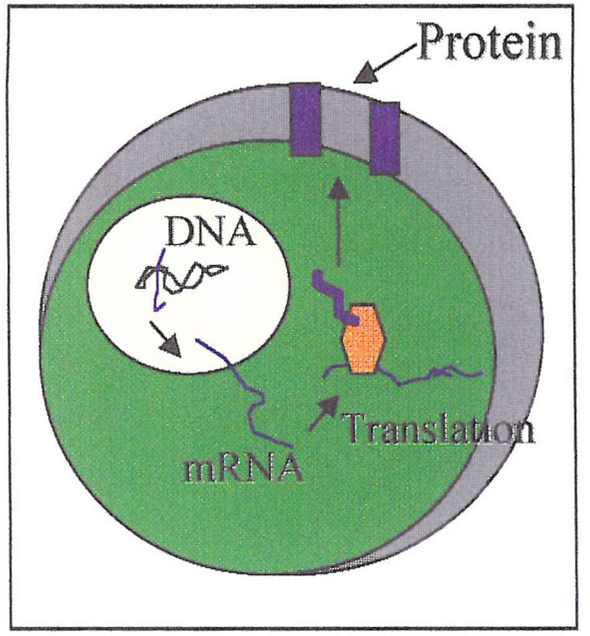
However, despite the fairly extensive uses of antisense oligonucleotides, the exact mechanism by which they prevent protein expression by translation is unclear. It is known that in antisense strategies, the designed synthetic oligonucleotide is complementary to a particular mRNA sequence of the protein of interest and the binding of the antisense probe to this region brings about the inhibition of translation (see figure 1.5).

There are currently two main models for the action of antisense probes once they hybridise to the mRNA. In the first model, the antisense-mRNA double stranded complex acts to prevent ribosome binding or prevent ribosome progression along the mRNA hence stalling translation. It is believed that in this model the ribosome cannot bind or move along the mRNA due to steric reasons or the masking of important binding sites in the mRNA (Dean et al. 1994/1996, Feier 1992, Helene and Toulme 1990)

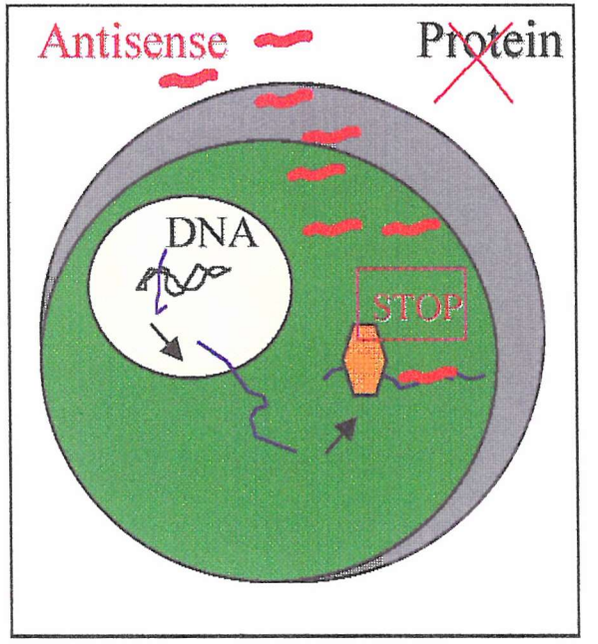
The second model involves the double stranded antisense-mRNA duplex stimulating the activity of the cellular nuclease, ribonuclease-H, which digests the mRNA, thus preventing translation. The antisense-mRNA complex may act as a template for this nuclease or act to stimulate its activity, with the result of both being the removal of the mRNA of the protein of interest and hence no translation. The second model is probably the most likely or most common method by which antisense oligonucleotides act to bring about the inhibition of translation. However, there is evidence that both methods may be used in parallel or occur in a cell-specific manner (Altmann *et al.* 1996, Hunter *et al.* 1995, Wagner *et al.* 1993). Other possible methods of antisense function also exist but are not well characterised (reviewed in Hunter *et al.* 1999)

After the group's previous success in using an antisense approach to target signalling proteins, it was decided to design similar probes against the PKB isoforms and use these to elucidate the potential roles of PKB in cellular function (see Chapter 3).

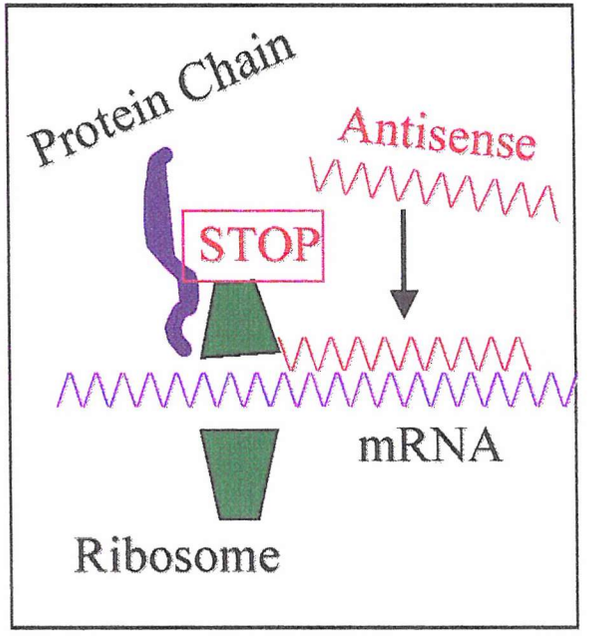
Figure 1.4 - Antisense Theory



NORMAL FLOW OF PROTEIN SYNTHESIS



ANTISENSE OLIGO BLOCKS EXPRESSION OF SINGLE PROTEIN



OLIGO HYBRIDIES TO TARGET mRNA BLOCKING PRODUCTION OF PROTEIN

CHAPTER 2 - MATERIALS AND METHODS

2.1 Materials

Glycerol, methanol, and TFA were obtained from Fisons Ltd, Loughborough England. Acetic acid, ammonium hydroxide (15M), SDS, BSA (for western blots) and AnalaR water were purchased from BDH Ltd, Poole, England. The 30% acrylamide solution was from National Diagnostics, Hull, England and the PBS tablets from OXOID, Unipath Ltd, Basingstoke, England.

The following chemicals were obtained from the Sigma Company Ltd, Poole, England. Materials for SDS-PAGE including acrylamide, N,N'-methylene-bis-acrylamide, Coomassie brilliant blue, pyronin Y, TEMED, ammonium persulphate, DTT, glycine, and molecular weight markers, Napthol blue black, Tween-20 and Kodak photographic solutions (developer, stopper, fixer) for western blotting protocols. Other materials purchased from Sigma were benzamidine, aprotinin, pepstatin A, leupeptin, antipain, BSA (for Protein standards and cell culture), β-glyceryophosphate, deoxycolic acid, nonidet P-40, sodium orthovanadate, PMSF, TPA, sodium fluoride, ATP, Triton X-100, HEPES and horse radish peroxidase (HRPO) conjugated donkey anti-sheep secondary antibody.

HRPO conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were purchased from Sera-lab, Crawley, England. The hyperfilm-MP and ECL reagent was from Amersham International plc, Buckingham, England. Pro-tran Nitrocellulose (0.2μm) was obtained from Schleicher and Schuell, Dassel, Germany and Immobolin-P PVDF from Millipore, Bedford, USA

For the synthesis of the GSK-3 kinase assay peptide substrates chemicals were obtained from a variety of sometimes specialist sources. The NH2-Wang resin was purchased from, Novabiochem, Notts; as were all the other N-α-Fmoc amino acids used. The coupling agents DIPEA, HOBt and Tbtu were also purchased from this supplier. The chemicals acetic acid, acetonitrile, DCA, toluene, DMF (which was maintained dry over size 4A molecular sieves and dried), TFA and DCM (maintained over anhydrous sodium carbonate and filtered using Whatman No.1 filter paper) were all obtained from Lancaster, Morecambe. Piperidine, ninhydrin, anisole, enthandithiol, dichlorodimethylsilane were all supplied by Simga-Aldrich. The pre-packed G-25 column (PD10) was from Pharmacia, Bucks.

For the synthesis of the GSK-3 phosphopeptide additional chemicals had to be obtained or synthesised. These included triethylamine, sodium hydrogen carbonate, sodium sulphate *meta*-perchlorobenzoic acid and tetrazole were obtained from Sigma-Aldrich. *di-tert* Butyl pyrocarbonate was purchased from Novabiochem. The N,N-diethylphoramidous chloride was kindly provided by Dr. Bob Broadbridge, who also helped greatly in the conversion of this into the required *bis-tert*-butyl N,N-phosphoramidite and general with all the peptide synthesis work.

For the peptide synthesis work all the reagents used were at least analytical grade, or HPLC grade in certain stated cases.

All the solvents used for HPLC were vacuum degassed and filtered through 0.22μM filters at 22°C prior to use. The HPLC model used in the analysis and purification of the peptides was the Gilson 805 Manometric model including 305/306 25ml pumps and an 811B dynamic mixer, Gilson 712/715 HPLC software, Vydac analytical and preparatory columns and an Applied Biosystems 759A absorbance detector. Mass Spectroscopy analysis of the peptides was performed with the help of Dr Broadbridge using a PerSpective Biosystems electron spray mass spectrometer.

Generally all chemicals and reagents were purchased from Sigma, BDH or Fisons unless otherwise stated. All used chemicals were of a high grade and were always used in accordance with the manufactures/suppliers instructions and general safety recommendations

2.2 Cell Culture.

2.2.1 Materials for cell culture

3T3-L1 fibroblasts were kindly provided by Dr. G. Gould (University of Glasgow, Scotland), Dulbeccos Modified Eagle Medium (DMEM $1gl^{-1}$ glucose with sodium pyruvate), BSA (99% pure), dexamethazone, 1-methyl-3-isobutylxanthine (IBMX) and dimethyl sulfoxide (DMSO) were obtained from Sigma. Glutamine, fungizone (amphotericin B), gentamycin, and trypsin-EDTA solution were purchased from GIBCO-BRL. Insulin (porcine) was from Calbiochem. Sterile multi-well plates, centrifuge tubes, flasks and sterile scrapers were from Bibby Sterilin and Helena Biosciences. Cryo-tubes were from Nunc and sterile filters from Sartorius.

Before being used insulin was made up as a 100μM stock in sterile 3mM HCl and IBMX was dissolved in sterile 0.35M KOH to give a 250mM solution. On arrival dexamethazone (1mg) was dissolved in 1ml ethanol and then diluted to a concentration of 50μM with DMSO prior to storage at -20°C

2.2.2 General Cell Culture Conditions.

All cell culture procedures were carried out under sterile conditions. Cell culture media was sterilised by filtering (0.22μM) prior to incubation with the cells, with any non-sterile solutions autoclaved (121°C, 15lbsin-2, 25mins) prior to use with cells. All work with cells was carried out in a class II cell culture cabinet to maintain a sterile environment.

3T3-L1 fibroblast cells were grown at 37°C / 5% CO_2 as monolayers in $75cm^2$ cell culture flasks in Dulbeccos Modified Eagle Media (DMEM), 10%(v/v) Foetal Bovine Serum (FBS), 2mM glutamine, $0.2mgml^{-1}$ gentamycin and $2.5mgml^{-1}$ fungizone. In each flask 20ml of this media was used and this was replaced every 48 hours. Once the cells had reached 80-90% confluencey they were either prepared for cryo-storage in liquid nitrogen or subcultured into $2.8cm^2$ multiwell plates (usually 12 well plates).

2.2.3 Cell Cryostorage and Resuspension.

When 80-90% confluent the cells were removed from the flask surface by first removing all the traces of media and then treating the cells with 5ml trypsin-EDTA (0.05%($^{w}/_{v}$) trypsin, 0.02%($^{w}/_{v}$) EDTA in modified PUCK solution) at 37°C / 5%CO_2 for 5 minutes. The resulting cell suspension was then placed in a sterile centrifuge tube and an equal volume of FBS added to neutralise the trypsin. The cells were then collected by centrifugation, (5 minutes / 1200rpm) and the supernatant removed. The cellular pellet remaining was then resuspending in cryoprotectant media (10% Sterile DMSO, 25% FBS in DMEM) using 3ml of this media for each 75cm^2 flask. 1ml aliquots of this suspension were then placed into each sterile cryo-tube and these were then slowly frozen overnight at -70°C before being placed in the cryogenic liquid nitrogen store.

In order to resuscitate these cells the cryoprotectant media containing the cells were first defrosted at 37°C and the cells collected by centrifugation (5min / 1200 rpm). The supernatant was then removed and the remaining cellular pellet resuspended in pre-warmed growth media and transferred to a suitable cell culture flask (usually 75cm^2 flask).

2.2.4 Subculturing of cells

When the cells in the 75cm^2 flask reach 80-90% confluent the growth media is discarded and 5ml of Trypsin-EDTA solution is added to release the monolayer of cells from the flasks surface. After this solution had been in the flask for 1 minute at room temperature 80% (4ml) of the solution was discarded and cells incubated for 4 minutes at 37°C to detach all the cells. Once the cells were fully detached 20ml of pre-warmed growth media was added to the flask to neutralise the trypsin and resuspend the cells. The cell suspension could then be divided into multiwell plates and if necessary new 75cm^2 flasks. The standard procedure for this was to place 2ml of the cell suspension into a new flask and add 18ml of pre-warmed media to this. For culturing in multiwell plates (usually 3.8cm^2 wells) the most common protocol was to add 0.5ml of the cell suspension and 0.5ml of our standard growth media to each well.

2.2.5 Differentiation of 3T3-L1 Fibroblasts into Adipocytes

For some of the experiments it was required to use 3T3-L1 adipocytes which could be obtained by differentiating our fibroblasts using a cocktail of factors. The standard protocol for this requires a specific combination of factors over an eight day time period as first detailed by Frost and Lane (Frost and Lane 1985). Firstly the fibroblasts were grown to confluence in 3.8cm^3 wells. Then at two days post confluence (day 0) the differentiation protocol was commenced. Firstly the standard media is removed from the cells and replaced with 1ml of standard media containing 175nM insulin, 0.5mM 1-methyl-3-isobutylxanthine and 0.25μM dexamethazone.

This media is then replaced 48 hours later (day2) with 1ml of our standard growth media containing 175nM insulin. This media is then removed after another 48 hours (day 4) and replaced with 1ml of standard growth media which is subsequently changed every 48 hours. The cells were then suitable to use as adipocytes 8-20 days post differentiation providing they were at least 70% differentiated as determined by lipid accumulation in the cells which could be observed by microscopy (Frost and Lane 1985).

2.3 Use of Cells

Once close to confluencey in the fibroblasts or fully differentiated (8 days post differentiation start) the cells were used in a variety of experiments including growth factor stimulation and antisense work via transfection.

2.3.1 Transfection of Cells with Oligodeoxynucleotides

Transfection techniques were used to introduce foreign DNA into the cells. In this case the DNA introduced was single stranded synthetic oligodeoxynucleotides designed to be used in antisense studies (see Chapter 3 for design theory/protocol). Before these oligonucleotides could be introduced into the cells they were first cleaved, purified and their concentration determined (see section 2.5). In my experiments the transfection techniques used were designed to transfect known concentrations of my probes into cells where I could observe and analyse any elicited effects.

In my antisense studies I initially used a liposome formulation of 1:1(w/v) cationic lipid, N-[1(2,3-dioleyloxy)proyl] n,n,n-trimetylammonium chloride {DOTMA} and dioceoylphophotidylethanolamine {DOPE}, known commercially as lipofectin™, to introduce the oligonucleotide into the cells (see section 3.1) using a well documented protocol. Firstly, the lipofectinTM and DMEM (supplemented with glutamine) are mixed together to give a 10% lipofectinTM solution (in a volume of 100µl) which was then left for 45 minutes at room temperature. The volume required to give the necessary concentration of the oligonucleotide (in a final volume of 400µl) was then diluted to 100µl with DMEM. The lipofectin™ and the oligonucleotide containing solutions were then mixed giving a volume of 200µl and left to stand for 20 minutes at room temperature.

The cells to be transfected are the removed from the incubator and washed 3 times with pre-warmed DMEM to remove all traces of serum and antibiotics. Following this 200µl of pre-warmed DMEM was added to each well along with the 200µl of the lipofectin™-DNA solution. The plate was then returned to the incubator and incubated at 37°C for 8 hours. After this period the lipofectin™ containing media was removed from the cells and replaced with 400µl of fresh media containing the same oligonucleotide concentration as before. The media used in this replacement was either supplemented with 5% heat inactivated FBS for the fibroblasts or 0.25% BSA for the adipocytes. The length of transfection varied depending on the experimental design but in all cases the media was changed every 48 hours and fresh oligonucleotide containing media (appropriately supplemented) was added.

2.3.2 Stimulation of Cells

The effects of a variety of stimulatory factors i.e. insulin, vanadate and various growth factors on our cells was also investigated both in conjunction with and separately from the antisense work. The exact protocol for these stimulations depended on the cell type and factor used and what had been previously done to the cells.

For non-transfected cells a period of serum starvation was required prior to the stimulation due to the fact that serum can act as a stimulatory factor in these cell lines. The length of serum starvation depended on the cell type used, for the 3T3-L1 fibroblasts it was between 20 and 24 hours whereas in the adipocytes cell line this serum starvation period was around 48 hours.

For these serum starvations the serum containing media was removed and the cells washed 3 times with DMEM (supplemented with glutamine). The cells were then incubated in 1ml of this serum free media supplemented with 0.25% BSA for the required time.

For cells which have already been transfected the serum starvation protocol is slightly different. With the adipocytes no additional serum starvation period is required since the cells are in serum free media during the transfection. However, prior to the stimulation the cells were washed twice in DMEM to remove any traces of oligonucleotide. With the fibroblasts the normal 5% FBS transfection media was removed 24 hours before the transfection endpoint/stimulation start and the cells washed twice with DMEM to remove any traces of serum. Fresh oligonucleotide was then added to the cells for the final 24 hours in 0.25%BSA / DMEM media to provide a serum free environment prior to the stimulation.

After the desired starvation time the cells were ready to be stimulated with the chosen factor. This involved removal of the media from the wells and replacement with 400µl of 0.25% BSA/DMEM containing the chosen factor at its required concentration and incubated for the required time point.

Once the cells had been incubated for the desired length of time they were removed from the incubator and placed on ice. The media was then removed from each well and the cells washed 4 times with ice cold phosphate buffered saline (PBS) before extraction (see section 2.4). For very short time points when the plate was removed from the incubator and the media poured off the cells were then quickly washed once with ice cold PBS to remove any residual BSA before the plate was quick frozen in liquid nitrogen.

2.4 Harvesting of Cells.

The extraction or harvesting of our cells followed a basically similar protocol with a few adaptations made to the extraction buffers used depending on what the harvested cells were required for. With all extraction's it was important that the harvesting be performed as quickly as possible with the cells kept cold throughout as this reduces loss of activity or cellular degradation.

2.4.1 Whole Cell Lysates

For cell extracts which were only used for protein visualisation or determination by western blotting, a whole cell lysis extraction was used. In this technique the PBS washed cells were scraped from the well into 50-100µl of SDS-lysis buffer (63.5mM Tris [pH7.4], 1% ($^{w}/_{v}$) SDS). This extract was then homogenised by passing the sample through a Hamilton syringe 10 times which resulted in a shearing of the cells' DNA thus reducing the viscosity of the extract.

The homogenised samples were then pulse centrifuged to settle the contents of the extraction vessel. The next step was to remove 10µl of the supernatant which was retained for protein concentration (see section 2.10). The remaining supernatant was then transferred to a new eppendorf and mixed with a one-quarter volume of 5x concentrated laemmli sample buffer. These samples were then boiled for 5 minutes at 100°C and stored at -70°C until required for western blotting.

2.4.2 Cell Lysates

For cell extracts which were required for other procedures, for example kinase assay the extraction protocol was a little more complex. The PBS washed cells were extracted by scraping as before however the lysis buffer used was somewhat different as it was always fresh made up and contained a cocktail of factors to prevent protein degradation and inhibit certain cellular proteins (i.e. other kinases or phosphatases).

In most cases the buffer consisted of 50mM β-glyceryophosphate (pH 7.5), 1mM benzamidine, 1mM DTT, 1mM sodium orthovanadate, 1mM PMSF, 2mM EDTA, 10mM sodium fluoride, 1.5mM EGTA, 1µgml-1 pepstatin, 1µgml-1 antipain, 1µgml-1 leupeptin, 20µM PKC inhibitor peptide (Calbiochem), 2µM PKA inhibitor peptide (Sigma), 20µM R24571 (calmodulin dependent protein kinase II inhibitor compound - Calbiochem), 1µM microcystin (Calbiochem). This cocktail of factors was made up in RIPA buffer solution. [50mM Tris (pH 7.5), 150mM sodium chloride, 0.25% ($^{v}/_{v}$) sodium deoxycholate, 1% ($^{v}/_{v}$) NP40] and unless stated all the chemicals used in this buffer were obtained from Sigma.

In these extractions the cells were extracted into 50-100µl of lysis buffer (depending on well size/ cell type) and then left to stand for 10 minutes on ice. The extracts were then homogenised 10 times using a 500µl Hamilton syringe and centrifuged at 10000g for 5 minutes in a pre-cooled centrifuge. A 10µl aliquot of the supernatant was then removed for protein concentration and a known volume of supernatant was also removed and processed for use in SDS-PAGE as described for whole cell lysates. The remaining supernatant was either rapid frozen with liquid nitrogen and stored at -70°C until required or more usually immediately used (or after protein standardisation) in the required assay (see section 2.9)

2.5 Preparation of Oligonucleotides

Possible antisense oligonucleotides and their matched controls were synthesised as phosphorothioate (deoxy)-oligonucleotides (ODNs) using an automated DNA synthesiser (Applied Biosystems 391). This was done using standard phosphorothioate chemistry with the nucleotides synthesised in a 3'-5' direction on a 10µM scale. This synthesis was initially done by Dr Mark Pickett at the department of Microbiology, Southampton General Hospital, but later in house synthesis was done by Dr Elizabeth Sale.

2.5.1 Cleavage and Deprotection of Oligonucleotides

Once synthesised these oligonucleotides had to be cleaved from their solid support and purified before they were suitable for use on the cells. To cleave the ODNs 1.5ml of 15M ammonia was passed up and down the synthesis column slowly for a period of 2 hours at room temperature. This ODN containing solution was then diluted to 3ml with 15M ammonia and sealed in a sterile vial which was then incubated at 55°C for around 18 hours to remove the ODNs protecting groups. After this time period the solution was cooled and the amount of ODN present determined by measuring the optical density at 260nM (OD260). The crude 5'-OH DMT ODN was then aliquoted (20OD units), dried down under vacuum (Univap) and stored at -20°C until required for further purification.

2.5.2 Purification of Oligonucleotides

In order to purify the crude 5'OH-DMT ODNs so they were suitable to use in transfections we employed the OPC cartridge system (applied biosystems). This purification method required the presence of the 5'-OH trityl group for the binding of the ODN to the solid matrix of the OPC cartridge. In order to prepare the OPC cartridge for use it was first washed with 5ml HPLC grade acetonitrile followed by 5ml of 2M triethylamine acetate (HPLC grade; applied biosystems). The crude oligonucleotide pellet was then resuspended in 1ml of 3.75M NH_4OH and slowly passed through the column (1-2 drops per second). This oligonucleotide solution was reapplied 5-6 times to the column to make sure the ODN is fully loaded.

The column was then slowly washed with two 5ml measures of 1.5M NH_4OH to remove any impurities and then the cartridge was flushed with two 5ml water washes. Following this the acid labile 5'OH-DMT group was removed by treatment with TFA (3% (%v) TFA/water 5ml). In this step 1ml of the TFA solution was passed through the column and left to stand for ten minutes before the further 4ml of TFA was passed through. Once this deprotection step was completed the column was washed with two 5ml water washes to remove all traces of TFA. The purified ODN was then eluted in 35% (%v) acetonitrile (1.8ml) and aliquoted (6 x 300µl) prior to being removal of the acetonitrile under vacuum (Univap). The dried down ODN was then stored at -20°C until required for rehydration.

The rehydration procedure involved dissolving up the dried down oligonucleotides in a total volume of 300µl (2 x 150µl) autoclaved and sterile filtered water and the concentration determined spectrophotometrically (A260). The purified ODN was then aliquoted into 20µl portions and stored at -20°C until required for transfections.

2.6 - Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.6.1 Solutions Required for Preparation of gels

SDS-PAGE was performed based on the method described by Laemmli et al. (1970). For SDS-PAGE techniques two different acrylamide solutions were used in gel preparation depending on the protein investigated and the resolution required. The first solution used for standard visualisation gels (slab gels) was a concentrated acrylamide solution (30% ($^{w}/_{v}$) Acrylamide / 0.8%($^{w}/_{v}$) N,N`-methylene-bis-acrylamide; National Diagnostics). The second solution which was required for band-shift gels had a lower bis-acrylamide concentration and was made up from solids (30% ($^{w}/_{v}$) acrylamide / 0.36% N,N'-bis-acrylamide). A two times concentrated resolving gel buffer (0.75M Tris/HCl (pH 8.8), 0.2% SDS) and stacking gel buffer (0.25M Tris/HCl (pH6.8), 0.2% SDS) was prepared in advance for use in gel preparation. The acrylamide solutions were diluted according to the % gel required which varied for the resolving gel but was always 4% for the stacking gel.

2.6.2 SDS-PAGE set up

Gels were 5cm x 8cm in size for normal visualisation gels (mini-gel, Biorad), however for the band-shift gels a longer resolving gel was required (8cm x 8cm; mighty-small, Hoeffer). The polymerisation of the bis-acrylamide and acrylamide was catalysed by the addition of 0.1% ($^{w}/_{v}$) ammonium persulphate and 0.2% ($^{v}/_{v}$) TEMED. The resolving gel was always poured first followed by an overlay of water to ensure the top of the gel was level. Once the resolving gel had set this water was removed and the stacking gel poured.

The gels were then positioned in the electrophoresis tank and the tank was filled with running buffer (0.5M Tris-HCl, 1M glycine, 0.2% SDS). Protein samples were prepared by boiling the cell extracts with one-quarter volume of 5x concentrated laemmli sample buffer (200mM Tris-HCl, 0.2%SDS, 2mg pyronin-Y) (100°C, 5mins). Samples were then loaded and electrophoresis performed at a constant current of 30mAmps (5cm x 8cm gel) or 18mAmps (8cm x 8cm) with water-cooling.

After electrophoresis gels were removed and either prepared for western blotting (see section 2.7) or stained (0.25%($^{w}/_{v}$) coomassie brilliant blue, 50%($^{v}/_{v}$) TCA) and destained (45%($^{v}/_{v}$) methanol, 5%($^{v}/_{v}$) acetic acid) to visualise the loaded protein.

2.7 Immunoblotting

2.7.1 Commercial Antibodies

The polyclonal sheep anti-PKBα antibody, polyclonal sheep anti-PKBβ, polyclonal rabbit anti-p70 S6 kinase antibody and the polyclonal rabbit anti-SHP-2 antibody were purchased from Upstate Biotechnology Incorporated (UBI); monoclonal mouse anti-ERK1/ERK2 antibodies (IgG clone No.Z033 and subsequently clone ERK7D8) were obtained from Zymed Laboratories Ltd and polyclonal rabbit anti-GSK3α/GSKβ antibodies were a kind gift from Dr. J. Woodgett (University of Toronto).

2.7.2 Western Blotting Protocol

Immunoblotting was performed based on standard protocols. Cell extracts were separated for blotting by SDS-PAGE (see 2.6) using different % acrylamide gels depending on the protein we would be blotting for. Membranes used were usually nitrocellulose (0.2uM; Pro-tran, Schleicher & Schuell), although sometimes PVDF (0.2μM; Immobilon-P, Millipore) were used. These PVDF membranes required pre-wetting with methanol prior to use, whereas the nitrocellulose was pre-wetted with water. Proteins were transferred from the gels to the membranes by means of a "transfer sandwich": The gel and the chosen membrane were pre-equilibrated along with pieces of 3MM paper (Whatman) in transfer buffer (25mM Tris, 192mM glycine and 20% methanol, pH8.3). The gel and membrane were placed together with 3 pieces of 3MM paper either side to form the transfer sandwich which was then placed in the transfer apparatus. Proteins were then transferred by semi-dry transfer using a Hoeffer Semi-PhorTM transfer apparatus (72mAmps, 120mins). After transfer, membranes were washed and blocked in appropriate buffers in accordance with the antibodies suppliers' protocol (see section 2.73). The transferred gel was then stained in the Coomassie Brilliant Blue solution and destained (see section 2.72) to determine whether uniform transfer had been achieved.

After blocking the membrane was incubated with the appropriate antibodies (see section 2.73.1) with constant agitation throughout the individual protocols to ensure the membrane remained wet. Subsequently the horseradish peroxidise (HRPO) conjugated secondary antibodies were visualised (in all cases) using an "enhanced chemiluminescence" method (ECL, Amersham). The membrane was incubated with the ECL reagents (2min, room temp) and then placed on a clean glass plate and wrapped in cling film. The membrane was then placed in an autoradiography cassette (Genetic Research Instruments Ltd) fitted with an intensifying screen (Dupont cronex lighting plus), and the light signal emitted recorded on photographic film (Hyperfilm-MP, Amersham) which was chemically developed and the image fixed.

In some cases the membrane filters were washed and stripped of antibodies so they could be re-probed with a different antibody. In this protocol the membrane was first washed twice with TBS-Tween (20mM Tris/HCl (pH7.4), 137mM NaCl, 0.05% Tween 20; 10ml, 5min) and then soaked in the stripping buffer (0.5M NaCl, 0.2M glycine, pH2.8; 20ml, 20min). The membrane was subsequently washed twice in 1M Tris base (pH7.4, 10min) and then 3 times in TBS-Tween (pH7.4, 5min) to neutralise the stripping buffer. After this the membrane could be washed and blocked in buffers appropriate for the new antibody to be tested.

After blotting the membrane filters were then washed in TBS-Tween and stained with amido black (45%($^{v}/_{v}$) methanol, 10%($^{v}/_{v}$) acetic acid, 0.1%($^{w}/_{v}$) Napthol blue black; 20ml, 2min). Destaining was performed (45%($^{v}/_{v}$) methanol, 5%($^{v}/_{v}$) acetic acid) in order to visualise the separated proteins and check the protein levels/loading. In all cases the western blots were quantitated by densitometry (section 2.10)

2.7.3 Protocols used for immunoblotting against specific antibodies

All the commercial primary antibodies were used following the manufactures basic protocol with occasional modification to enhance the obtainable resolution or diminish background and non-specific binding. In all cases the antibodies were stored at -20°C in appropriately sized aliquots until required for the incubations which were performed in sterile universals or polypropylene centrifuge tubes using a rolling platform to maintain constant membrane coverage.

2.7.3.1 Anti-PKBα and Anti-PKBβ Western Blots

Transfer was to nitrocellulose which was subsequently washed twice with water (10ml, 1min) and then pre-blocked in TBS-Tween (20mM Tris/HCl (pH7.4), 137mM NaCl, 0.05% Tween 20; 10ml, 10min). The membrane was then blocked in 3%(w/v) Non-fat milk protein (NFMP), TBS-Tween blocking buffer (15ml, 60min, room temp). The membranes were then incubated in blocking buffer containing either the PKBα or PKBβ primary antibody (1 in 2000 dilution) for 16-20 hours at 4°C. Before incubating with the secondary antibody the membranes were washed twice in TBS-Tween (10ml, 2min).

The secondary antibody was a HRPO conjugated donkey anti-sheep antibody (1 in 1000 dilution in blocking buffer) and the membrane was incubated with this for 90 minutes at room temperature with constant agitation. Following this the membrane was washed twice with water (10ml, 2min) and then with TBS-Tween (10ml, 5 min) before being rinsed 5 times in water (10ml, 1min). The membranes were then ready to be treated with the ECL reagents and the PKB protein bands visualised.

2.7.3.2 Anti-p70S6 Kinase Western Blots

Transfer was to nitrocellulose which was then washed twice with water (10ml, 2min) and then pre-blocked in TBS-Tween (20mM Tris/HCl (pH7.4), 137mM NaCl, 0.05% Tween 20; 10ml, 15min). The membrane was then blocked at 4°C overnight (16-20 hours) in 3% NFMP blocking buffer. The membranes were then incubated with the p70S6 kinase antibody (1 in 2000 dilution, in blocking buffer) for 2 hours at room temperature with constant agitation. Following this the membranes were washed 3 times in TBS-Tween (10ml, 2min) and then incubated at room temperature for 90 minutes with the HRPO conjugated goat anti-rabbit secondary (1 in 2000 dilution in blocking buffer). Extensive TBS-Tween washing (4 x 5mins, 10ml) was then performed before the membrane was treated with the ECL reagents and the blot developed.

2.7.3.3 Anti-Erk1/Erk2 Western Blots

Transfer was to PVDF (Poly vinyl diethyl fluoride) membrane which was then blocked overnight in 5% BSA (%/v) blocking buffer (20mM Tris/HCl (pH7.4), 137mM NaCl, 0.1%

Tween 20) at 4°C. The membrane was then incubated for 2 hours at room temperature with BSA blocking buffer containing the anti-Erk1/2 antibody (1 in 10000 dilution). After these incubation the PVDF membrane was washed extensively in BSA blocking buffer (3 x 10mins, 10ml). The secondary antibody used was a HRPO conjugated goat anti-mouse (1 in 10000 dilution in BSA blocking buffer) and the incubation was at room temperature for 90 minutes. The membranes were then washed 5 times in TBS-Tween (20mM Tris/HCl (pH7.4), 137mM NaCl, 0.1% Tween 20; 10ml, 5mins) prior to ECL treatment and visualisation.

2.7.3.4 Anti-SHP-2 Western Blots

After protein transfer to nitrocellulose membrane the membrane was blocked overnight at 4°C in 5%($^{w}/_{v}$) BSA blocking buffer (20mM Tris/HCl (pH7.4), 137mM NaCl, 0.1% Tween 20). Membranes were then incubated at room temperature in blocking buffer containing the anti-SHP-2 antibody (1ug.ml-1) for 2 hours. The membrane was then washed in BSA blocking buffer (3 x 5min, 10ml) and incubated with the HRPO conjugated goat anti-rabbit secondary (1 in 10000) for 1 hour at room temperature. Further washing of the membrane with TBS-Tween (20mM Tris/HCl (pH7.4), 137mM NaCl, 0.1% Tween 20; 5 x 5mins) was then performed before the ECL treatment and visualisation steps.

2.7.3.5 Anti-GSK3 α/β Western Blots

After transfer to nitrocellulose the membrane was blocked overnight in 5%($^{w}/_{v}$) NFMP blocking buffer (20mM Tris/HCl (pH7.4), 137mM NaCl, 0.1% Tween 20). The membrane was then incubated in NFMP blocking buffer containing either the GSK3α or GSK3β antibody (1 in 1000 dilution) for 90 minutes at room temperature. The membrane was subsequently washed 3 times in 1% BSA ($^{w}/_{v}$) blocking buffer (10ml, 20mins) before being incubated for 1 hour at room temperature with a HRPO conjugated goat anti-rabbit secondary antibody (1 in 4000 dilution in 1% BSA ($^{w}/_{v}$) blocking buffer). After this incubation the membrane was washed in 1% BSA blocking buffer (10ml, 10min) and then 5 times with TBS-Tween (20mM Tris/HCl (pH7.4), 137mM NaCl, 0.1% Tween 20; 10ml, 5mins) before treatment with the ECL reagents.

2.8 Synthesis of GSK-3 Assay Peptide Substrate

2.8.1 Overview of Solid Phase Peptide Synthesis

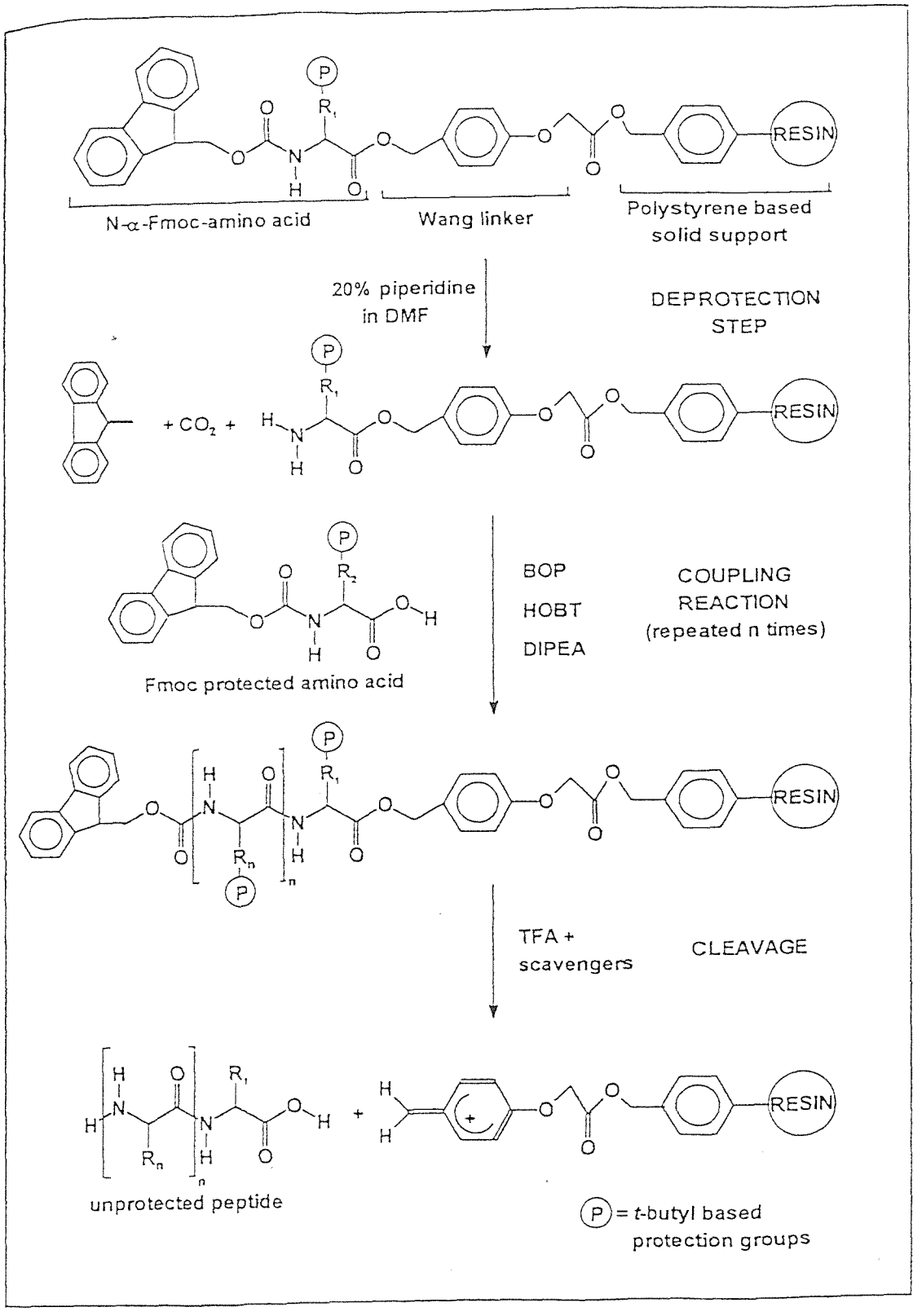
In order to directly assay for GSK-3 activity in the crude lysates of the cell lines tested suitable specific peptide substrates had to be synthesised. The GSK-3 specific phosphopeptide and its non-phosphorylated matched control were synthesised according to the design established by Proud *et al* (Proud *et al* 1997) which is further detailed and discussed in Chapter 6.

The synthesis protocol is based on the solid phase synthesis technique first developed by Merrifield in 1963 and then adapted by Sheppard in 1971 (reviewed in Sheppard 1989). In this method elongation of the growing polypeptide chain is via the addition of an Fmoc protected amino acid to the first C-terminal amino acid. The first C-terminal amino acid is conjugated to a p-benzoxybenzyl alcohol linker via its carboxyl moiety. This linker is then connected to an insoluble cross linker polyacrylamide or polystyrene carrier (resin) which acts to immobilise the peptide chain during synthesis.

The extension of the polypeptide chain which therefore occurs in a C-N manner, involves successive rounds of deprotection and coupling to add each amino acid in sequence (see Figure 2.1). Deprotection of the of these amino acids for subsequent coupling involves the release of the N-terminal Fmoc group from the immobilised polypeptide chains end terminal amino acid which is achieved by cleavage using piperidine. The exposed N-terminus can then be effectively be coupled to the next amino acid.

The coupling reaction involves the activation of the C-terminus of the incoming amino acid which in this study was achieved using the coupling agent TBtu. In the presence of the base DIPEA and the catalyst HOBt, TBtu activates the free carboxyl function through the formation of an active ester. This ester then undergoes nucleophilic attack at the Nα-function to form the desired peptide bond and thus extend the polypeptide. In this study to ensure effective and efficient coupling excess regents are used. The rounds of deprotection and coupling are continued until the entire peptide is synthesised and the complete peptide can then be further processed if required (i.e. phosphorylation) and then cleaved from the resin and purified by HPLC.

Figure 2.1 General Overview of Solid Phase Peptide Synthesis



In order to effectively use this solid phase approach to generate my polypeptides certain other synthesis factors need to be considered. Firstly, suitable groups need to be used to block the functional side chains of certain amino acids to prevent unwanted side reactions. In general these side chain-protecting groups need to be chemically resistant to the normal peptide synthesis conditions and yet easily removed when the peptide is cleaved from the resin. In this study, most side chain protecting groups used were either Trityl or *tert* Butyl as these are stable to successive round of piperidine/DMF deprotection. In this synthesis protocol certain special; side group protection considerations needed to also be accounted for in the synthesis of the phosphopeptide and these are detailed in the synthesis protocol.

Other important considerations include the type of resin/linker used. In these experiments Nα-Fmoc amino acid substituted WANG resins were employed. In these a WANG bridge or linker was used between the resin and the peptide to increase the stability of the synthesis. Also a method of qualitatively analysing the degree of deprotection and coupling is required in order to ensure efficiency of synthesis. This was monitored by looking for either the presence or absence of free amine present in the peptide by ninhydrin based assay.

2.8.2 General Protocol for the Synthesis of the GSK-3 Peptides.

The following methods were used for the synthesis of both peptides on a 0.15mmol scale. In all reactions a 15ml reaction vessel fitted with a sintered glass filter and tap to allow soluble reagents to drain from it under slight positive (N_2 gas) pressure was used.

This system allowed the soluble reagents to be effectively removed from the insoluble resin and attached polypeptide chain which is retained in the vessel and also allows for efficient/effective washing of the peptide/resin during the synthesis. After each addition/wash the vessel was flushed through with N_2 using positive pressure to remove any trace solvents. Generally all reactions were carried out at room temperature and the reaction vessel was shaken throughout the reaction.

Prior to use the reaction vessel was silated to prevent adsorption of the peptidyl-resin to the glass walls of this container. This coating was accomplished by the incubation of the vessel with 15ml (%) dichlorodimethylsilane in toluene (10ml) for six hours. The vessel then underwent subsequent extensive washing with DMF (10ml, 1min x2) followed by 50% (%)

methanol:DCM (10ml, 1min x2) and finally DMF (10ml, 1min, x2). The resin (0.15mmol of Fmoc Leucine) was added to the vessel and washed twice with DMF (10ml, 1min) before being incubated for 30 minutes in DMF (10ml) to allow the resin to fully swell. The resin was then washed 5 times in DCM prior to the first deprotection step and subsequent coupling of the next amino acid.

1) Deprotection:

In order to remove the N terminal Fmoc protecting group the resin was incubated firstly for 5 minutes with 20% ($^{v}/_{v}$) piperidine in DMF (10ml) and then subsequently for 10 minutes in the fresh 20% piperidine ($^{v}/_{v}$) in DMF. The resin was then washed 3 times in DMF (10ml, 1min) and the extent of free NH2 terminus available for coupling was assessed by qualitative ninhydrin assay (see step 3).

2) Coupling:

The next Fmoc amino acid (i.e. glutamate) was added to the resin containing vessel in a 3 molar equivalent (0.45mmol) excess in the presence of the coupling agents; HOBt (3 molar equivalents / 0.45mmol), TBtu (3 molar equivalents / 0.45mmol) and DIPEA (9 molar equivalents / 1.35mmol) again in excess and dissolved in DCM. The coupling reaction was the allowed to proceed for 45 minutes with constant shaking. After this step the resin was extensively washed with DMF (10ml, 1min, x4) and a small quantity (1mg) of the resin was removed for ninhydrin assay to cheek that efficient coupling had occurred.

3) Qualitative Ninhydrin Assay:

The extent of coupling of each amino acid was monitored at each stage of peptide synthesis using the ninhydrin assay. For this assay, 1-2mg of the resin was removed from the vessel, dried and placed in a small test tube. Regent A (100µl) and 25µl Reagent B (see below) were added and the mixture was heated to 100°C for 5 minutes. A pale yellow colour indicated protection and efficient coupling of the amino acid onto the peptide chain, whereas a blue colour indicated deprotection of the amino acid or inefficient coupling.

Reagent A: 40g of phenol were slowly dissolved in 10ml ethanol with warming. To this were added 4g of amberlite MB-3 resin and the mixture was stirred for 45 minutes and then filtered. KCN (65mg) was dissolved in 100ml water and 2ml of this was added to 100ml pyridine. This solution was combined with the amberlite resin/phenol/ethanol solution.

Reagent B: Ninhydrin (2.5g) was dissolved in 50ml ethanol.

4) Re-coupling and capping:

If the coupling efficiency was deemed to be insufficient (<99%) or when certain amino acids (i.e. arginine and proline) were to be added the coupling procedure was repeated using the same protocol as before. The reason for this was that arginine coupling can be notoriously inefficient and the coupling of proline cannot be monitored by ninhydrin assay due to proline's secondary amine nature. However if sufficient coupling was not achieved after two re-couplings, any free amine groups remaining were capped by acetylation.

Acetylation of the free amine was achieved by incubating the peptidyl-resin in 50% ($^{v}/_{v}$) acetic anhydride/pyridine (10ml) for 30 minutes. The resin was then washed 4 times in DCM (10ml, 1min) and the ninhydrin assay was repeated to confirm the effectiveness of the acetylation.

Once any coupling, re-coupling and capping procedures had been successfully performed and their effectiveness confirmed by qualitative ninhydrin assay the next round of deprotection and subsequent amino acid addition could commence.

5) Cleavage of the Peptide:

Once the peptide synthesis was complete the terminal Nα-Fmoc group was removed by the normal deprotection procedure and the peptide washed as before with DMF (10ml, 1min, x3). The peptide could then be cleaved from the resin in the presence of 96% ($^{v}/_{v}$) TFA, 2% ($^{w}/_{v}$) phenol, 2% ($^{w}/_{v}$) anisole and 2% ($^{w}/_{v}$) ethandithiol for 2 ½ hours. The cleavage solution which now contains the dissolved peptide was then collected and the TFA removed by rotary evaporation. The peptide was then cold precipitated/triturated using diethyl ether (15 hours, 4°C) and the peptide fragment recovered by filtration. This crude peptide was then dissolved in 0.1% ($^{v}/_{v}$) TFA and subjected to freeze drying to remove other impurities. The generated peptide was then ready for further processing (i.e. de-salting and HPLC purification) to yield a highly pure peptide for use (see section 2.8.4).

2.8.3 Generation of the GSK-3 Specific Phosphopeptide

Whilst the general synthesis protocol for the GSK-3 phosphopeptide and its non-phosphorylated control peptide are very similar in the main there are several important

differences used for the generation of the required phosphoserine. The synthesis of this peptide utilises phosphoramidite chemistry to provide the necessary phosphate group (Pullen 1994). The first task for this synthesis was therefore the generation of the *bis-tert*-butyl N,N-diethylphosphoroamidite from its precursor N,N-diethylphosphoramidous chloride which had been previous prepared by Dr. Bob Broadbridge but was freshly distilled prior to use.

2.8.3.1 Preparation of *bis-tert*-butyl N,N-phosphoramidite

N,N-diethylphosphoramidous chloride which had been previous prepared by Dr Broadbridge from phosphorous chloride and diethylamine in accordance with the standard synthesis protocols of Perick and Johns (1988) and Pullen (1992) was freshly distilled under reduced pressure using an oil bath to yield the pure product (0.1Torr, 34-36°C, 30.9g, 71% yield). N,N-diethylphosphoramidous chloride (7.5g, 0.043mol) was dissolved in dry ether (25ml) and added drop wise to potassium hydroxide-dried triethylamine (ET_3N)(9.21g, 12.68ml, 0.091mol) and anhydrous *tert*-butol (6.7g, 8.6ml, 0.091mol) in 100ml dry ether. This reaction was allowed to proceed for 24 hours at room temperature with constant stirring (see figure 2.2).

The hydrochloride was then filtered off onto Celite 521 using positive pressure and the residue was carefully washed with 100ml ether. The filtrate was then washed with 10% ($^{w}/_{v}$) $NaHCO_3$ (30ml, x2) and subsequently dried over Na_2SO_4. The drying agent was then removed and the filtrate reduced *in vacuo* and distilled under reduced pressure using an oil bath (52°C, 0.2Torr, 9.47g, 88%). These distilled fractions of *bis-tert*-butyl N,N-phosphoramidite were subsequently stored at -20°C over silica gel until used for the phosphitylation reaction in the GSK-3 phosphopeptide synthesis. A general mechanistic view of this synthesis is detailed in figure 2.2.

2.8.3.2 Phosphitylation of the GSK-3 substrate peptide

The general synthesis of the peptide was as detailed previously up to and including the final amino acid addition prior to deprotection and cleavage. Before the phosphitylation of the required serine residue could be performed the final Nα-Fmoc protection group had to be converted to a *tert*-butyl group as any incorporated phosphate groups are likely to be removed during normal Fmoc deprotection with 20% piperidine/DMF but are more stable towards *tert*-butyl deprotection using mildly acidic conditions.

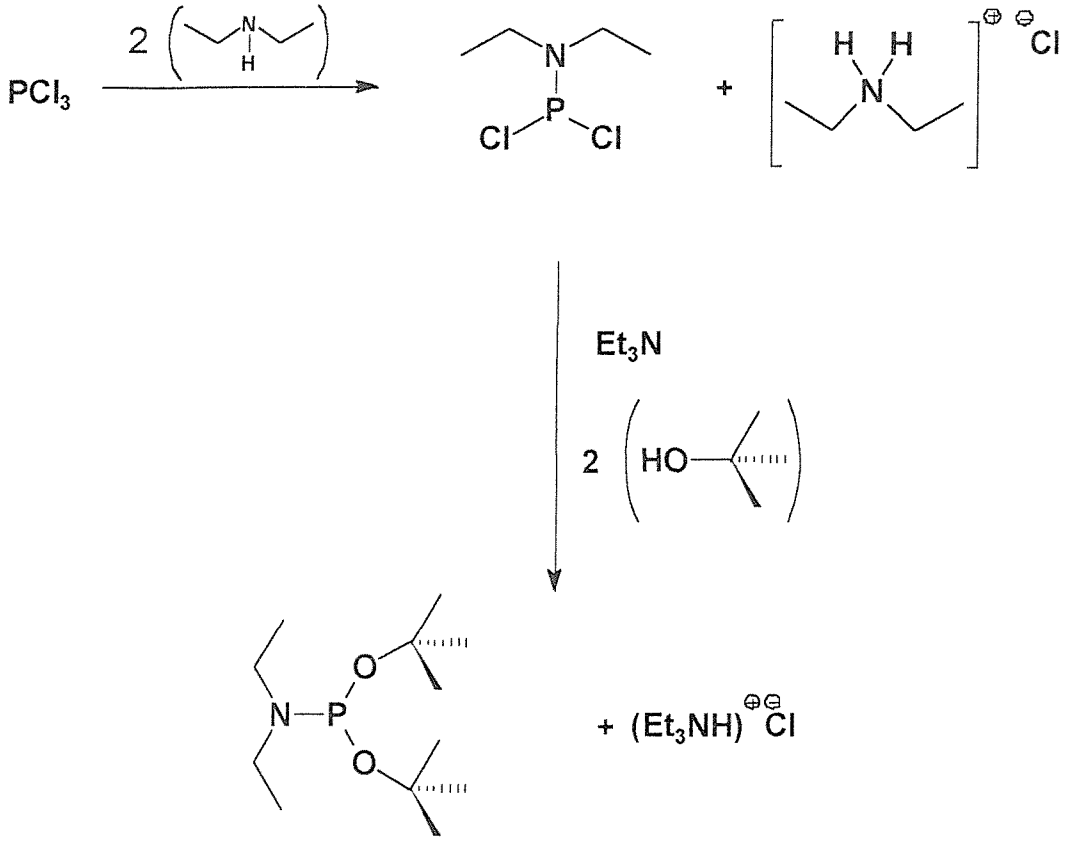


Figure 2.2 Generation of the *bis-tert*-butyl N,N-phosphoramidite. Phosphorous chloride and diethylamine were used to generate the N,N-diethylphosphoramidous chloride. This was subsequently converted into the bis-tert-butyl N,N-phosphoramidite by the addition of triethylamine and *tert*-butol.

The Nα-Fmoc group was therefore deprotected using 20% (%) piperidine/DMF and could subsequently be converted to the *tert*-butyl group by the action of *di-tert*-butyl pyrocarbonate and triethylamine in distilled DMF for 1 hour at room temperature. The side chain trityl protecting group on the desired serine could then be deprotected ready for phosphitylation. This deprotection was performed using 20% (%) DCA in DCM (10ml, 30 sec, x8) with intermittent DCM washes (10ml, 1 min). The release of the trityl group was monitored as a function of a decrease in $A_{432.5}$ until all of it was removed. Once this step was completed the peptide was ready to be phosphorylated using the previously prepared *bis-tert*-butyl N,N-diethylphosphoamidite.

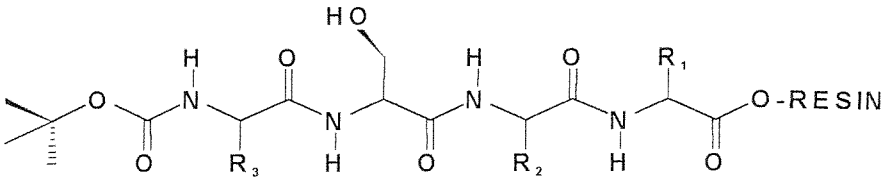
Prior to the phosphitylation reaction the peptide was washed with DMF (10ml, 1 min, x3). The phosphitylation was carried out by incubating the peptidyl-resin for 2 hours with *bis-tert*-butyl N,N-diethylphosphoamidite (10 equivalents) and excess tetrazole (40 equivalent) in distilled DMF (2ml) with constant shaking. The resin was then washed with DMF (10ml, 1 min, x2) and the phosphitylation reaction repeated twice more. The generated phosphite was then oxidised by incubation with dry *meta*-pherchlorobenzoic acid (85% (%), 20 equivalents, 1hr) in DCM (10ml) (see figure 2.3).

In order to confirm the efficiency of this phosphitylation reaction a small sample of the peptidyl resin was removed and the peptide, cleaved and analysed using mass spectroscopy (see section 2.8.4). If the yield of phosphopeptide was sufficient (i.e. effective phosphitylation had occurred) then the peptide was cleaved from the resin as previously described and the peptide was ready for further processing.

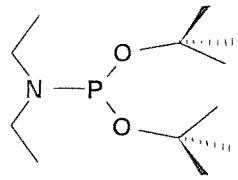
2.8.4 Peptide Purification

Before the synthesised peptides could be used in any GSK-3 kinase assays they had to be purified to remove any synthetic impurities formed during the solid phase peptide synthesis. Several purification steps were therefore performed to generate pure peptide products.

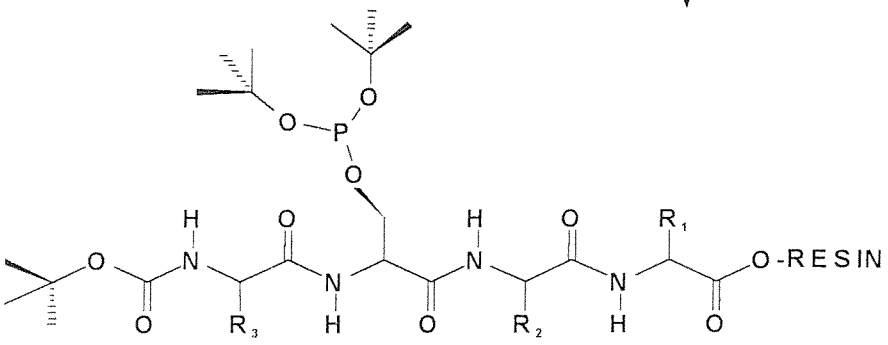
Figure 2.3 General scheme for the generation of the phosphopeptide. The *bis-tert*-butyl N.N-diethylphosphoamidite is added with excess tetrazole to the reaction. Nucleophilic aatack occurs, with subsequent cleavage with TFA generating the free unprotected phosphopeptide.



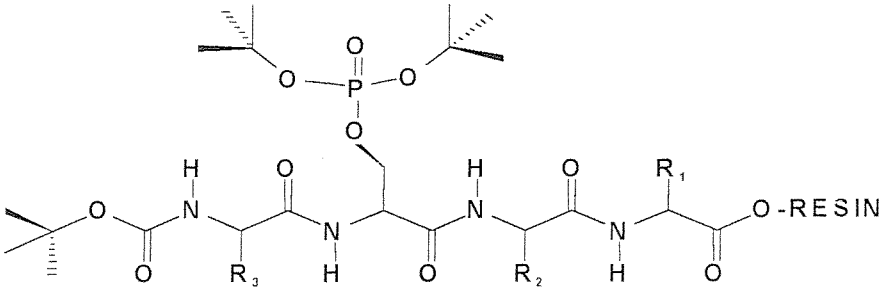
Prepared peptide- WANG-resin with side-chains protected, except Serine (or Threonine). N-terminal is *t*-Boc protected.



+ H-tetrazole



oxidation



Cleavage with TFA achieves the free unprotectected phospho-peptide

2.8.4.1 Peptide desalting

The initial purification step for the peptides was the removal of "salts" which form during the cleavage procedures previously described. Initially peptide (10mg) was dissolved in 1ml of 0.1% (%v) TFA in water. This solution was pulse centrifuged briefly (10,000 x g) and the supernatant applied to a G-25 Sephadex column which had been pre-washed and equilibrated in 50ml 0.1% (%v) TFA in water. The flow rate was maintained at 1ml min^{-1} and the peptide solution was washed through the column with 1ml fractions collected. Each fraction was then analysed by ninhydrin assay and the ninhydrin positive fractions (i.e. those containing the peptide) were retained. This procedure was repeated until all of the peptide had been desalted with the retained peptide fractions pooled for further processing.

2.8.4.2 Peptide Purification by Reverse Phase High Performance Liquid Chromatography

The impure desalted peptides were then further processed by HPLC. For this HPLC purification the dissolved peptide was applied to an inert stationary phase, and eluted by the progressive mixing of 0.1% (%v) TFA in acetonitrile (Solvent B) with 0.1% (%v) TFA in water (Solvent A). The elution profile of the peptides were monitored by measuring the absorbance at 216nm

The peptide was dissolved in 0.1% (%v) TFA in water, vortexed and then pulse centrifuged (10,000 x g). Peptide was injected onto a C-18 analytical (0.2-0.5mg peptide), or a C-18 preparative column (10mg peptide) with an injection volume of 200µl or multiples thereof. The peptide was eluted using a 20 minute acetonitrile elution gradient between 0 and 80% (%v) solvent B with flow rates of either 1ml min^{-1} for the analytical or 15ml.min^{-1} for the preparative columns.

Purification was initially carried out on a small sample of peptide using the analytical column to establish the elution profiles of the peptides. Large-scale purification on the bulk of the peptide was then performed using the preparative column. The acetonitrile was subsequently removed from the peptide containing fractions using a rotary evaporator and the peptide was lyophilised by freeze-drying. The purified peptides could then be characterised for their purity by analytical HPLC and mass spectroscopy (see section 2.8.4.3).

2.8.4.3 Analysis of Peptides by Mass Spectroscopy

For accurate analysis of the peptides Electron-spray mass spectroscopy was used to confirm the generated peptides were correctly synthesised and were in a highly pure state. A small sample of the peptide (1-2mg) was dissolved in 5µl 0.1% (%v) TFA in water. 1ml aliquots were subsequently injected into the electron-spray mass spectrometer and the generated "flight" profiles were analysed with the help of Dr. Bob Broadbridge to determine the molecular weight and purity parameters of the peptides.

The molecular weights of the peptides were calculated based on the molecular weights of the amino acids present, with 18 mass units added to the final figure to account for the H and OH groups at the peptide ends. For the phosphopeptides 80 units were added to this value to account for the phosphate group. The final calculated value for the peptides' molecular weight was ±1. Sample mass spectroscopy and HPLC traces for the peptides are shown in chapter 6 along with a detailed explanation/treatment of peptide purity studies.

2.9 Glycogen Synthase Kinase (GSK-3) Kinase Assay

Crude cell lysates treated with a variety of stimuli or antisense probes could be assayed to assess the activity of Glycogen synthase kinase-3. The lysates used were extracted in the "inhibitor cocktail rich" cell lysis buffer described in section 2.42. Crude cell lysates could routinely be assayed for GSK-3 activity without the requirement of further processing (i.e. immunoprecipitation of the GSK-3 isoforms) due to the abundance of Protein kinase/phosphatase inhibitors present in the lysis buffer and the use of a very specific substrate peptide for GSK-3 (see section 2.8). The development of this assay is further discussed in chapter 5. However, the established protocol I used in the majority of my GSK-3 kinase assays is detailed below.

Firstly into a 1.5ml Eppendorf 10µl of assay dilution buffer (20mM MOPS (pH 7.2), 25mM β-glycerophosphate, 5mM EGTA, 1mM sodium orthovanadate, 1mM DTT) also referred to as ADB, was mixed with a 10µl of inhibitor cocktail solution (20µM PKC inhibitor peptide, 2µM PKA inhibitor peptide, 20µM R24571, all in assay dilution buffer) and then placed on ice. To

this was added 10µl of either 1mM of GSK-3 phospho-peptide solution (in ADB) or 1mM of control peptide solution (in ADB). A 10µl aliquot of crude cell lysate was then added to the tube and the solution was pre-incubated on ice.

The kinase reaction was commenced by the addition of a 10µl aliquot of [γ-32P] ATP stock solution mixture (75mM Magnesium Chloride, 500µM ATP, 1µCi [γ-32P]ATP (~3000 Ci/mmol) all in ADB). The reaction was then allowed to proceed for 10 minutes at 30°C. The reaction was stopped by removing two 20µl aliquots of the mixture and pipetting these onto two strips of P81 phosphocellulose paper (4cm x 1cm). After allowing the radiolabelled substrate to bind to the filter paper for 1 minute the strips were immersed in 1% (v/v) phosphoric acid solution and washed for 5 minutes. The strips were then washed twice more in fresh 1% (v/v|) phosphoric acid solution (5 minutes each) before being washed once in acetone (3 minutes).

The strips were then allowed to air dry for 10 minutes before being folded and placed in a scintillation vial containing 3ml of Scintillation cocktail (Optiphase, Hi-Safe). The radioactivity bound to the paper was then quantitated by counting for 2 minutes on a Beckman Scintillation counter. The amount of radioactivity incorporated into the peptide could then be calculated and corrected for protein concentrations.

In all GSK-3 kinase activity experiments suitable blanks were used and these values were subtracted in final calculations. For each cell lysate examined, reactions were done using both the specific phospho-peptide and the non-phosphorylated control peptide with the value for this non-specific substrate subtracted from the value for the phospho-peptide to give the specific GSK-3 activity of the lysate tested. Lysis buffer only controls with each peptide were also performed and the values for these also used to account for non-specific binding of [γ-32P]ATP and its breakdown products to the phosphocellulose paper. A more though treatment of these control measures and the activity calculations is detailed in chapter 6.

2.10 Miscellaneous Methods

2.10.1 Quantitation of Western Blots by Densitometry

In order to determine the intensity of bands on western blots or stained membranes they were scanned using an Epson scanner and its appropriate software. The generated images could then be quantified and compared to other bands using "Phoretix" densitometry software. This enabled a measure of test proteins' levels within the cell after different treatments to be obtained and subsequently these levels compared to see what affects the treatment (i.e. antisense) may have on individual and total protein levels.

2.10.2 Protein Assay of Cell Extracts

The protein concentration of cell lysates containing SDS were determined using the dc microplate assay (BioRad). Cell extracts (5µl) were placed in each well of the microplate and 25µl of reagent A' was added followed by 200µl of reagent B. The plates were then gently shaken and left to incubate for 15 minutes at room temperature before being read at 630nm using a plate reader (Dynatech). By use of the Revelation software package and appropriate BSA standards the protein concentration in each well was automatically calculated and was used to determine the loading of each sample required.

For cell lysates-containing detergents other than SDS the Protein concentration determination method of Bradford was used. In this cell extract (4µl) was placed in a cuvette and made up to a 1ml final volume with diluted (20% (%v)) Bradford reagent (BioRad). The colour change at 595nm compared to lysis buffer only blanks was noted and the protein concentration determined from a BSA protein concentration standard curve.

CHAPTER 3 - DESIGN AND DEVELOPMENT OF AN EFFECTIVE PKBα ANTISENSE PROBE

3.1 Design and Development of Effective PKBα Antisense Probes

3.1.1 Designing Effective Antisense probes

The potential importance of the PDK/PKB pathway within the field of cell signalling makes it an ideal one to study and attempt to decipher. Many functions of PKB within this pathway and possible cellular roles have already been postulated. However, to date, all of the accumulated evidence is based on overexpression studies, which are not related to endogenous PKB levels/activity and may not be a true physiological representation of the possible roles of PKB. Also, these overexpression studies have, to date, not looked in any detail at possible specific roles of the individual PKB isoforms.

At present there are no direct and specific inhibitors of PKB functioning. The existence of selective inhibitors against of some cell signalling components, for example, the fungal inhibitor wortmannin or the compound LY294002 which inhibit PI3K, a likely upstream activator of PKB, have helped to propose possible roles for the PI3K pathway in cells. However understanding the function of downstream proteins in this pathway and PKB in particular is key in unravelling this complex pathway. Recently, the p38 MAP kinase inhibitor SB203580 was also found to inhibit the PDK1/PKB pathway at the level of PDK1 (Lali *et al.* 2000). Whilst this compound represents the first more direct inhibitor of the PDK1/PKB cascade, the fact it also inhibits the p38 MAPK cascade means that its use in analysing the functions of PKB is very limited and should be viewed with caution (Lali *et al.* 2000). The non-specific inhibitory effects of this compound emphases the need for highly specific inhibitors in order to establish the individual roles of PKB. A second inhibitor ML-9 has also been shown to powerfully inhibit PKB activity (Smith *et al.* 2000). However again this compound is a non-specific PKB inhibitor since it also powerfully inhibits PKA and p90S6K activity (Smith *et al.* 2000). Therefore this compound again emphasises the difficulty in targeting the protein kinase activity of PKB in the development of effective and specific inhibitors of PKB.

Hence, it is of primary importance to directly target PKB and by inhibiting its function unravel the complex signalling processes it appears to be involved in. Towards this objective I have looked into

developing antisense oligonucleotides against PKB. Antisense provides an ideal tool for the targeting of PKB, as it is possible to target probes against unique regions of mRNA in order to generate a highly specific inhibitor of PKB expression. The highly specific targeting of antisense probes also enables the design of isoform specific probes against the major PKB isoforms, in order to investigate not only the general role of PKB, but also any specific roles the individual isoforms may have.

The design of suitable antisense oligonucleotides is of critical importance to maximise the inhibition or "knock-out" achievable against the target protein. In order to obtain these goals potential probes need to meet certain specific criteria. Firstly, the potential probe must bind to the complementary mRNA with a high affinity. Therefore, the targeted sequence mRNA of the protein of interest must be GC rich, to give the theoretical maximal binding affinity possible for the potential antisense probe. However, the chosen probe should not bind non-specifically to non-target sequences. Therefore, a balance between high affinity and lack of specificity needs to be attained (reviewed in Bennett *et al.* 1999, Stein 1999, Agrawal 1999).

In fact it has been established that the optimum GC rich composition is between 65-70%, as this gives a high affinity binding whilst not being too "sticky" to other sequences. Any potential probe should also be specific for only the target mRNA sequence and not be too similar to any other sequences of mRNA. This is because the more sequence similarities present in the target mRNA to other mRNA sequences, then the more likely the antisense probe will bind to other non-targeted sequences. This will decrease the probes antisense potency and could cause non-antisense effects which may results in an incorrect assignment of roles (Brown *et at* 1994, Hunter *et al.* 1995, reviewed in Bennett *et al.* 1999).

The mRNA sequence targeted must also be accessible to the designed antisense probe, so that the probe can access the complementary sequence and be able to hybridise to it. Therefore, it is important that the targeted sequence region should not fall within the secondary structure of the mRNA (i.e. hairpin region) as this also reduces or even prevents hybridisation (Stein *et al.* 1999, Agrawal *et al.* 1999). The antisense probes themselves should also not have any secondary structure (self-hairpins) or form dimers with each other (self-complementation). Such probe sequence related issues would lead to a decrease in the antisense potency, as well as leading to possible uptake and

degradation problems. The selected antisense probe needs to evoke one of the possible hypothesised antisense mechanisms within the cell and so the design rationale including the chosen targeted sequence and the probes chemical composition, needs to account for this necessity of action (reviewed in Hunter 1995).

The length of the selected probe is also of extreme importance, as although longer probes form stronger more stable hybrids, increasing the length of the probe increases the risk of both non-specific mRNA being targeted and probe self-complementation. Longer probes may also be more difficult to get into the cell to start with (Hunter *et al* 1995, Coleman 1990).

The cellular uptake/distribution and stability of the antisense probes are themselves issues that also need to be addressed in the design brief for possible probes. Antisense oligonucleotides need to get into the cells easily and fairly rapidly. The mechanism of oligonucleotide uptake by cells is still not clearly defined but is likely to be by adsorptive endocytosis, pinocytosis or some other form of membrane crossing and distribution via heparin binding proteins. Once in the cells, the probes need to be delivered effectively to the targeted mRNA and need to be fairly resistant to nuclease attack, so they can bring about their affects with maximum potency and for a prolonged period (Bennett *et al.* 1992, Wagner *et al*, 1993).

Whilst the uptake, subcellular distribution and stability/longevity of an oligonucleotide is not strictly sequence driven, GC rich sequences and charged or modified oligonucleotides can enhance these parameters and therefore increase the potency of a probe. Artificial oligonucleotide carriers can also be used to further increase uptake and distribution as shall be seen later (Bennett *et al.* 1992).

Whilst these considerations deal with the general rules for antisense design it is also important to think about the part of the sequence targeted. Since antisense works by inhibition of translation, it is believed that targeting the initiation start (AUG) codon of the mRNA probe is the most effective method (Liu *et al.* 1995, Chaikof *et al.* 1995). This has been shown to be true in many cases; for example, all previous antisense probes developed in this group have been directed against this region (Sale *et al.* 1995, Arnott *et al.* 1999). It is believed that by targeting this region of the mRNA, not only can possible nuclease based antisense mechanisms be stimulated, but also the antisense potency is enhanced (reviewed in Crooke et al. 1999). This occurs by preventing the binding of the

ribosome/translation initiation apparatus due to steric hindrance or recognition site masking by the antisense probe (reviewed in Baker *et al.* 1999).

However, despite the apparent advantage of targeting the initiation codon sequence in the mRNA, there is mounting evidence that antisense oligonucleotides targeted against other regions of the mRNA can be as effective, providing the other design points are stringently met (reviewed in Baker *et al.* 1999). It is believed that probes directed against other areas utilise other antisense mechanisms most notably the activation of RNase-H which subsequently digests the single stranded mRNA (Altmann *et al.* 1996). Thus, it is critical to fulfil all the above elements in order to obtain the best possible probes and this is achieved using a variety of methods.

It is now considered best to adopt a "gene-walk" strategy in the design of antisense probes in which many different regions of the mRNA are targeted and the antisense probes directed against each of these assessed. By covering regions throughout the target mRNA this method of design can not only allow for the potential different antisense mechanisms that may be evoked, but also minimise the possible effects of mRNA secondary structure, bound proteins or variations in RNase accessibility, on antisense potency. Therefore, it is best to analyse several different probes which encompass the various design criteria.

In order to design antisense probes which fulfil the above criteria a variety of techniques including computer programs and databases are used. It has been found that probes between 15 and 22 nucleotides in length best achieve these size requirements since they are long enough to give strong binding, but still of a short enough length for uptake and specificity. Shorter probes form less stable hybrids and so show weaker binding. Therefore short oligonucleotides may not be able to hybridise sufficiently to the target to elicit an antisense effect. Short probes also have specificity problems since shorter regions of mRNA are less likely to be unique to the target. Longer probes on the other hand have an increased risk of hairpin formation and may have problems associated with cellular uptake and distribution. Also longer sequences have a greater chance of containing short stretches of consecutive nucleotides which could hybridise to non-target mRNA. This means probes outside the optimum length are far more likely to bind to sequences other than the target, which may ultimately lead to the cleavage of non-specific mRNA due to unplanned antisense effects against these non-targeted sequences (Hunter *et al.* 1995, reviewed in Coleman 1990).

To develop potential probes, the mRNA sequence for the protein of interest in the desired species is first obtained from a database. This sequence is then "run-through" a probe program which produces a list of potential probe sites of the selected size (usually 18mers) throughout the sequence, based on pre-set binding affinity (GC content) and binding ability (not within hairpin regions) parameters (Bloomfield and Giles 1992).

When selecting these potential target sequences, there are several motifs that should be avoided, as they can lead to non-antisense effects that may cause complications when assigning the physiological consequences of the probe. One such motif is a stretch of more than 3 contiguous guanines, as these can lead to a stacked secondary structure which can prevent Watson and Crick hybridisation and cause non-antisense effects including protein interactions. Unmethylated CG sequences should also not be targeted as this can lead to immunostimulatory problems. Palindrome sequences (e.g. GACGTC, AGCGCT) containing at least one guanine can induce interferon signalling and therefore are also best avoided.

Having accounted for these potential pitfalls and by analysing the computerised data for the theoretical target areas, suitable regions are selected based on position and high mRNA-probe complex melting temperatures (i.e. binding). These are converted into the complementary antisense sequence. Potential probes are then checked by computer program to ensure they are not self-complementary (i.e. don't form dimers) or form self-hairpins which would greatly reduce their potency or perhaps hinder their entry into the cells (Bloomfield and Giles 1992).

Probes meeting these requirements are analysed using a similarity search to establish whether they may also bind to mRNA sequences of other proteins. The program used lists all known sequences which have four or less base differences to the targeted sequence in both the forward and reverse directions. From this analysis, any potential probe region which has 3 or less base differences to other sequences is discarded from further analysis due to the possibility that this probe could hybridise to non-targeted mRNA sequences. As a further level of control, the first choice selections have at least 4 base differences to any known sequence. In general any potential probe selected will have more than 4 base differences to sequences which correspond to signal transduction or other important cellular proteins. Antisense sequences which pass all these set theoretical parameters can

then be synthesised and transfected into cells to establish how successful they really are at targeting the desired protein.

In order to increase the uptake and potency of the antisense probes selected, one can use chemically modified phosphorothioate oligonucleotides (synthesised at SGH or in house), in which one of the non-bridging phosphate linked oxygens in each nucleotide is replaced by sulphur. These modified oligonucleotides are resistant to cellular nucleases and, are therefore much more stable than normal/unmodified oligonucleotides which are rapidly metabolised within the cell (reviewed in Stein 1999, Rou 1997). The improved stability gives an enhanced potency meaning that shorter transfection times or lower concentrations of phosphorothioate oligonucleotides can be used to elicit an antisense effect. Phosphorothioate oligonucleotides also exhibit similar uptake and hybrid kinetics to unmodified oligonucleotides and so act in a similar way (Stein 1996). Phosphorothioate oligonucleotides also are known to support and even further stimulate RNase-H activity within the cell so the use of these modified oligonucleotides may enhance any potential antisense effect (Altmann *et al.* 1996, reviewed in Crooke *et al.* 1999).

Using a specialised delivery system to transfect the probes into the cells can enhance the potential potency of the selected probes. Studies have shown that using the cationic liposome transfection system (lipofectin^{TM}) to deliver the oligonucleotides to the cell greatly enhances the cellular uptake of the probes and improves the subcellular distribution and therefore improves antisense potency (Bennett *et al.* 1992, Wagner *et al.* 1993). These lipid-based compounds form a complex with the oligonucleotide prior to transfection; the liposome structure is rapidly taken up and processed by the cells, thus rapidly and effectively delivering the probe to its site of action. However this lipid-based system can only be used for over a short transfection time scale, up to 24 hours depending on cell type, since it can become toxic to the cells over longer periods (Bennett *et al.* 1992, Wagner *et al.* 1993). This method of assisted oligonucleotide delivery to cells can also only be used for adherent cell cultures and therefore is inappropriate for cell lines grown in suspension. Also, several of the older generation systems, including lipofectin^{TM}, are not resistant to serum and so can only be used in serum free conditions (reviewed in Stein 1999).

In order to analyse the potency of a potential antisense probe, a noticeable effect on the target gene/protein must be clearly shown. This needs to be in the form of either a significant decrease in

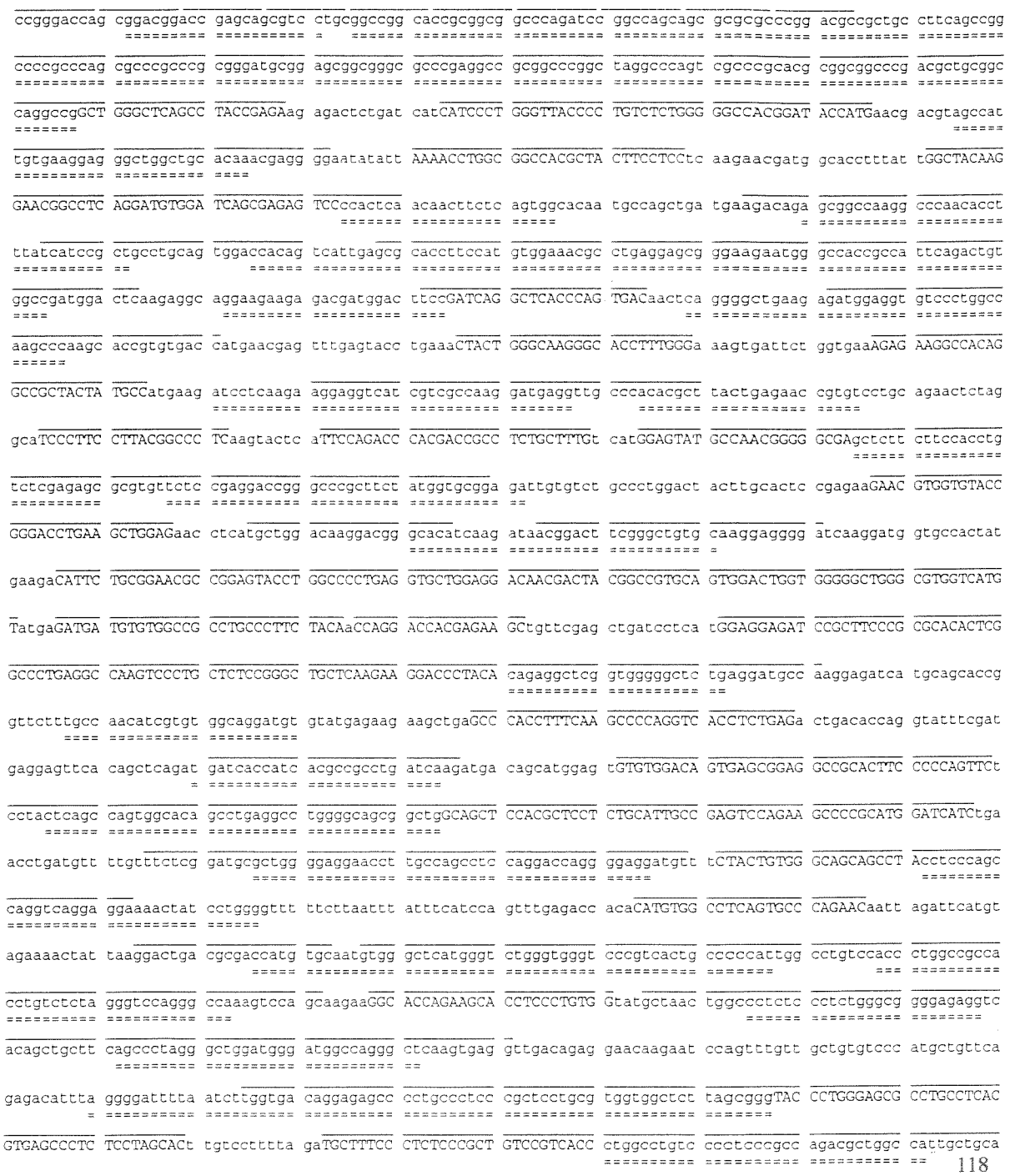
the mRNA level of the target or by a considerable depletion at the protein level, when compared to cells untreated with the antisense. In both cases, the greater the reduction in the level of the analysed component compared to the control, then the more effective the antisense oligonucleotide. In the case of changes in mRNA level of the target, a quantitative PCR approach can be used to assess the level of depletion whereas in the case of protein level analysis western blotting is used to assess the reduction. Only probes causing a significant depletion in the target, usually at least an 80% reduction at the protein level are considered for further analysis into their potential as an antisense. This usually consists of initially optimising their transfection profile by establishing the most effective transfection time and probe concentrations.

Potential probes passing the initial test for antisense effects should then be further characterised to show the observed effect is a genuine antisense effect. This can be proved firstly by showing a dose response effect for the selected probe with higher concentrations of the probe eliciting a greater reduction in target levels (reviewed in Bennett *et al.* 1999). Secondly the chosen probe should be shown not to affect the mRNA or protein levels of non-targets thus indicating that it does not bind to non-specific sequences. Finally, it is important to show that only the specifically designed antisense probe causes a depletion in the target levels and that suitably designed control oligonucleotides usually sense, scrambled and mismatch do not affect the target (Bennett *et al.* 1992, Wagner *et al*, 1993). Once this has been achieved the potential probe is deemed suitable and effective as an antisense and can be used to assess the cellular roles of the target. Thus by adhering to these strict rules for the development and transfection of antisense probes it should be possible to design suitable probes to target PKB.

3.1.2 Design and Development of an Effective Antisense Probe Against PKBα

In order to design potential antisense probes for PKBα, the mouse mRNA sequence for the protein was retrieved from the EMBL database (accession number X686168) and the sequence was run through the probe finder program to highlight potential target regions (see figure 3.1). Probe regions throughout the mRNA sequence which were deemed to be high affinity (GC rich) and not within predicted mRNA secondary structure were therefore selected for further processing.

Figure 3.1 - Potential probe regions in the mouse PKBα sequence. Mouse PKBα mRNA sequence, analysed from 1 to 2626, with the G/C Rich regions, Hairpins & potential Probes region shown: CAPITAL LETTERS - Probe regions; Single Dash (_) - G/C rich regions, Equals Sign (=) - Hairpin regions.



These potential antisense "hot spots" were subjected to further computer analysis to eliminate self-complementary and less specific probes. From this a short list of possible probes was drawn up. It was decided from this list to pick the most suitable theoretical probe in each of the different regions of the sequence instead of only around the initiation codon and hence test the effectiveness of various antisense probes directed against points throughout the sequence.

The rationale behind this more spread targeting is due to a number of factors based on the PKBα sequence. Firstly, although the initiation region of PKBα mRNA can be targeted by antisense probes it does not lie in a GC rich region and hence probes against this region would have a lower affinity binding and so may be less potent antisense agents. All the possible probes directed at this area are also likely to form complementary dimers (with 4 hydrogen bonds). This may reduce the probes' binding to its target and the initial cellular uptake of the probe and so decrease antisense. However, probes targeted against other regions of the sequence have a higher theoretical binding affinity and so could elicit a potent antisense effect due to this increased hybridisation potential.

Another reason for the more spaced targeting is that other regions of the mouse PKBα sequence have been found to be identical to the sequences of the other PKB isoforms, hence setting up the possibility of multi-isoform probes (discussed later). Finally, evidence suggests that targeting many regions of the sequence the so called "gene walking" strategy increases the chance of identifying the most potent and effective antisense probe. This may also lead to the design, development and use of more than one potent antisense probe directed against different regions of the target and subsequently to using the two or more region specific probes at one time to achieve maximum antisense effect.

I initially selected and synthesised four possible PKBα antisense phosphorothioate oligonucleotides directed against different regions of the mouse sequence (see figure 3.2). Firstly, αAS1 which was directed against the initiation codon (bases 272-289) but had the potential problem of inter-probe dimerisation (4 hydrogen bonds) that may reduce its potency. Secondly, the kinase domain targeting probe αAS2 (1317-1334), a possible multi-isoform probe with homology to similar regions in mouse PKBβ and PKBγ. Finally, the probes αAS3 which is directed against the 3' translated region (1665-1682) and αAS4 directed against the 3' untranslated region (1769-1786) were selected as they are both high affinity probes based on the sequence composition and free energy of binding.

Figure 3.2 - Potential mouse PKBα antisense probes. Position, Sequence, binding and affinity data of selected PKBα antisense probes: αAS1, αAS2, αAS3 and αAS4 (higher numbers indicating stronger probe).

Position	Sequence	Dimers (No. H bonds)	DG(kcal)	Tm (°C) NN GC
Initiation Codon (272)	GTTCATGGTATCCGTGGC αAS1	4	2.3	- -
Middle Kinase (1317)	TGTAGAAGGGCAGGCGGC αAS2	-	1.3	71 60
Carboxyl Terminal (1665)	GCCTCCGCTCACTGTCCA αAS3	-	1.4	66 58
3'Untranslated (1769)	CGGGGCTTCTGGACTCGG αAS4	-	1.4	73 62

The mRNA position, sequence and affinity/thermodynamic properties of these first wave probes are summarised in figure 3.2 along with the possible dimerisation problems and other PKB isoform similarities. In each of these four probes, all the basic antisense characteristics are met to a greater or lesser degree and with the spread of these probes throughout the sequence it was felt that this rationale should enable the establishment of the best region for antisense and most important antisense design parameters needed to generate the most effective PKBα probe.

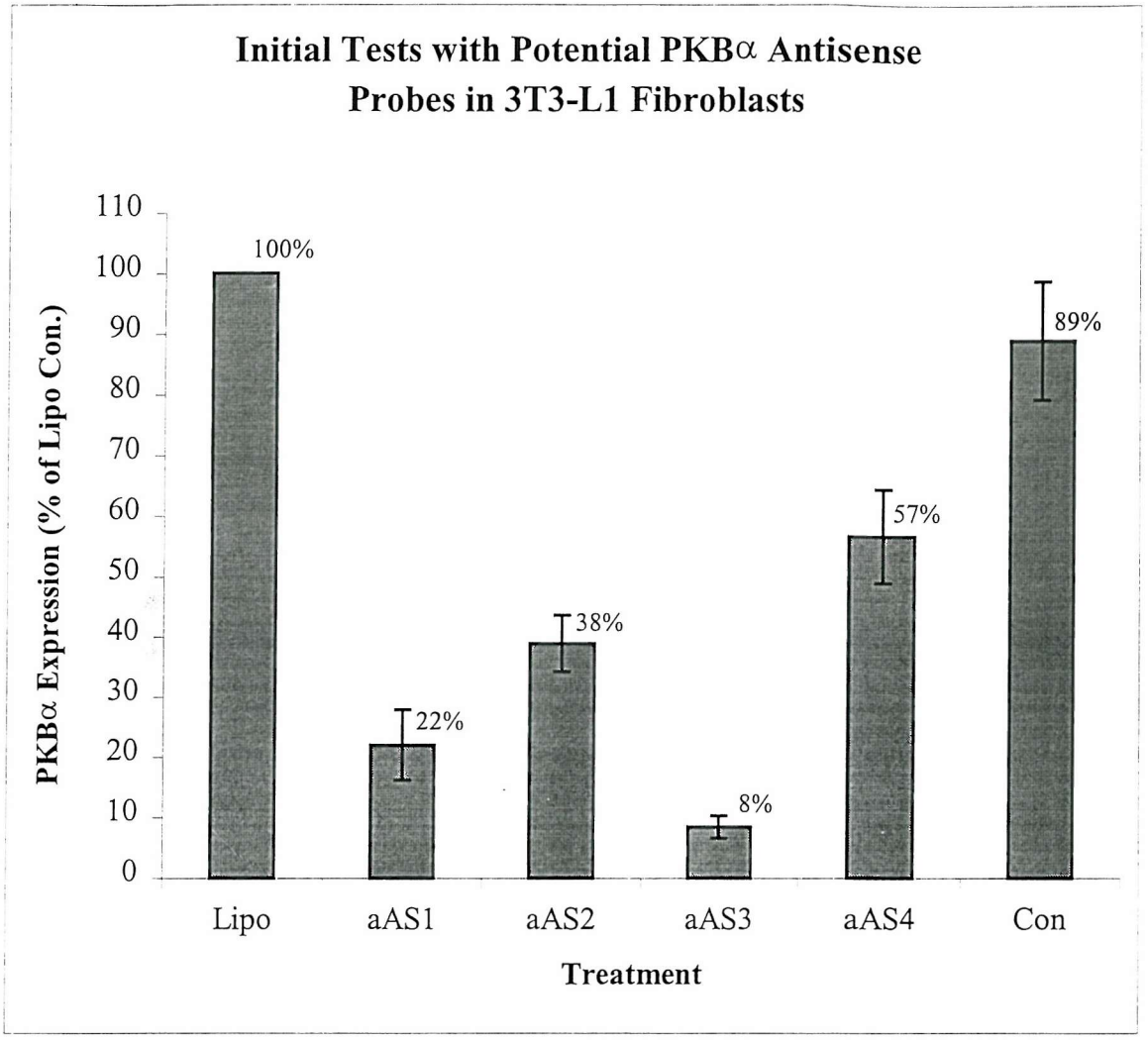
In order to test the antisense potential of the selected probes, it was necessary to select a suitable cell line for antisense transfection and establish a method of detection. A PKBα antibody that was found to be suitable for detecting both small amounts of PKB and changes in protein expression by western blotting (not shown) was obtained from Upstate Biotechnologicals. Therefore, it was decided to analyse the effectiveness of the probes by looking for changes in protein expression after antisense treatment using western blotting. For the initial test studies the mouse 3T3-L1 fibroblast cell line was chosen for transfection with the potential antisense probes. This cell line was found to express PKBα in easily detectable amounts under normal conditions and is also a cell line previously used for antisense studies. Therefore, 3T3-L1 fibroblasts were known to be fairly straightforward to transfect, and thus, a suitable cell type to use in these studies.

Initially the 4 probes were transfected into the cells independently using lipofectin™ as a delivery system for the first 8 hours. Control (untreated cells) and lipofectin™ only (8 hours) treated cells were set up, with which to compare the expression of PKBα protein seen in the antisense treated cells. In the first transfection 5μM of each oligonucleotide (per well) and a 4 day transfection protocol were used, as these condition were similar to those used for previous antisense studies on the fibroblasts. Once extracted, an equal amount of total protein from each condition was run on a SDS-PAGE gel and the levels of PKBα expression analysed by western blotting (see Figure 3.3).

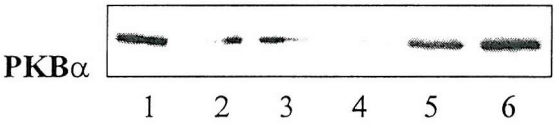
The initial transfections using the potential PKBα probes showed that whilst all the probes depleted the levels of PKBα protein by a significant level, the 3'-translated region probe αAS3 was by far the most effective antisense probe (see figure 3.3) giving around 90% depletion of PKBα at the protein level. The αAS1 initiation codon directed probe was also shown to be a fairly effective antisense probe despite the apparent sequence specific shortcomings (i.e. inter-probe dimerisation), with this probe giving a depletion of around 80% of the PKBα protein level.

Figure 3.3 -Treatment of 3T3-L1 fibroblasts with possible PKBα antisense probes leads to varying but significant reductions in PKBα. 3T3-L1 fibroblasts were incubated with 5μM of the potential PKBα antisense probes for 96 hours with lipofectin ($8\mu g ml^{-1}$) present for the first 8 hours. These cells and control (no ODN, +/- lipofectin) cells were then extracted as a whole cell lysate and prepared for SDS-PAGE. **A.** Depletion of PKBα was quantitated by densitometry and results expressed graphically where 100% is taken to be the intensity in the lipofectin only control. **B.** Representative PKBβ Western blot of whole cell extracts. Lane 1, Lipofectin only; Lane 2, αAS1; Lane 3, αAS2; Lane 4 αAS3; Lane 5, αAS4. Lane 6, Untreated. (n=3)

A.


Initial Tests with Potential PKBα Antisense Probes in 3T3-L1 Fibroblasts
PKBα Expression (% of Lipo Con.)
110
100
90
80
70
60
50
40
30
20
10
0
100%
22%
38%
8%
57%
89%
Lipo
aAS1
aAS2
aAS3
aAS4
Con
Treatment

B.


PKBα
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4
5
6

The other two probes αAS2 and αAS4 whilst showing some depletion of PKBα at 60% and 40% respectively were discounted as PKBα antisense agents at this stage as they did not appear to be effective antisense agents. The multi isoform probe αAS2 was however, saved for future use against the other PKB isoforms, which it is also targeted against. An increased concentration of this probe could be needed in order to give be effective antisense probe against not only the PKBα isoform but also other PKB isoforms

Before deciding on αAS3 as the antisense probe which would be used in future experiments, this probe and the second most effective probe, αAS1 (directed against the initiation codon), were tested at 2,5 and 10μM concentrations. These simple concentration dependent studies were performed to see if any significant difference in the potency of either antisense probe could be obtained at different concentrations of each probe. Also these experiments would show which of these two antisense probes is the most effective agent against PKB and at what range of concentrations this probe is most effective. Both probes showed a concentration dependent antisense effect with both giving a significant depletion of PKBα at all concentrations (for example, αAS1 80% depletion and αAS3 91% depletion at 5μM). However, these tests confirmed that αAS3 was the most effective probe at significantly depleting PKBα in 3T3-L1 fibroblasts, giving a greater than 90% depletion of the endogenous PKBα (see figure 3.4)

Having established αAS3 as the main PKBα antisense probe the next step was to establish the optimum time and concentration conditions for use of this probe in the fibroblasts. Initially it was important to establish the most suitable length of transfection in order to give the best possible depletion of PKBα without using too much oligonucleotide or damaging the cell viability. Initially the 3T3-L1 fibroblasts were transfected with either 5μM αAS3, the lipofectin™ delivery system or left untreated. One well for each condition was extracted every day for six days. This enabled changes in PKBα protein levels with or without αAS3 antisense treatment with time to be compared. From this the optimum length of transfection with αAS3 could be determined (see figure 3.5). A 96 hour (5μM αAS3) transfection time was found to be the most effective length of time for antisense treatment. Transfection of the fibroblasts with αAS3 for this time period yielded the maximal depletion of PKBα with a 91% reduction in the protein levels of PKBα. In these studies the 50% protein depletion time of PKBα was established to be around 40 hours.

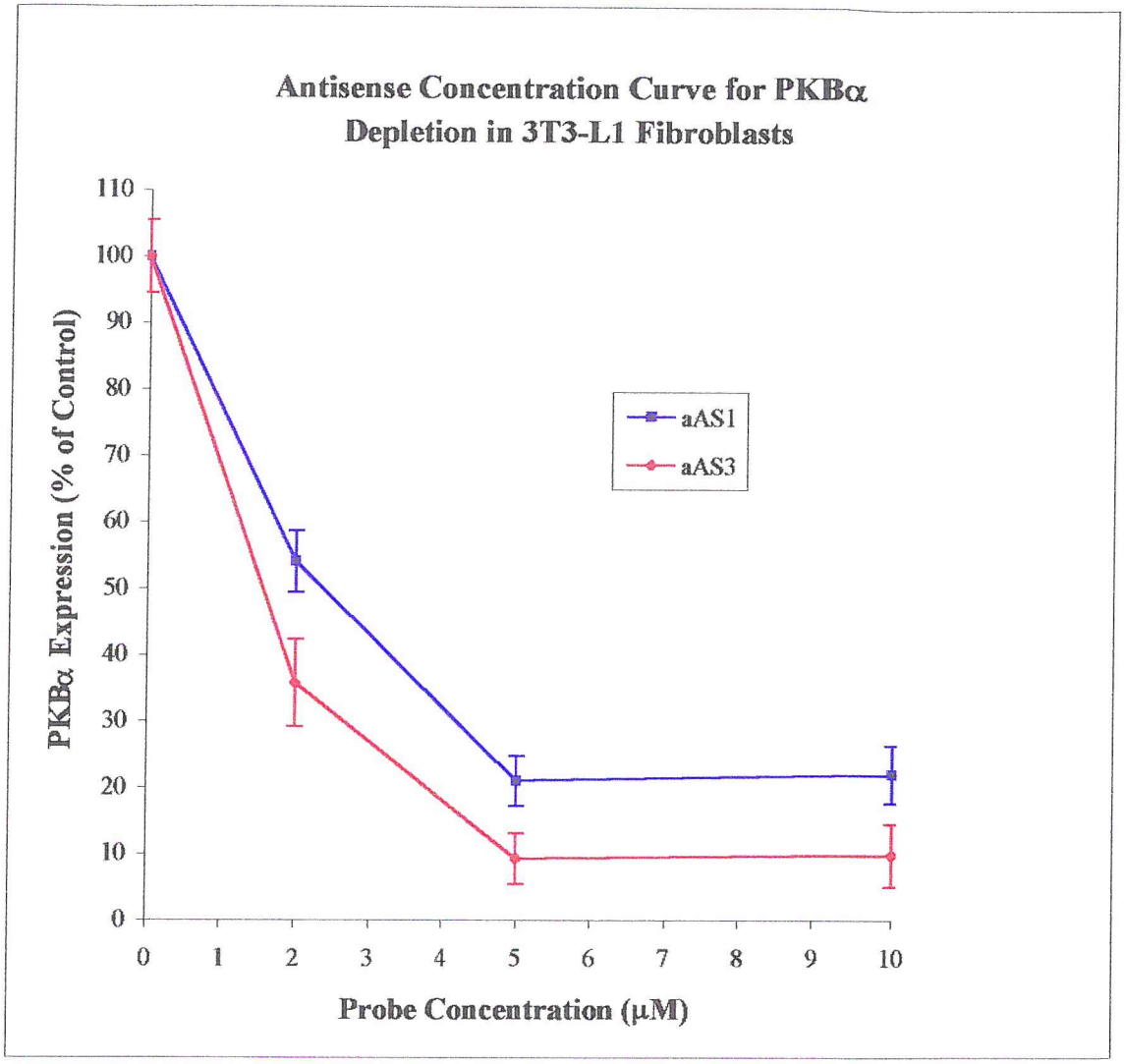


Figure 3.4 - αAS1 or αAS3 treatment of 3T3-L1 fibroblasts caused a concentration dependent depletion of PKBα. 3T3-L1 fibroblasts were treated over a 96-hour transfection period using a range of PKBα antisense concentrations. Extracts were analysed by SDS-PAGE and Western blotting and the PKBα levels quantitated by densitometry. 100% PKBα expression is taken to be the level of PKBα in no ODN controls (n=3).

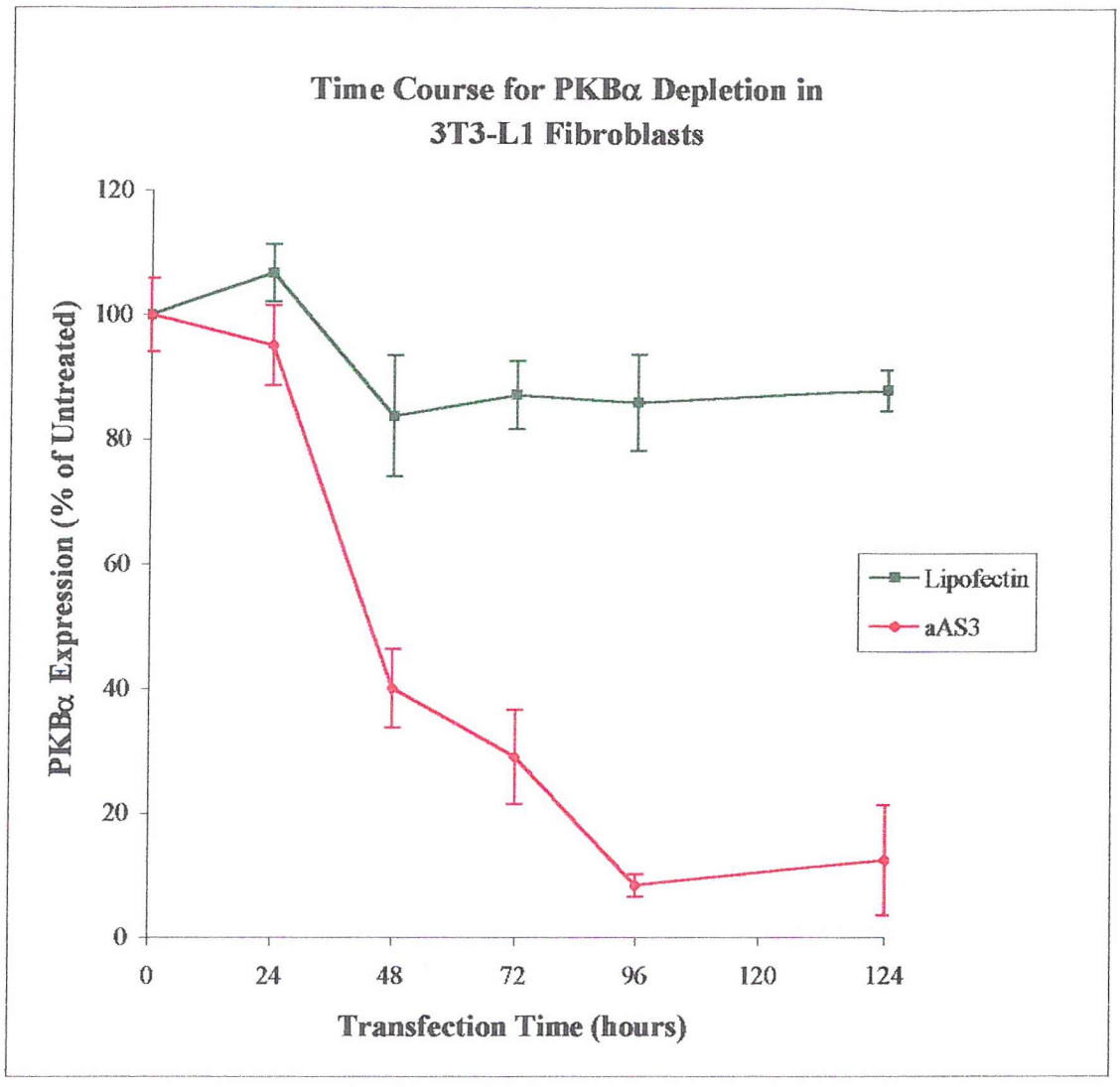


Figure 3.5 - αAS3 treatment of 3T3-L1 fibroblasts causes a significant depletion in PKBα in a time dependent manner. 3T3-L1 fibroblasts were transfected with 5μM αAS3. At various time points one transfected sample and one control sample were extracted. The samples where analysed by SDS-PAGE and Western blotting and the PKBα levels quantitated by densitometry. PKBα levels are expressed as % of the PKBα levels band in cells extracted on day 0 (i.e. before the transfection) (n=3).

Having determined the optimum transfection time for αAS3 it was important to establish the concentration of αAS3 which yields the maximum depletion of PKB without using unnecessarily high concentrations of probe which may adversely affect other cellular functions The fibroblasts were transfected using the optimum 96 hour time period over a range of αAS3 concentrations of between 1 and 10μM. The depletion of PKBα obtained using these concentrations was then compared to the levels in untreated and lipofectin™ only treated controls and the optimum αAS3 concentration determined (see figure 3.6). A concentration dependent depletion in PKBα levels was observed up to a concentration of 4μM of αAS3, which yielded a 92% depletion of PKBα. At higher concentrations than this the level of PKBα remained constantly low at around 10% of the control levels.

From these studies, it was established that 4μM was the lowest concentration that gave effective depletion, typically yielding a 92% depletion of PKBα. The IC50 of αAS3 was 0.9μM. In summary the optimum conditions for transfection of 3T3-L1 fibroblasts with αAS3 was established as 4μM of probe for 96 hours.

To establish that the depletion of PKBα by αAS3 was a genuine and specific antisense effect, various control oligonucleotides, namely sense, random (same base composition different arrangement) and mismatch (similar sequence with 4 base changes) were designed. These control oligonucleotides were designed to have similar characteristics to αAS3 including, similar binding affinities and kinetic/thermodynamic parameters in order to minimise any potential non-attributable sequence specific effects and allow direct comparison between the oligonucleotides. Each control oligonucleotide was analysed for potential dimerisation and hybridisation to other sequences (up to 3 mismatches) using appropriate computer programs in order to remove any potential non-specific effects (see figure 3.7). Having designed these suitable controls, they were used to show whether only the αAS3 antisense oligonucleotide caused a depletion in PKBα levels.

These control oligonucleotides and the αAS3 antisense were tested at 4μM in the 3T3-L1 fibroblasts and the depletion of PKBα analysed by western blotting and compared to PKBα levels observed in lipofectin™ only and untreated cells (see figure 3.8). From these tests, it was shown that only the αAS3 antisense probe causes a significant depletion of PKBα protein levels in these cells.

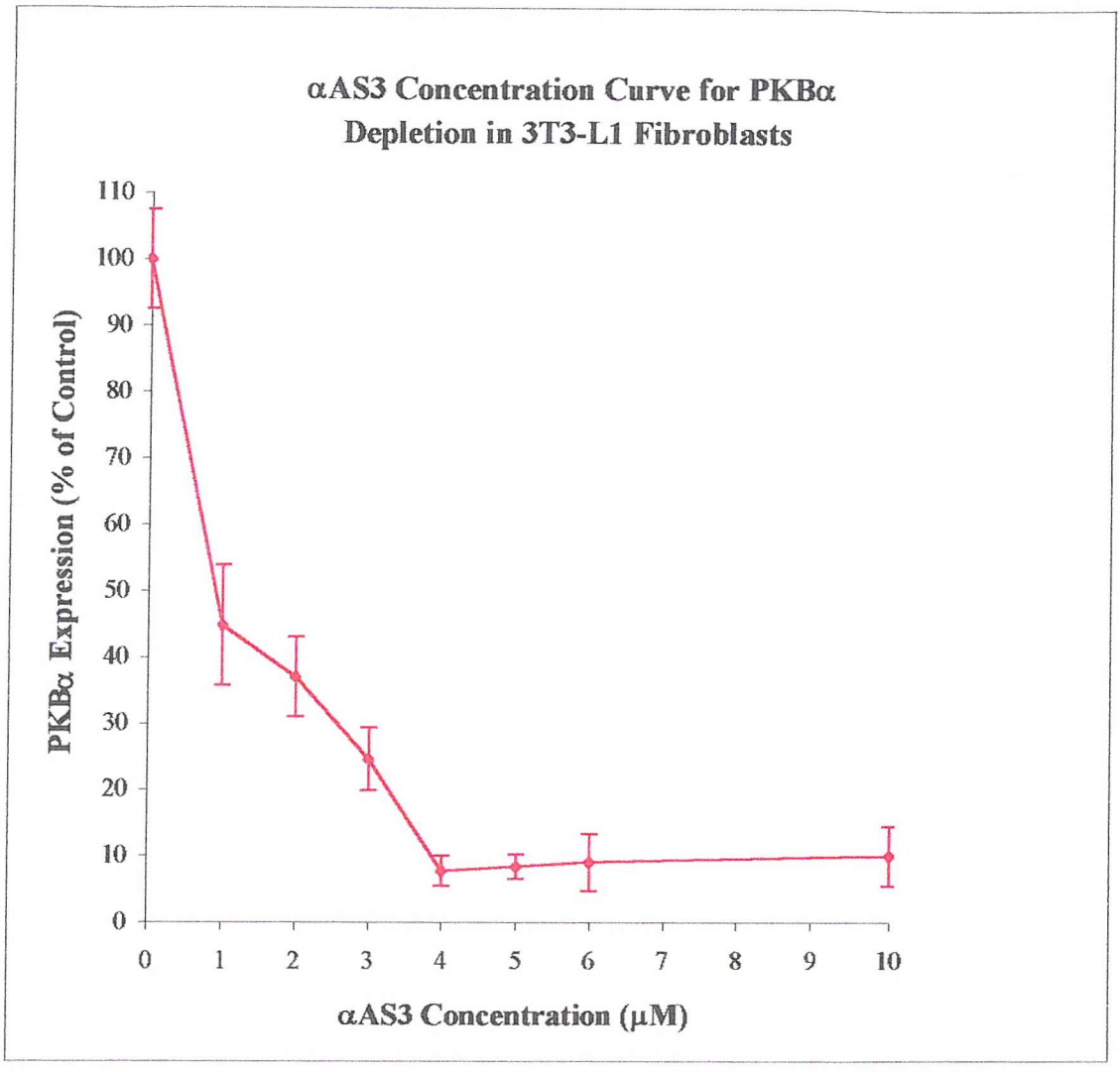


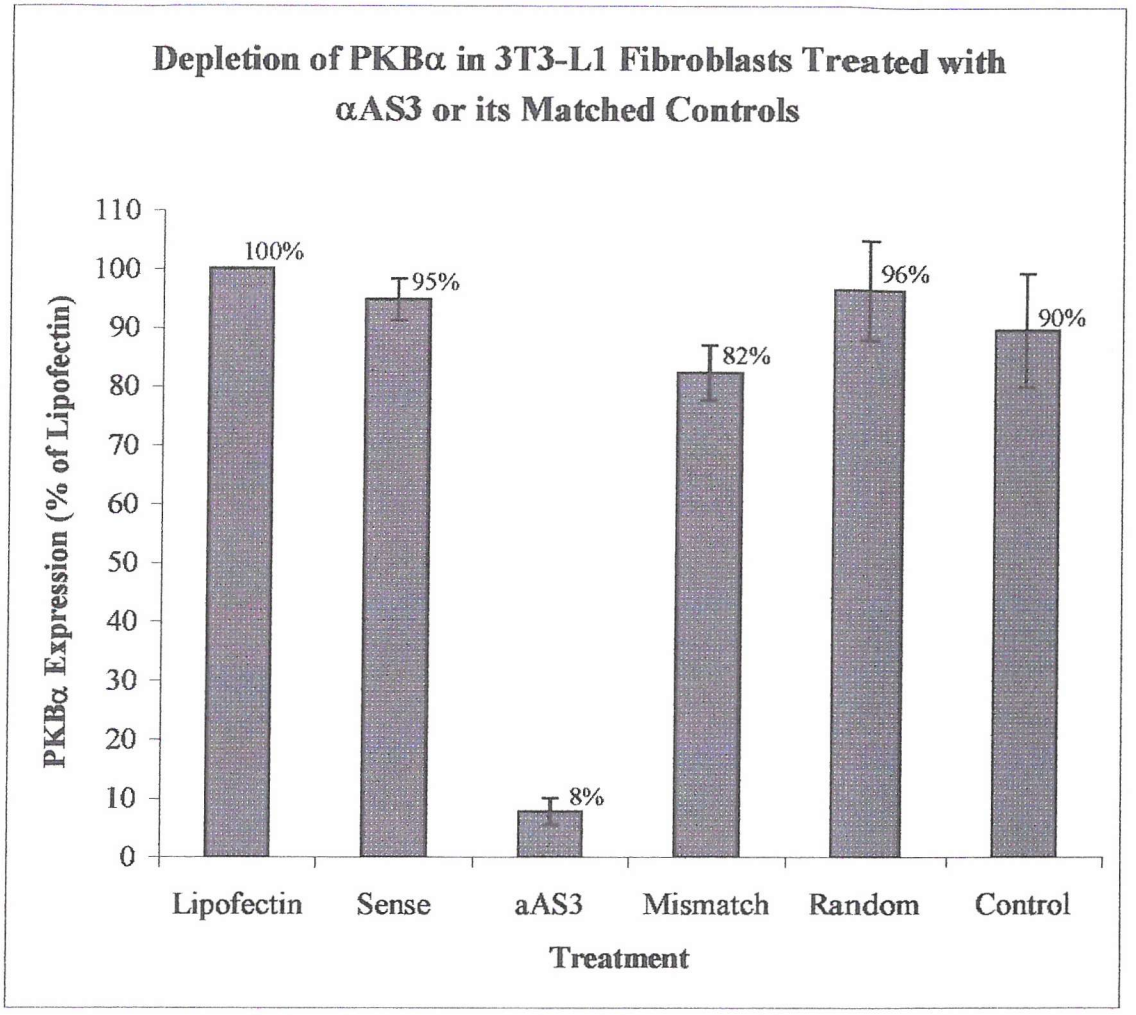
Figure 3.6 - αAS3 treatments of 3T3-L1 fibroblasts causes a significant depletion in PKBα in a concentration dependent manner. 3T3-L1 fibroblasts were transfected for 96 hours using a range of αAS3 concentrations. Samples were analysed by SDS-PAGE and Western blotting and the PKBα levels quantitated by densitometry. 100% expression of PKBα is taken to be the level of PKBα in no ODN (lipofectin only) controls (n=4)

Figure 3.7 - PKBα antisense probes αAS3 and its matched control oligonucleotides. Position, sequence, binding and affinity data of selected PKBα antisense probes; αAS3, and its control oligonucleotides which were design to possess similar theoretical parameters as αAS3

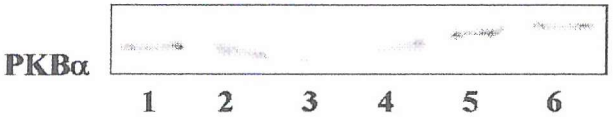
Probe	Sequence	Dimers (No. H bonds)	DG(kcal)	Tm (°C) NN	GC
αAS3	GCCTCCGCTCACTGTCCA	-	1.4	66	58
Sense	TGGACAGTGAGCGGAGGC	-	1.4	-	-
Random	CGGACTTTCGCCCCTCAC	-	0.7	-	-
Mismatch (4 base changes -)	GACTCCTCCCAATGTTCA	-	3.6	-	-

Figure 3.8 - Depletion of PKBα in 3T3-L1 Fibroblasts is specific for αAS3 antisense probe. 3T3-L1 fibroblasts were treated with 4μM oligonucleotide over a 96-hour transfection period. The results were analysed by SDS-PAGE and Western blotting and the PKBα levels quantitated by densitometry. **A.** Graphical representation of levels of PKBα levels where 100% Expression is the intensity of the PKBα band in no ODN Control (lipofectin only). (n=4) **B.** Representative PKBα Western blot showing strong depletion of PKBα only in αAS3 treated cells, Lane 1, lipofectin/no ODN; Lane 2, Sense; Lane 3, αAS3; Lane 4, Mismatch; Lane 5, Random; Lane 6, no lipofectin/no ODN.

A.


Depletion of PKBα in 3T3-L1 Fibroblasts Treated with αAS3 or its Matched Controls
PKBα Expression (% of Lipofectin)
110
100
90
80
70
60
50
40
30
20
10
0
100%
95%
8%
82%
96%
90%
Lipofectin
Sense
aAS3
Mismatch
Random
Control
Treatment

B.


PKBα
1
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The control oligonucleotides did not significantly affect the PKBα levels compared to the levels observed in untreated or lipofectin™ only cells. These experiments, therefore showed, αAS3 to be a potent and unique antisense probes directed against PKBα

Having established that αAS3 is an effective antisense probe against PKBα compared to various controls, it was important to confirm that αAS3 acts specifically against PKBα and does not act against the mRNA coding for other proteins. Therefore the effect of the αAS3 probe and its matched controls on the protein levels of other signalling proteins including MAP kinase, p70S6 Kinase and SHP-2 was analysed by Western blotting (see figure 3.9). The αAS3 probe also did not deplete the other major PKB isoform PKBβ suggesting that αAS3 is an isoform specific antisense probe against PKBα (see figure 3.10).

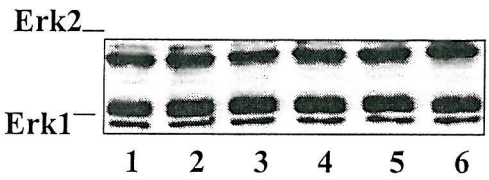
These western blots showed no change in the expression levels of these other proteins indicating that the probe αAS3 or its controls did not have any non-specific effect on these cells. Therefore, these control western blots showed that the αAS3 antisense probe acts only on the desired mRNA sequence and depletes only PKBα.

The affect of αAS3 on overall cellular protein levels and cell function was also investigated. Treatment with the probe αAS3, or the various controls was found not to affect overall cellular protein levels, as shown by amido black staining of the nitrocellulose blotting membrane (see figure 3.9). The overall cell function and viability of the 3T3-L1 Fibroblast cells was not affected by the various antisense and control oligonucleotide treatments, since, stimulation of non-linked pathways in treated cells could still be achieved. For example, vanadate or EGF induced activation of the MAP kinase pathway could still occur (data not shown).

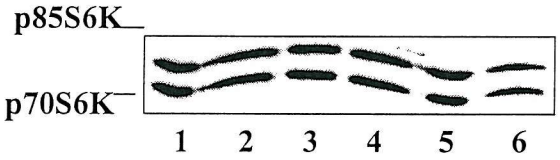
It is clear that an effective PKBα antisense probe had been established and the optimum conditions for its use in the 3T3-L1 Fibroblast cells determined. The selected PKBα probe, αAS3 was found to be a very potent antisense probe giving a depletion of PKBα protein of around 92% when used at the optimum conditions of 4μM for 4 days. Suitable control oligonucleotides (Sense, Random and Mismatch) and control western blots (i.e. PKBβ, MAPK and p70S6K) have also shown that this probe exerted a specific antisense effect for PKBα.

Figure 3.9. Use of the PKBα antisense αAS3 and its matched controls does not affect the levels of other cellular proteins. A. Erk1/2 blot, **B** S6K blot, **C.** SH-PTP blot, **D** Amido black staining show general protein levels are unaffected. **Lanes; 1** Lipofectin only, **2** Sense, **3** αAS3 antisense, **4** mismatch, **4.** Random, **6** Untreated.

A


Erk2
Erk1
1 2 3 4 5 6

B


p85S6K
p70S6K
1 2 3 4 5 6

C


SH-2PTP
1 2 3 4 5 6

D

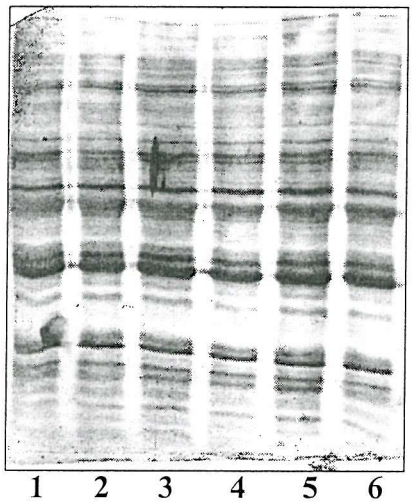
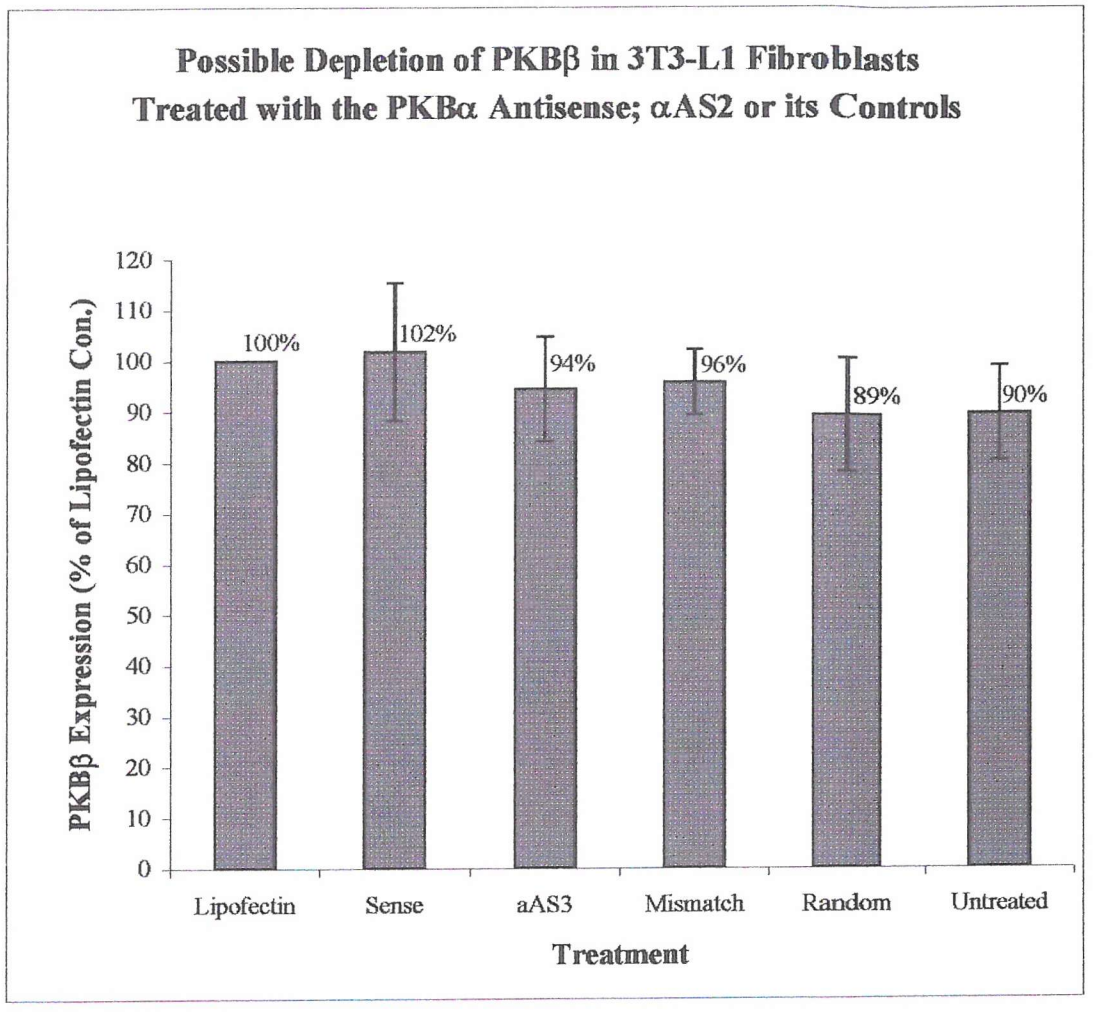
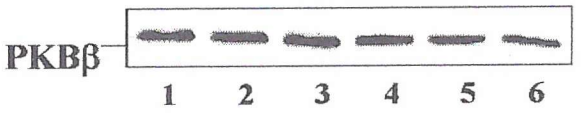

1 2 3 4 5 6

Figure 3.10 - The PKBα antisense probe αAS3 does not deplete of PKBβ in 3T3-L1 Fibroblasts. 3T3-L1 fibroblasts were treated with 4μM oligonucleotide over a 96-hour transfection period. The results were analysed by SDS-PAGE and Western blotting and the PKBβ levels quantitated by densitometry. **A.** Graphical representation of levels of PKBβ levels where 100% Expression is the intensity of the PKBβ band in no ODN Control (lipofectin only). (n=3) **B.** Representative PKBβ Western blot showing no depletion of PKBβ with any treatment, Lane 1, lipofectin/no ODN; Lane 2, Sense; Lane 3, αAS3; Lane 4, Mismatch; Lane 5, Random; Lane 6, no lipofectin/no ODN.

A.


Possible Depletion of PKBβ in 3T3-L1 Fibroblasts
Treated with the PKBα Antisense; αAS2 or its Controls
PKBβ Expression (% of Lipofectin Con.)
120
110
100
90
80
70
60
50
40
30
20
10
0
100%
102%
94%
96%
89%
90%
Lipofectin
Sense
aAS3
Mismatch
Random
Untreated
Treatment

B.


PKBβ
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4
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The PKBα antisense probes αAS1 and αAS3 were also tested in a mouse 3T3-L1 adipocytes cell line which can be obtained from the fibroblasts by cell differentiation (data not shown). These cells were shown to contain easily detectable levels of PKBα protein by western blotting with the UBI antibody (not shown). Initially, the PKBα probes αAS1 and αAS3 were tested at 5μM for 4 days in the adipocytes with both probes found to be as effective as antisense agents in these cells as they were in the fibroblasts, giving PKBα protein depletion of 70% and 90% respectively (data not shown).

Further processing of the more effective PKBα probe: αAS3 was performed by testing this probe using the same time course and concentrations optimised for use in the fibroblast cell transfections namely 4μM for 4 days. The αAS3 antisense transfections were compared to the designed PKBα control oligonucleotides (sense, random, 4 mismatch) as well as to lipofectin™, only and untreated cells and analysed by western blotting. As in the fibroblasts the αAS3 probe was shown to deplete the protein level of the PKBα isoform by more than 90%, whilst the control oligonucleotides had no effect on this translation when compared to the lipofectin™ only and untreated controls. Therefore at the same conditions as in fibroblasts, the probe αAS3 proved to be a very effective antisense agent in the related mouse 3T3-L1 adipocyte cell line.

To confirm the potential usefulness of this antisense, the αAS3 antisense probe was then shown to be isoform specific (i.e. not deplete PKBβ protein levels) and not to affect the levels of other cellular proteins (e.g. MAPK) or general cell function in the adipocytes (data not shown). From this it was apparent, that the αAS3 probe was not only an effective antisense agent in the fibroblasts but could also be used as a potent antisense tool in adipocytes.

It is therefore clear that with the antisense probe αAS3 I have developed an effective isoform specific antisense tool against mouse PKBα which when used at its optimum transfection conditions of 4μM for 4 days has been shown to significantly the deplete (>90%) the protein levels of this isoform in two mouse cell lines namely 3T3-L1 fibroblasts and adipocytes. A second PKBα antisense probe αAS1, was also shown to effectively deplete the PKBα protein levels by more than 70% in both the cell lines and may with further study (i.e. optimisation and control experiments) prove to be a useful secondary antisense. It is clear that with the design of these two probes, I have

developed the first effective and isoform specific agents which can directly alter the protein levels and, hence, potentially the function of PKBα.

3.2 Discussion of PKBα Antisense Probes Development

3.21 Design of PKBα Probes

From the results presented in this chapter it is clear that with αAS3 and to a lesser extent αAS1, I have designed effective antisense probes that individually target the mRNA of the α isoform of PKB and causes significant depletion of the protein levels of this isoform. These two antisense probes are not only the first known direct modulators of endogenous PKB protein levels but also the first functionality inhibitor to directly target this important cellular signalling component. Not only is this the case but also by targeting just the α isoform of PKB these probes enable research into isoform specific differences of PKB functioning. Hopefully the development of these probes will open up the study of the roles of PKB.

These effective antisense probes were designed and tested following similar methods to ones previously used in the design of effective antisense tools within the group for example in the development of effective antisense probes against Erk1/2 (Sale *et al.*1995) and the phosphatase PTPα (Arnott *et al.* 1999).

The most successful PKBα antisense probe αAS3 was targeted against the 3'translated region of PKB mRNA which would translate to the carboxyl terminal of the protein. As will be discussed later in this section, the 3'-translated region targeted by this probe represents a fairly novel location for antisense targeting. Previous probes designed in this research group and most antisense probes in general are directed towards the initiation codon, which was the region the other effective PKBα antisense probe αAS1 targets.

In the 3T3-L1 fibroblasts, the effective PKBα antisense probe αAS3 was found to give around a 92% depletion of PKBα protein levels compared to controls when used at the experimentally

determined optimum levels of a 96-hours/4μM transfection protocol. As with the fibroblasts the αAS3 probe was also shown to be an effective antisense tool against PKBα in 3T3-L1 adipocytes. The same optimum conditions namely 96 hours/4μM used in the 3T3-L1 fibroblasts also proved to be the most effective conditions for use of αAS3 in the adipocytes given around a 95% depletion of PKBα protein levels. Therefore, the probe αAS3 is effective as an antisense probe in both the mouse 3T3-L1 fibroblast and adipocyte cell lines.

From the transfection time experiments (5μM αAS3) the time required to deplete PKBα by 50% was estimated to be around 40 hours. A 96 hour transfection time protocol was required for maximal depletion of PKBα, during which it was necessary for fresh oligonucleotide to be reapplied every 48 hours. This may suggest that PKBα protein has a fairly long turnover before endogenous protein degradation is completed. The re-addition of the oligonucleotide required during this transfection may also be a measure of the degradation rate of the antisense probe. The fairly high αAS3 concentration of 4μM required for maximum depletion of PKBα protein may be some indication of the levels of PKBα in the cells or a measure of the uptake/distribution parameters of the probe.

It would be of great interest to study the possible uptake, distribution and extra/intracellular processing of the αAS3 oligonucleotide, to see what the characteristics of these factors are for αAS3 and other antisense probes. This could possibly be studied by the use of fluorescently tagged versions of the oligonucleotide probes and analysing their processing by confocal microscopy. Hopefully, this would not only provide a useful insight into the functioning of my PKBα antisense probe αAS3 but may also be of some use in the design and development of future antisense protocols. It would also be interesting to investigate the half-lives of the oligonucleotide and PKBα.

Previous antisense agents developed show that the optimum transfection conditions to yield the maximum antisense effect vary greatly between the protein targeted and the cell type studied. Therefore, it is important to establish the optimum transfection conditions for each different protein targeted and each antisense probe designed. A previously designed Erk1/2 antisense probe, EAS-1, required a 48 hour/2μM transfection protocol in the fibroblasts and a 96 hour/4μM transfection protocol in the adipocytes for maximum depletion. Another antisense agent directed against PTPα required a 192-hour/15μM transfection schedule in the adipocytes to elicit the maximum antisense

response. In the present study the optimum conditions for use of the PKBα antisense probe αAS3, found to be identical in the fibroblast and adipocytes. Therefore, it would be interesting to see if these similar transfection conditions reflect a similar PKBα expression or turnover, or similar uptake, distribution or processing of αAS3 between the 2 cell lines.

Another developed PKBα antisense probe αAS1, which targets the initiation codon of the PKBα mRNA was also shown to be a fairly effective antisense in the fibroblasts and the adipocytes. Initial experiments with this probe showed it to be a fairly effective antisense agent against PKBα, although not as effective as the selected PKBα probe αAS3 at the tested concentrations. A mini dose response curve (2, 5 and 10μM) for this probe already showed that 10μM was the most effective concentration for this probe yielding a depletion of >80% of the PKBα protein levels. If required, αAS1 could still prove to be an effective single isoform antisense probe against PKBα. This may be useful in the study of PKBα in other cell lines if αAS3 did not yield a significant antisense effect in these cells. The initiation codon region targeted by this probe showed no significant homology to either the β or the γ PKB isoforms so is specific for only the α isoform. The PKBα sequence targeted by this probe also showed little similarity to any other mRNA sequences at 3 or less mismatches, so the probe αAS1 should also not affect the levels of any other proteins. Possible further study to determine the optimum transfection conditions for αAS1 could establish how effective and useful as a PKBα antisense agent this probe could be.

In the studies into developing an effective PKBα antisense, the initiation codon directed probe, αAS1 was not as effective as my selected probe αAS3. In fact, even when used at its optimum concentration of 10μM which is more than double the 4μM optimum for αAS3 this probe only gave a 80% depletion of PKBα protein levels compared to the 92% depletion seen with the more potent αAS3 probe. This is likely to be due to the fact that the initiation region was located in a non GC rich area of sequence which would lead to reduced affinity probes which would not bind the target as strongly.

Potential probes targeted against the initiation codon were found to be self-complementary and self-dimerisation problems would be likely to reduce the potency of the probe. The reduced antisense potency of this probe may occur via hindering the probes cellular uptake/distribution and

accessibility to the target sequence. This may explain the need for a higher concentration of this probe to generate an effective antisense effect. Therefore, in the case of PKBα, the initiation region is not the most effective region to target with an antisense strategy. This indicates that despite previously held design briefs for the development of effective antisense probes, targeting the initiation codon region may not be the ideal site for maximal antisense potency and other site/design rationales may be more important in antisense design.

The successful and potent targeting of the 3'-translated region with potent antisense probes against the α isoform of PKB suggests that the most important parameters in the design of antisense are a high binding ability and no self complementation rather than the sequence position of the probe. Therefore, GC rich regions of sequence wherever they occur within the mRNA look to be the best regions to target as they provide a high affinity-binding site for the probe. The PKBα probe αAS3 has a high free energy of binding leading to potentially high affinity binding and is also GC rich again giving stronger binding to the target mRNA.

It is possible that the 3'translated region of the PKBα mRNA targeted by aAS3 may be more accessible for probe hybridisation, as this region contains no secondary structure. Likewise the aAS3 probe targeting this region also has no secondary structure problems which may affect the uptake and distribution of other probes. The increased binding and accessibility of the probe to its target act to increase the antisense potency. These parameters may also aid the access and activity of RNase-H, a nuclease which digests mRNA in many antisense strategies. The synthesis of αAS3 as a phosphorothioate oligonucleotide is one method by which the potency of the probe can be increased, as phosphorothioate oligonucleotides stimulate the activity of RNase-H.

The successful targeting of the 3'translated region leads to the possibility of designing potent antisense probes against other proteins where the initiation region of the mRNA sequence is an unsuitable site due to the presence of a hairpin region or low GC content. Previously these proteins had not been thought possible to target with antisense strategies. However, the potential to target any GC rich regions of the sequence that are accessible to the probe, should enable these proteins to now be targeted by antisense. This may open the way for a resurgence in antisense techniques as a tool for elucidating many components of the signal transduction cascades.

Targeting different regions throughout the mRNA sequence with potential antisense probes is rapidly becoming the way forward for antisense design. The so-called "gene walking" strategy of antisense design in which an array of probes are designed and tested for their antisense potency is gaining increasing support as the best rationale for the design of effective antisense probes. This high output technique allows for the selection of potential probes throughout the target sequence, each with unique properties which may increase the potency of these potential antisense agents. These varying selection criteria including position targeted and sequence content may affect delivery, binding and antisense action (i.e. RNase-H stimulation).

This form of sequence and position driven antisense design should lead to the generation of several effective antisense probes against different regions which can then be further characterised. It may be possible to use two or more probes directed against different points of the mRNA sequence to bring about a combined antisense effect possibly by eliciting several different antisense mechanisms. This synergistic multi-probe approach could lead to the design of even more potent antisense tools giving an even higher depletion of endogenous protein levels. It may therefore, be possible to use the PKBα probe αAS3 in conjugation with another PKBα probe. For example, the fairly successful PKBα antisense probe αAS1 could be used in tandem with αAS3, in order to enhance the effective depletion of endogenous PKBα protein levels further.

The possibility of effectively targeting other regions of mRNA with antisense also leads to the possibility of designing multi-isoform probes. Although αAS2; the potential multi-isoform probe was not very successful in initial tests, it did have some antisense effect on PKBα, perhaps further investigation with this probe at different concentrations or for a longer transfection time is warranted to see if it could be used as an effective PKBα antisense agent.

This probe could theoretically act against the α, β and γ sequences, therefore, it may need to be used at higher concentrations or for a longer transfection time to achieve a maximal antisense effect and deplete both isoforms effectively. When used in a 96 hour transfection at 5μM in the 3T3-L1 fibroblasts, αAS2 yielded a 60% depletion of PKBα. This suggest αAS2 has a small antisense effect against PKBα and so may warrant further study. The possible use of this probe against the β isoform of PKB is discussed in Chapter 4 (see 4.2.1).

The synthesis of the oligonucleotides as the more stable, nuclease resistant phosphorothioate form was designed to enhance the potency of the antisense probes as has been previously reported (Brown *et al.* 1994, Dean *et al.* 1994). Also, phosphorothioate oligonucleotides have been shown to stimulate the nuclease RNase-H, which is the most frequently used cellular mechanism by which antisense oligonucleotides exert their effects on the target mRNA/protein levels. This means that phosphorothioate oligonucleotides have an increased potency as they remain active for longer and also increase the stimulation/action of RNase-H the major antisense directed effector. Phosphorothioate oligonucleotides, however, have lower affinity for binding to the target mRNA than unmodified oligonucleotides. This may explain why only very high affinity binding sequences, including the one utilised by the PKBα antisense, αAS3 prove to be effective targets for phosphorothioate oligonucleotides.

The use of unmodified oligonucleotides is not recommend as they tend to have a reduced cellular uptake and are very unstable and rapidly metabolised. Not only does this rapid degradation of unmodified rapidly reduce their half life and potency, but also this degradation leads to the generation of free nucleotide bases which can be damaging to the cell with dGMP being particularly cytotoxic.

As well as phosphorothioate oligonucleotides, other so-called "second generation" antisense agents have been developed and effectively used. These include O'methyl modified oligonucleotides which have a methyl group at the C3' position and which despite their failure to support RNase-H mediated mRNA cleavage are still effective antisense agents possibly due to their high affinity binding.

It is possible to combine the positive attributes and reduce the negative shortcomings of these two modifications by synthesising oligonucleotides which contain both elements. In this strategy, the O'methyl modified nucleotides occupy the last three positions at either end of the oligonucleotide, with phosphorothioate-modified bases occupying the positions in-between. The O'methyl modified end bases in this antisense increase the probe's binding affinity. The phosphorothioate-modified bases in the middle of the probe increase uptake and stability of the probe thus prolonging its antisense effect. The phosphorothioate modification also acts to stimulate RNase-H mediated cleavage thus increasing antisense potency. Therefore, the combined effect is to increase the affinity,

stability and activity of the antisense. The increased effectiveness of this combined modification is something that would be interesting to research further, to see whether this modification could enhance the antisense potency of the PKB antisense probes. Other alternative oligonucleotide modifications worth considering in order to enhance the overall effectiveness of future antisense probes are known as "third generation" antisense oligonucleotide modifications. These include, peptide nucleic acids (PNAS), where a peptide modification is added to the oligonucleotide to increase antisense potency and half-life whilst minimising unwanted potential side effects. At present, the use of these and other modifications is limited, however, it is important to be aware of the continuous evolution of antisense agents to maximise the effectiveness of antisense as tool in biological research.

Phosphorothioate oligonucleotides can sometimes exhibit non-specific and non-antisense side effects. Therefore, it was important to demonstrate that the PKBα antisense probe, αAS3 was exhibiting genuine antisense effects, with the use of suitable control oligonucleotides. The selected control oligonucleotides (sense, random and 4 mismatches) for the αAS3 probe were found to be without effect on PKB protein levels when compared to lipofectin™ only and untreated cells. Therefore, it was evident that only αAS3 caused any inhibition of PKB translation and hence was acting as an effective antisense agent. This indicates that the specifically targeted antisense phosphorothioate probe αAS3 was having a genuine antisense effect and the changes in protein expression observed were not due to non-specific effects.

Analysis of the PKBα probe αAS3's antisense potency against PKBα by western blotting showed a clear depletion of the PKBα protein level with the antisense treatment. This was compared to the PKBα protein levels seen in the various control oligonucleotides which all showed no change in expression to that in lipofectin™ only/untreated cells. This indicates that these controls were ineffective against PKBα and only the probe αAS3 exerted a genuine antisense affect against this protein.

Database analysis revealed that the αAS3 probe and its respective control oligonucleotides were not complementary to the mRNA sequences of other proteins, with at least 4 base differences to any other mRNA sequences. Therefore, these oligonucleotides were unlikely to hybridise to other sequences and elicit non-specific/targeted antisense effects. Western blotting analysis against other

proteins including the β isoform of PKB, MAPK and p70S6K confirmed this to be the case.

Results showed that my PKBα, αAS3 probe and its matched control oligonucleotides did not affect total cellular protein levels as indicated by amido black staining of the transfer membrane. The lack of non-specific effects of αAS3 and its controls on cell viability could clearly be seen by light microscopy, which showed similar cell morphology with all treatments (not shown). Also, growth factor stimulation of non-PKBα pathways, for example MAP kinase stimulation by vanadate was unaffected by any of the oligonucleotide treatments (not shown).

Having discussed the rationale behind the sequence specific design of my PKBα antisense probe and its control oligonucleotides, it is now appropriate to consider the actual use of these probes including the transfection protocol, optimisation of the probe's use and general conditions for use.

3.22 Use of PKBα Antisense Oligonucleotides

The lipofectin™ liposome delivery system was used in transfection protocols for the first eight hours, to improve the uptake and delivery of the antisense to the cell and hence increase its potency. However prolonged use of lipofectin™ can lead to it becoming toxic to the cell. The use of lipofectin™ for more than 8 hours would be unsuitable in 3T3-L1 fibroblasts as they are more susceptible to its toxic effects. In the 3T3-L1 adipocytes which are a more stable and resilient cell line however, lipofectin™ has previously been used for longer period to enhance the uptake of the oligonucleotide. This system has previously been employed to deliver antisense probes into a variety of cells including the 3T3-L1 fibroblasts and adipocytes without any adverse effect on the function of the cell (Sale *et al.* 1995, Bennett *et al.* 1992).

In these experiments the lipofectin™ had no affect on the levels of either PKB isoform tested or any of the control proteins blotted against. It was found that lipofectin™ did not cause any alterations to cellular functions or overall protein levels. In the 3T3-L1 fibroblasts the use of lipofectin™ for the first eight hours minimised any side effects the lipofectin™ may have on the cells. In other cells lines, it may be useful to test the probes over a lipofectin™ time course to optimise the conditions for its use.

The concentration of lipofectin™ used in all transfections was $8\mu gml^{-1}$, the standard concentration previously used in antisense transfections. However, it may be necessary to vary the concentration of this delivery system in other cell lines to maximise antisense potency by altering its uptake and distribution parameters. Effective use of lipofectin™ at higher concentrations could be particularly useful when using more than one antisense probe in a single transfection for example in the targeting of more than one PKB isoform with separate antisense probes.

It should be noted that with lipofectin™ based transfections, only adherent cell lines can be successfully transfected and that cells grown in suspension cannot be transfected by this method. Lipofectin™ can only be used in serum free conditions since it is not serum stable, and is therefore unsuitable for use in totally serum dependent cell lines. In transfection of the 3T3-L1 fibroblasts, it was found that after the initial eight hours transfection using lipofectin™ in serum free media, 5% heat inactivated FBS had to be added for the remainder of the transfection period, usually 86 hours. This FBS addition acted to stabilise the fibroblasts, so the recovery of protein was sufficient for using in Western blots. When the serum free media was used throughout the study the protein recovery was much lower and the cells appeared to have lost some viability as judged by microscopy.

In experiments using the αAS3 antisense and control probes to test the cellular roles of PKBα, it will be necessary to use serum-free conditions for some of the transfection in order to down regulate cellular activity before growth factor stimulation. Therefore, for the final 20 hours of transfections the cells were treated with fresh oligonucleotide in a serum free media supplemented by 0.25% BSA, which acts to stabilise the fibroblasts and provide the correct serum starved environment for subsequent growth factor stimulation. In the antisense transfections of the more stable/resilient 3T3-L1 adipocytes, it was not necessary to use heat-inactivated serum as a stabilisation factor during the transfection period. These cells could be incubated for the full 4-day transfection protocol with the oligonucleotides in a serum free media containing a 0.25% BSA supplementation after the first eight hours.

By establishing the optimum conditions for using the PKBα probes, it enabled them to be used to the maximum effect in subsequent transfections in the mouse 3T3-L1 fibroblast and adipocyte cell lines. Any further optimisation that could be established involving the use of lipofectin™ or other

delivery systems and the exact transfection conditions would be obviously advantageous for future use of the PKBα antisense probe, particularly when using it in combination with other antisense probes or in other cell lines

The PKBα antisense probe, αAS3, has been shown to be an effective antisense agent in two mouse cell lines namely the 3T3-L1 fibroblasts and adipocytes with the probe showing similar optimum conditions and potency in the two cell types. It would be interesting to test the effectiveness of this antisense probe in other cell lines. Such studies would be particularly important in determining whether the antisense probe is as effective in other cell lines and particularly other species. This may be useful in finding suitable cell lines in which the probe is a potent antisense to use in the study of various possible roles of PKB particularly cell lines which are effective models of apoptosis.

Mouse PKBα mRNA is 86% identical to the rat PKBα mRNA sequence and 80% identical to the human PKBα mRNA sequence throughout the entire length. At the level of the mRNA region targeted by the probe αAS3, the rat mRNA sequence is identical and the human mRNA has only 1 base difference to the mouse mRNA PKBα sequence. Therefore, it is likely that this probe could be used to effectively deplete PKBα, not only in other mouse cell lines but also in rat cells and human cells after 1 base change. The high degree of homology of the probe target region shows to these other mammalian species also raises the chances that the PKBα antisense probe, αAS3 could also be used in other mammalian cell lines for example, dog, monkey and hamster cell lines which are frequently used as models for various conditions. New time course and concentration responses would have to be established for each cell type and different parameters may be more important in different cells. Therefore, it is important to thoroughly characterise the probes and its matched controls affects in each cell line tested

The optimum conditions for use of the use of the probe should be reassessed periodically to confirm that it is still functioning as it was designed. Within my transfection experiments I have noticed some variation in the potency of the αAS3 probe between different batches of cells. Therefore, it is important to test the probe is working to its maximum levels at regular intervals in every cell line it is used in.

Optimum conditions for the design of antisense probes have been established and used in the design and development of an effective antisense probes against the α isoform of the signalling protein PKB. With these important design briefs it is now possible to use these techniques to develop other antisense tools against other signalling components. Targeting mRNA at regions other than the initiation codon could be used to generate effective antisense probes against other isoforms of PKB. Initially it will be important to target the other isoforms of PKB particularly the other major PKB isoform, PKBβ (see chapter 4), before perhaps targeting other related proteins. One such possible target is serum and glucocorticoid induced kinase (SGK) which shows a similar substrate motif to PKB and so may act on a similar set of substrates to ones PKB is proposed to phosphorylate. Other signalling proteins related to the PKB pathway which could include the upstream PKB activators PDK1 and PI3K could also be targeted with antisense probes to establish PKB dependent and independent effects and general cellular roles of these proteins. After this potential downstream components of the PKB pathway including GSK3, p70S6 Kinase, PFK-2 and the pro-apoptotic BAD protein could be directly targeted with antisense to fully determine the exact roles of the PKB pathway and its individual components.

In summary, with αAS3, an effective antisense probe against PKBα has been designed, developed and tested in 3T3-L1 fibroblasts and adipocytes. This probe not only is the first known direct modulator of endogenous PKBα protein levels, but also is the first antisense probe developed in this group which is not targeted against the initiation codon of the mRNA. A second potential isoform specific PKBα antisense probe αAS1, which is directed against the mRNA initiation codon has also been shown to have great promise as an antisense agent. These probes can now be used to establish the cellular functions of PKB and particularly the roles the α isoform plays in these potentially critical cellular roles.

CHAPTER 4 - DESIGN AND DEVELOPMENT OF AN EFFECTIVE PKB ANTISENSE PROBE AGAINST PKBβ

4.1 Design and Development of an Effective PKB Antisense Probe Against PKBβ

4.1.1 Development of PKBβ Antisense

Having successfully designed a potent PKBα antisense probes the attention turned to the other major PKB isoform, PKBβ, and the development of an antisense strategy for this protein. A suitable and specific antibody (UBI) for detection of PKBβ levels by western blotting was obtained and PKBβ was found to be present in easily detectable quantities in the 3T3-L1 fibroblasts (not shown). Therefore, by following a similar design protocol to that of the PKBα antisense, I should be able to design, test and analyse the effectiveness of possible PKBβ antisense probes.

As with the design of the PKBα antisense, the mouse PKBβ sequence was obtained from the database (Accession number M95936) and this mRNA sequence was run through the Findprobe program (see figure 4.1). This produced a list of possible 18mer probeable regions throughout the mRNA sequence which could potentially be targeted by an antisense strategy. Using the PKBα design rationale the 3'-translated region of the PKBβ sequence was targeted for antisense attack. This more direct targeting approach was deemed to be most suitable based on experience and theories drawn from the design of the PKBα probes.

Firstly, the initiation codon region of the sequence again falls in a portion of the sequence which is not GC rich and probes against this region were likely to be self-complementary. Therefore this was a similar situation to the PKBα sequence, where targeting the initiation region with antisense was not nearly as effective as targeting the 3'-translated region of the mRNA with the probe αAS3. The 2 PKBα probes against other regions of sequence, αAS2 (kinase domain directed multi-isoform probe) and αAS4 (3'-untranslated region direct probe) were not particularly successful as antisense agents when compared to αAS3. Therefore, efforts were concentrated on the same region of sequence where the αAS3 probe so effectively attacked, namely the 3'translated region.

Figure 4.1 - Potential probe regions in the mouse PKBβ mRNA Sequence.
Mouse PKBβ mRNA sequence, analysed from 1 to 1741, with the G/C Rich regions, Hairpins & potential Probes shown: CAPITAL LETTERS - Probe regions; Single Dash (_) - G/C rich regions; Equals Sign (=) - Hairpin regions; - antisense probe region.

The sequence data from the 3'-translated region of the mRNA of PKBβ, as with the same region of PKBα, indicated this area would be a good target zone for an antisense strategy against the β isoform, as it contained a high GC content and so it should be possible to generate high affinity probes. Also this region of the PKBβ mRNA was found not to possess any secondary structure which could reduce probe accessibility or hybridisation to the target sequence which should again aid design.

Therefore, two PKBβ probes were initially devised against the 3'translated region of mouse PKBβ, namely βAS1 and βAS2. These 2 potential probes were selected, because of a high theoretical affinity for the target sequence and lack of self-complementation (intra/inter-probe dimerisation) between probes. The two designed probes were also shown to be specific for only the targeted PKBβ mRNA sequence when analysed using the probe mismatch database scan. Hence these two probes were deemed suitable for experimental analysis (see figure 4.2).

These probes were synthesised as phosphorothioates and their effectiveness as antisense probes tested initially in mouse 3T3-L1 fibroblasts. For the preliminary transfection experiments these two probes and αAS2; the possible multi isoform probe, were tested at a 5μM (mid-range) concentration over a 96 hour time period. From these results, βAS2 appeared the most promising antisense probe at 5μM giving around a 70% depletion of PKBβ protein expression. The βAS1 probe and the αAS2 multi-isoform probe were found to be weak antisense agents giving depletion of the PKBβ protein levels of around 37% and 28% respectively (see Figure 4.3).

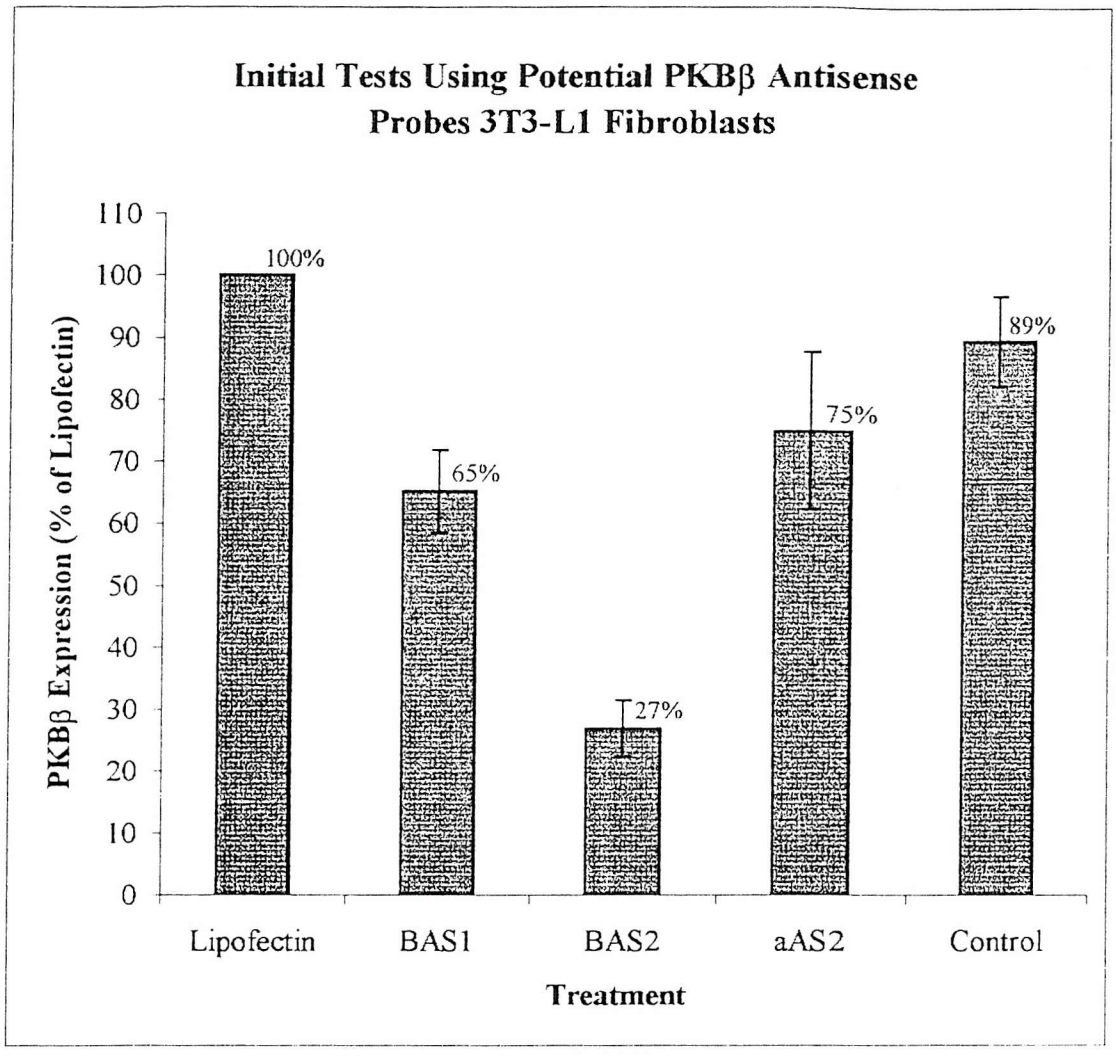
These initial findings showed that at 5μM these 3 potential PKBβ antisense probes were not very effective antisense agents. This is especially true when compared to the antisense potency the PKBα probes showed in initial tests. Therefore, the two specific PKBβ probes, βAS1 and βAS2 were characterised over a range of concentrations to establish their possible effectiveness. βAS1 and βAS2 were tested at 2,5 and 10μM concentrations over a 96-hour transfection period to establish the most effective PKBβ antisense probe (see figure 4.4). The probe βAS1 was found to give a small dose responsive depletion in PKBβ protein levels up to a maximum depletion of 50% when used at a concentration of 10μM. Therefore this probe was deemed unsuitable for further study. The other tested probe βAS2 on the contrary, was found to exhibit a marked dose responsive depletion, a 10μM concentration giving the maximal depletion of PKBβ protein expression at around 89%. These experiments confirmed that βAS2 was the most effective PKBβ antisense of the potential probes tested and so this probe was selected for further characterisation.

Figure 4.2 - Potential mouse PKBβ antisense probes. Position, Sequence, binding and affinity data of selected PKBβ antisense probes; βAS1 and βAS2, which does target the 3'translated carboxyl terminal region of PKBβ mRNA (higher numbers indicating stronger probe).

Position	Sequence	Dimers (No. H bonds)	DG(kcal)	Tm (°C) NN GC
Carboxyl Terminal (1565)	GCCTCCGCTCACTGTCCA βAS1	-	2.6	67.58
Carboxyl Terminal (1642)	CGGGGCTTCTGGACTCGG βAS2	-	2.6	67.58

Figure 4.3 -Treatment of 3T3-L1 fibroblasts with possible PKBβ antisense probes leads to varying but significant reductions in PKBβ. 3T3-L1 fibroblasts were incubated with 5μM of the potential PKBβ antisense probes for 96 hours with lipofectin ($8\mu gml^{-1}$) present for the first 8 hours. These cells and control (no ODN, +/- lipofectin) cells were then extracted as a whole cell lysate and prepared for SDS-PAGE. **A.** Depletion of PKBβ was quantitated by densitometry and results expressed graphically where 100% is taken to be the intensity in the lipofectin only control. **B**. Representative PKBβ Western blot of whole cell extracts. Lane 1, Lipofectin only; Lane 2, βAS1; Lane 3, βAS2; Lane 4 αAS2; Lane 5,Untreated. (n=3)

A


Initial Tests Using Potential PKBβ Antisense Probes 3T3-L1 Fibroblasts
PKBβ Expression (% of Lipofectin)
110
100
90
80
70
60
50
40
30
20
10
0
100%
65%
27%
75%
89%
Lipofectin
BAS1
BAS2
aAS2
Control
Treatment

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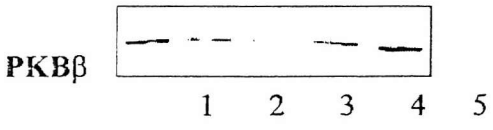
PKBβ
1
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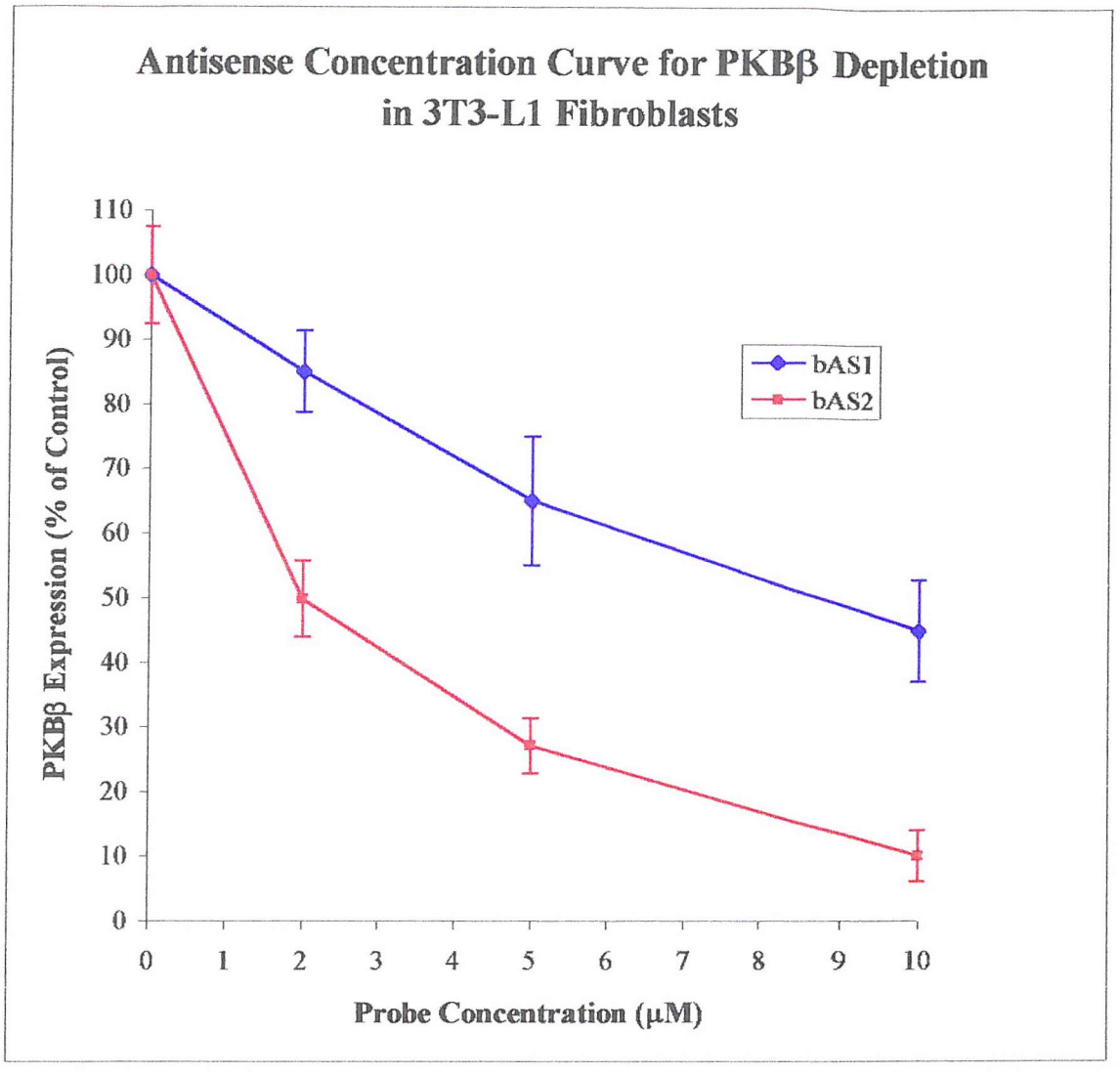


Figure 4.4 - βAS1 or βAS2 treatment of 3T3-L1 fibroblasts caused a concentration dependent depletion of PKBβ. 3T3-L1 fibroblasts were treated over a 96-hour transfection period using a range of PKBβ antisense concentrations. Extracts were analysed by SDS-PAGE and Western blotting and the PKBβ levels quantitated by densitometry. 100% expression is taken to be the level of PKBβ in no ODN controls (n=4).

In order to establish the optimum concentration of βAS2 to use in a 96-hour transfection, βAS2 was tested over a range of concentrations between 0.5 and 12 μM (see figure 4.5). Initial tests indicated that the optimum concentration would be around 10μM and so experiments were focused around this region (7-10μM). These studies revealed that 8μM was the most effective βAS2 concentration to use, as the protein levels of PKBβ were depleted by over 90% (90.9%) at this concentration. From these tests the IC50 of βAS2 was determined to be 2μM.

Transfection of mouse 3T3-L1 fibroblasts for 96 hour with 8μM βAS2 yielded a very high depletion of PKBβ protein levels (91%) as analysed by western blotting. Since this level of depletion was similar to the reduction of PKBα levels yielded over a 96-hour transfection time for the α probe αAS3, it was decided to continue to use a 96-hour transfection time for the β probe βAS2. The fact that the 96-hour transfection time for βAS2 is the same as the optimum time for the PKBα probe, also means use of the two probes in tandem to deplete both the major isoforms of PKB should be straightforward.

Therefore, the optimum conditions for using the PKBβ antisense probe βAS2 were defined as 8μM for 96 hours. Next the specificity and selectivity of βAS2 was tested using suitable control oligonucleotides and western blots for selected control proteins.

Suitable sense, random and mismatch (4 base changes) control phosphorothioate oligonucleotides were designed for the PKBβ probe βAS2. These controls all possessed similar affinity, thermodynamic and specificity/mismatch characteristics to the antisense probe βAS2 (see figure 4.6).

In control tests, βAS2 significantly depleted PKBβ levels in the mouse 3T3-L1 fibroblasts (> 90%) whilst the control oligonucleotides gave similar levels of the PKBβ to levels in untreated or lipofectin™ treated cells (see figure 4.7). In these experiments, the sense oligonucleotide was found to decrease the levels of PKBβ protein by around 25% when compared with lipofectin™/untreated controls, however, this was deemed not to be significant to the overall levels of PKBβ. Therefore, it was clear that βAS2 was having a sequence specific antisense effect on the PKBβ mRNA leading to an inhibition of protein translation and a depletion in the cellular levels of PKBβ.

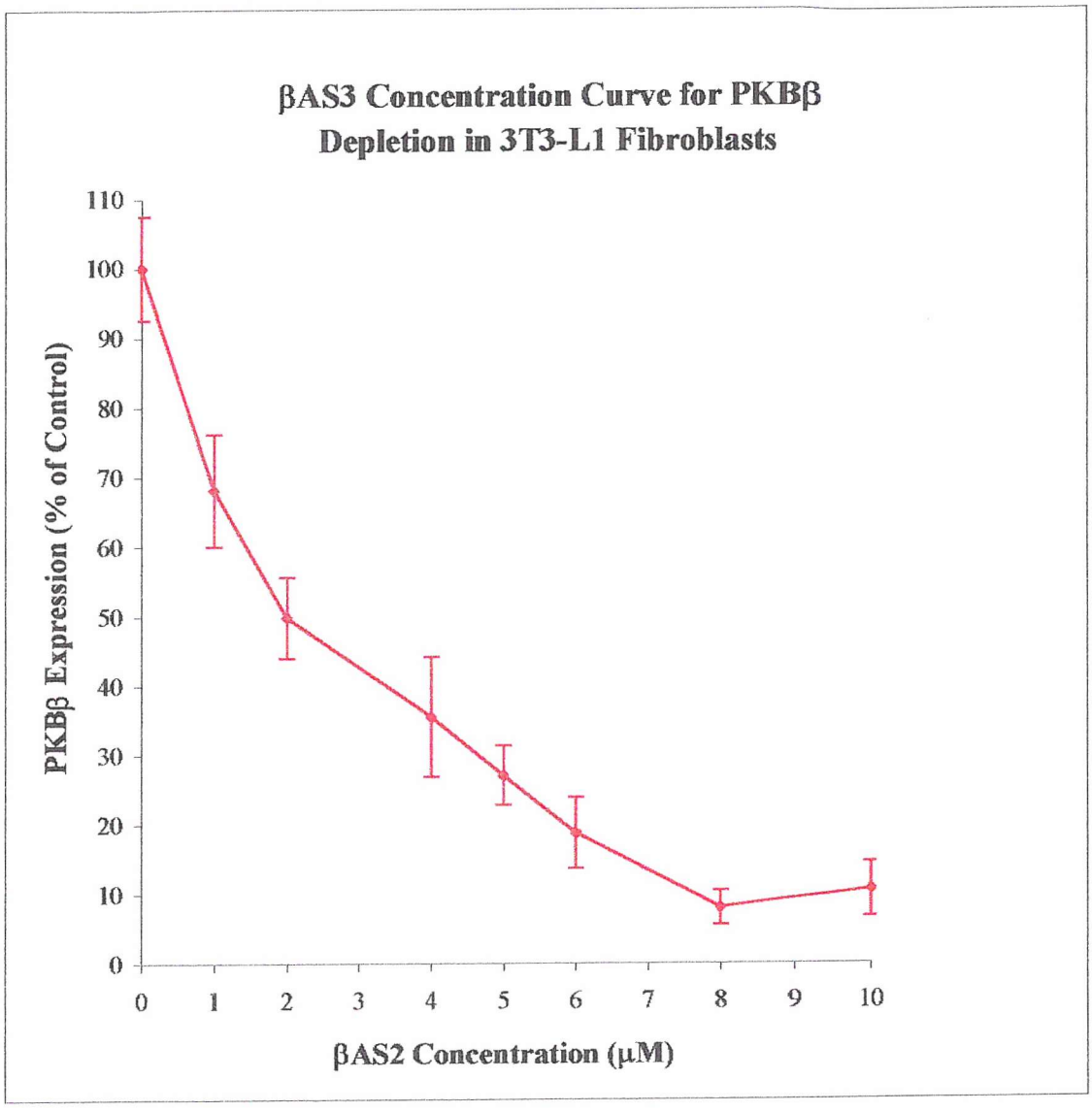


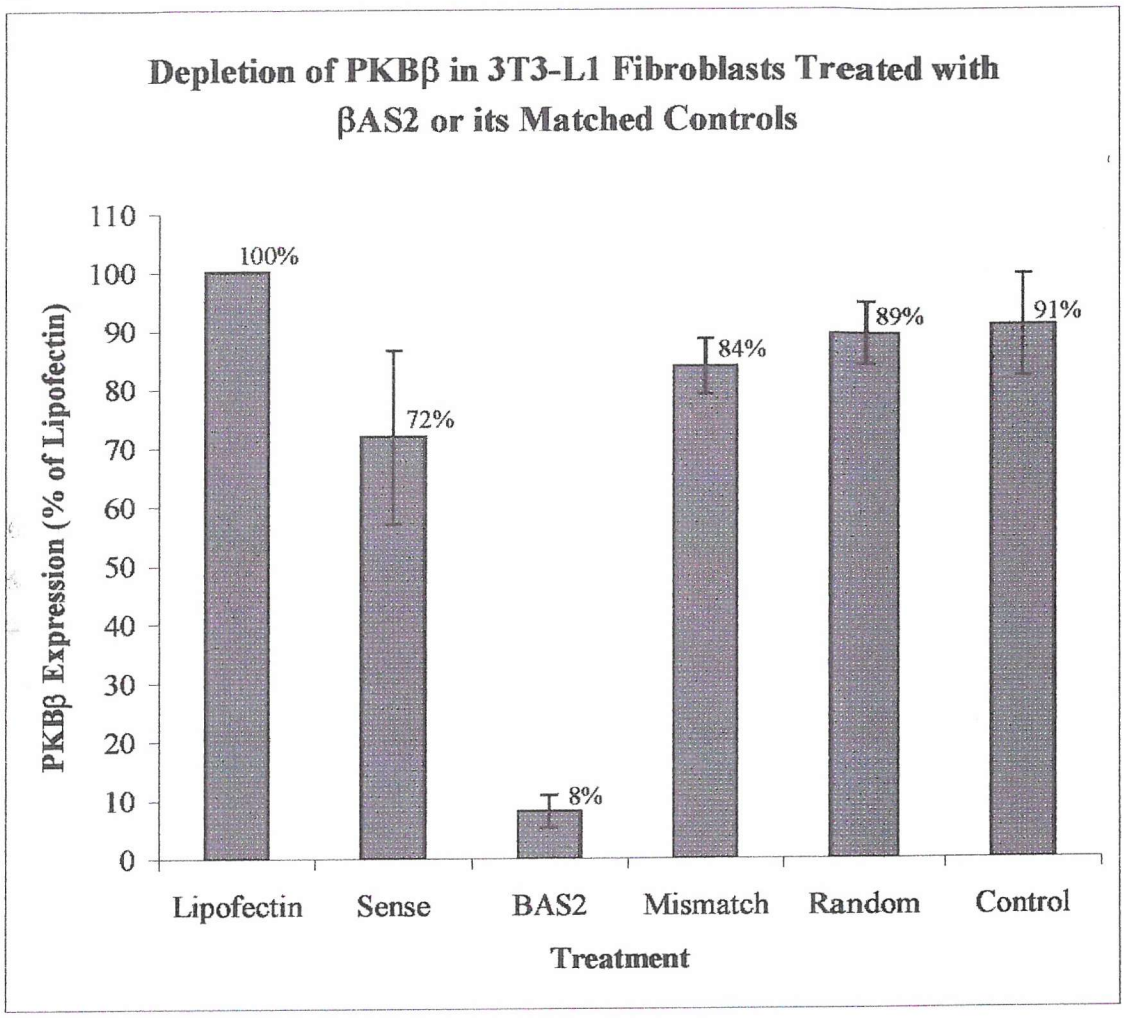
Figure 4.5 - βAS2 treatments of 3T3-L1 fibroblasts causes a significant depletion in PKBβ in a concentration dependent manner. 3T3-L1 fibroblasts were transfected for 96 hours using a range of βAS2 concentrations. Samples were analysed by SDS-PAGE and Western blotting and the PKBβ levels quantitated by densitometry. 100% expression of PKBβ is taken to be the level of PKBβ in no ODN (lipofectin only) controls (n=3)

Figure 4.6 - PKBβ antisense probes βAS2 and its matched control oligonucleotides. Position, sequence, binding and affinity data of selected PKBβ antisense probes; βAS2, and its control oligonucleotides which were design to possess similar theoretical parameters as βAS2

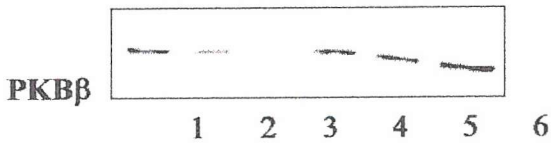
Probe	Sequence	Dimers (No. H bonds)	DG(kcal)	Tm (°C) NN GC
βAS2	CTCTCGGATGCTGGCTGA	-	1.4	66 58
Sense	TCAGCCAGCATCCGAGAG	-	1.4	- -
Random	AGTGAGCGGTTTCCCTGC	-	1.0	- -
Mismatch (4 base changes -)	CTATTGGATGATGGATGA	-	2.5	- -

Figure 4.7 - Depletion of PKBβ in 3T3-L1 Fibroblasts is specific for βAS2 antisense probe. 3T3-L1 fibroblasts were treated with 8μM oligonucleotide over a 96-hour transfection period. The results were analysed by SDS-PAGE and Western blotting and the PKBβ levels quantitated by densitometry. **A.** Graphical representation of levels of PKBβ levels where 100% Expression is the intensity of the PKBβ band in no ODN Control (lipofectin only). (n=3) **B.** Representative PKBβ Western blot showing strong depletion of PKBβ only in βAS2 treated cells. Lane 1, lipofectin/no ODN; Lane 2, Sense; Lane 3, βAS2; Lane 4, Mismatch; Lane 5, Random; Lane 6, no lipofectin/no ODN.

A.


Depletion of PKBβ in 3T3-L1 Fibroblasts Treated with βAS2 or its Matched Controls
PKBβ Expression (% of Lipofectin)
110
100
90
80
70
60
50
40
30
20
10
0
100%
72%
8%
84%
89%
91%
Lipofectin
Sense
BAS2
Mismatch
Random
Control
Treatment

B.


PKBβ
1
2
3
4
5
6

βAS2 was found to cause only depletion in the protein levels of PKBβ and not to exert any non-specific or non-antisense effects. The probe βAS2 did not affect general levels of total cellular protein as indicated by amido black staining of transfer membrane (see figure 4.8). This antisense probe βAS2 did not alter the levels of the specific cellular proteins; MAP kinase, SHP-2 or p70S6 Kinase, indicated by western blotting using suitable antibodies (see Figure 4.8). Western blotting also showed that the PKBβ specific antisense probe βAS2, did not affect the protein levels of PKBα when compared with the various controls (see figure 4.9). Therefore βAS2 was deemed to be an isoform specific antisense agent against PKBβ. In summary an effective and specific antisense probe (βAS2) against the β isoform of PKB has been designed. When, βAS2 is used at its optimum transfection conditions of 8μM for 96 hours a >90% depletion of PKB is achieved.

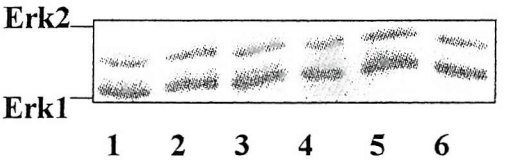
The PKBβ antisense probe, βAS2 was also tested in the related mouse cell line 3T3-L1 adipocytes. This probe was initially tested using the same time course and concentrations devised for the 3T3-L1 fibroblast transfection and the PKBβ depletion observed compared to the oligonucleotide controls (sense, random, 4 mismatch) and to lipofectin™ only and untreated cells. As in the fibroblasts, this probe was shown to deplete the protein levels of the β isoform by >90% whilst the control oligonucleotides had no effect on the translation of PKBβ (data not shown).

In the adipocytes the βAS2 probe did not affect the levels of other cellular proteins (i.e. MAPK) total cellular protein or cell viability (data not shown). The βAS2 antisense probe also showed an isoform specific depletion of PKBβ. Therefore, the βAS2 probe was found to an effective antisense probe against the PKBβ isoform in the adipocytes using the same conditions (96 hours/8μM) optimised in the fibroblasts. From this it was apparent that the β isoform probe βAS2 was not only an effective antisense agent in the 3T3-L1 fibroblasts but could also be used as potent antisense tools in adipocytes depleting PKBβ by >90% in both cell lines.

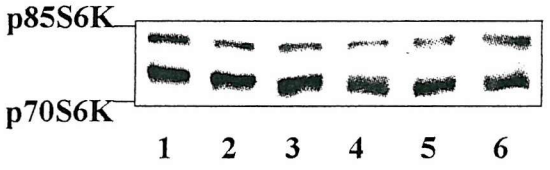
Therefore with βAS2, an effective PKBβ antisense probe had been developed. Various controls show that βAS2 is specific for the β isoform of PKB and gives a protein depletion of 90%. This specific PKBβ isoform antisense probe βAS2, is also the first known direct and specific modulator of endogenous PKBβ protein levels and should be a major breakthrough in research into this protein and its cellular roles.

Figure 4.9. Use of the PKBβ antisense βAS2 and its matched controls does not affect the levels of other cellular proteins. A. Erk1/2 blot, **B** S6K blot, **C.** SH-PTP blot. **D** Amido black staining show general protein levels are unaffected. **Lanes;** 1 Lipofectin only, **2** Sense, **3** βAS2 antisense, **4** mismatch, **4.** Random, **6** Untreated.

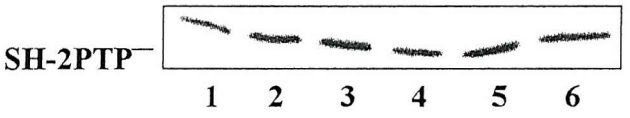
A


Erk2
Erk1
1 2 3 4 5 6

B


p85S6K
p70S6K
1 2 3 4 5 6

C


SH-2PTP
1 2 3 4 5 6

D

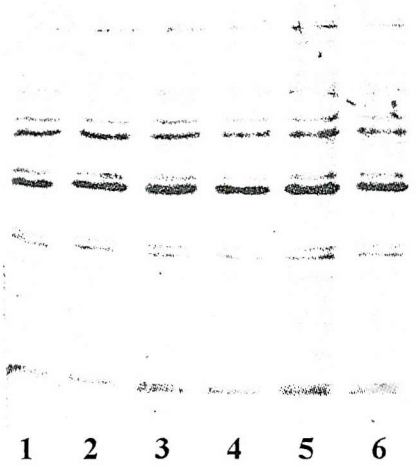
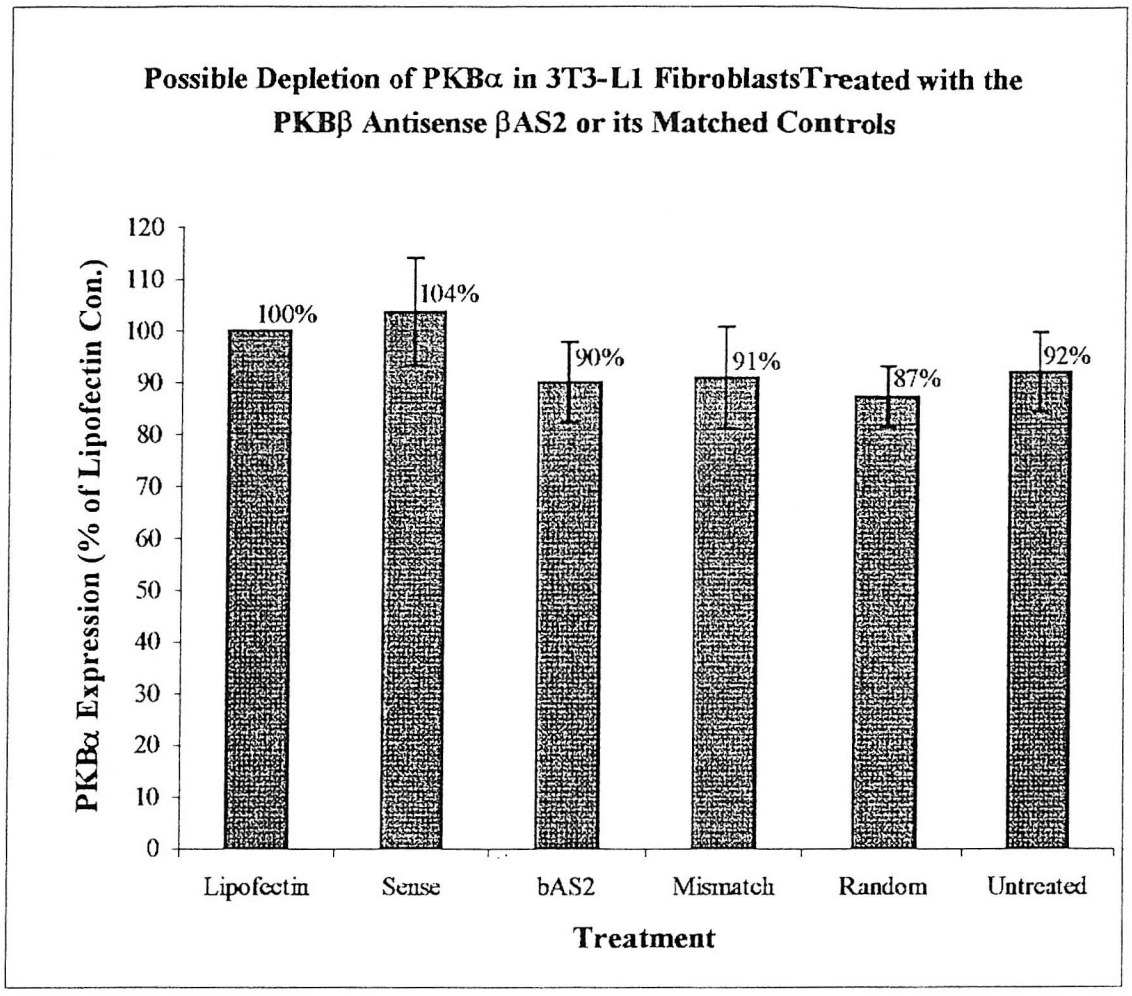
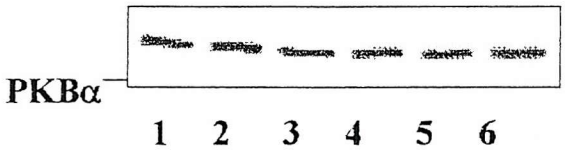

1 2 3 4 5 6

Figure 4.9 - The PKBβ antisense probe βAS2 does not deplete of PKBα in 3T3-L1 Fibroblasts. 3T3-L1 fibroblasts were treated with 8μM oligonucleotide over a 96-hour transfection period. The results were analysed by SDS-PAGE and Western blotting and the PKBβ levels quantitated by densitometry. **A.** Graphical representation of levels of PKBα levels where 100% Expression is the intensity of the PKBα band in no ODN Control (lipofectin only). (n=4) **B.** Representative PKBα Western blot showing no depletion of PKBα with any treatments, Lane 1, lipofectin/no ODN; Lane 2, Sense; Lane 3, βAS2; Lane 4, Mismatch; Lane 5, Random; Lane 6, no lipofectin/no ODN.

A.


Possible Depletion of PKBα in 3T3-L1 FibroblastsTreated with the PKBβ Antisense βAS2 or its Matched Controls
PKBα Expression (% of Lipofectin Con.)
120
110
100
90
80
70
60
50
40
30
20
10
0
100%
104%
90%
91%
87%
92%
Lipofectin
Sense
bAS2
Mismatch
Random
Untreated
Treatment

B.


PKBα
1 2 3 4 5 6

Therefore with the two antisense probes, αAS3 and βAS2, isoform specific antisense agents had been developed against the 2 major isoforms of PKB. Experiments show these probes to be very effective in depleting their respective PKB isoforms by >90% in both the mouse 3T3-l1 fibroblast and adipocyte cell lines. These probes can now be used to verify the precise cellular roles of PKB and establish if any isoform specific differences in PKB functioning exist. It is also important to design an effective and specific antisense strategy against PKBγ, yielding specific antisense agents against all 3 known isoforms of PKB.

4.1.2Design of Potential Antisense Probes against PKBγ

The other known isoform of the PKB family of protein kinases is PKBγ. This isoform has been recently cloned in mouse, human and rat and has been found to be similar to the other major PKB isoforms with around 85% homology at the protein level. This isoform is less widely distributed throughout many tissues but is however abundant in certain tissues including testis and brain. This isoform has also been shown to be overexpressed in certain cancers (reviewed in Datta *et al.* 1999). For example PKBγ is overexpressed in 40% of pancreatic cancers (Jones *et al.* 1998). Therefore it is important to target this recently cloned isoform with an antisense probe in order to establish the overall roles of PKB and particularly any isoform specific functions PKBγ may have in cells/tissues where it is expressed.

The publication of the mouse PKBγ sequence has enabled this isoform to be targeted by the similar antisense strategy previously used in the design of effective antisense probes against the other PKB isoforms. PKBγ has also been shown to be expressed at low levels in 3T3-L1 fibroblasts and adipocytes which makes these cell lines suitable for use in the development of an antisense oligonucleotide against PKBγ (Cronus *et al.* 1997).

The mouse PKBγ sequence was obtained from the EMBL database and run through the Findprobe computer program to generate a list of potential probable regions through the sequence (see Figure 4.10). As was found when designing the antisense probes against the α and β isoforms, the 3'translated region of PKBγ mRNA seems to be the most promising area to target with antisense probes. This region falls within an area of high affinity (GC rich) sequence, with little or no secondary structure, and so should be easily accessible to, and strongly bound by an antisense. Since this region proved to be so effective with the previous PKB antisense strategies, it was felt that this region should be used in order to design a highly effective PKBγ antisense probe.

Possible probes directed against selected regions of the 3`translated area have been processed via the self-complementation/dimer programme and mismatch/similarity database to check their potential as effective antisense probes. From these studies, 2 potential antisense probes have been selected and synthesised ready for testing in the two chosen cell lines (3T3-L1 fibroblasts and adipocytes) (see Figure 4.11). These probes were selected based on their high binding affinities, lack of secondary structure at the probe and mRNA level as well as their positions at the 3`translated region. These 2 PKBγ antisense probes, termed γAS1 and γAS2, are currently undergoing testing in the laboratory.

Figure 4.10 - Potential Probe Regions in the Mouse PKBγ Sequence

Below is a listing of the DNA sequence, analysed from 1 to 1760, with the G/C Rich regions, Hairpins & potential Probes shown

KEY:- CAPITAL LETTERS - Probe regions

Single Dash (_) - G/C rich regions

Equals Sign (=) - Hairpin regions

gaattcggca cgaggggggc tcagagggga gccatcatga gcgatgttac cattgtgaaa gaaggttGGG TTCAGAAGAG GGGAGaatat ataaaaaact

ggaggccaag atacttcctt ttgaagacag atggctcatt cataggctat aaggagaaac ctcaagatgt ggacttacct tatcccctca acaacttctc

agtggcaaaa tgtcagttaa tgaaaacaga acgaccaaag ccaaatacat ttattatcag atgtcttcag tggaccactg ttatagagag aacatttcat

gtagatacac cagaggaaag agaagagtgg aCGGAAGCTA TCCAAGCCGT AGCCGACCGA TTGCAGAGGC AAGAGGAGGA GAGGAtgaat tgtagcccaa

cctcacagat tgataatata ggagaagaag agatggatgc gtctacaacc catcataaaa gaaagacgat gaatgatttt gactatttga aactactagg

taaaggcact tttgggaaag ttattttggt tcgagagaag gcaagtggaa aatactatgc tatgaagatt ctgaagaaag aagtcattat tgcaaaggat

gaagtggcac acactcttac tgaaagcaga gtactaaaga acaccagaca tccatttttа acatccttga aatattcctt ccagacaaaa gaccgtttgt

gttttgtgat ggaatatgtt aatggcggag agctgttttt ccatttgtcg agagagcgag tgttctctGA GGACCGCACA CGTTTCtatg gtgcagaaat

tgtctctgct ttggactatc tacattctgg aaagattgtg taccgtgatc tcaagttgga gaatttgatg ctagataagg atggccatat aaaaattacg

gattttgggc tttgcaaaga agggatcaca gatgcagcta ccatgaagac attctgtggc acaccagagt acctggcacc agaggtatta gaagataATG

ACTATGGCCG AGCCGTGGAC TGGTGGGGCT TAGGTGTTGt catgtatgaa atgatgtgtg gaaggttgcc tttctacaac caggatcatg agaaactctt

tgaattaata ctaatggaag acattaaatt CCCCCGAACA CTCTCTTCag atgcaaaatc attgctttca gggctcttga taaaggatcc aaatAAACGC

CTTGGTGGAG GGCCAGatga tgcaaaagaa atcatgaggc atagtttttt ttctggagta aactggcaag atgtatatga caaaaagctt gtacctcctt

ttaagcctca agtaacatct gaaacagaca cccgatattt tgatgaagaa tttacagctc agactattac aataacacca cctgaaaagt ATGACGACGA

CGGCATGGAC GGCATGGACA ACGAGCGGCG GCCACACTTC CCTCAGTtCT CCTACTCTGC AAGCGGACGG GAATaagttc ctttcagtct gtttctacac

tgtcatctta gactttgcct gagactgatt cctggacatc tctaccagtc ctcgctctta cagttagcag gggcaccttc tgacatccct gACCAGCCAA

GGGTCTTCAC CCTCACCACC tttcactcac atgaaaccat atacacagac actccagttt tgtttttgca tgaaattgta tctcagtcta aggtctcatg

ctgttgctgc tactgtctta ctattatagc aaccttcaga agtaatctcg tgccgaattc

Figure 4.11- Potential mouse PKBγ antisense probes. Position, Sequence, binding and affinity data of selected PKBγ antisense probes; γAS1 and γAS2, which targets, the 3'translated carboxyl terminal region of PKBγ mRNA (higher numbers indicating stronger probe).

Position	Sequence	Dimers (No. H bonds)	DG(kcal)	Tm (°C) NN GC
Carboxyl Terminal (1414)	CGGCCGCTCGTTGTCCAT γAS1	-	1.6	74.60
Carboxyl Terminal (1453)	CCCGTCCGCTTGCAGAGT γAS2	-	1.3	71 60

4.2 Discussion PKBβ Antisense Probe Development

4.21 Design of PKBβ Probes

Having successfully established an effective antisense strategy for targeting the α isoform of PKB it next seemed appropriate to try to do the same with the second major isoform of PKB, namely PKBβ. This was subsequently successfully achieved with the design and development of the probe βAS2 which effectively depletes PKBβ protein levels by >90% when used at optimum levels in 3T3-L1 fibroblasts and adipocytes.

The design and development of the PKBβ probe was based on the design rational laid down in designing the PKBα probe αAS3 and discussed in chapter 3. Firstly, in designing the β isoform specific antisense only the 3' translated region of the PKBβ mRNA was targeted, as this was the region so successfully attacked by the PKBα probe αAS3. As previously seen in the mRNA sequence of PKBα, the 3'translated region of PKBβ mRNA is GC rich and contains no secondary structure, so should be an ideal area to target for antisense attack.

Initially two potential antisense probes were directed against this region and tested at various concentrations. These experiments established βAS2 as a potentially very effective antisense probe against PKBβ. As with the PKBα isoform probe αAS3, the designed PKBβ probe βAS2 has a high GC content with a high free energy of binding. Therefore, this probe would be expected to hybridise strongly to its target sequence in the PKBβ mRNA and this was clearly borne out by the strong antisense effect seen with this probe.

The PKBβ antisense probe βAS2 targets the 3'translated region of PKBβ mRNA which is the same region to the PKBα mRNA so successfully targeted by αAS3. In both these sequences, the 3'translated region targeted, is highly GC rich and contains no secondary structure. This enables the design of high affinity, complementary probes which have easy access to the target mRNA, they then hybridise to eliciting an antisense effect. The strong probe binding and access these target areas provide leads to effective stimulation of a potent antisense effect and hence inhibition of protein translation.

The successful targeting of the 3' translated region in both the PKBα and β antisense strategies emphasises the potential of this region as a potent mRNA target site for antisense attack. Previously

most antisense probes target of the initiation codon suggesting that the position of the mRNA sequence targeted is of key importance. However the results presented here, suggest that in design of effective antisense probes the sequence targeted and hence the binding affinity of the potential antisense probe is the most important consideration. The base sequence of the targeted mRNA is therefore critical, with a GC rich target sequence key in achieving high affinity binding and antisense potency. The portion of mRNA targeted is a secondary consideration but should not be within a region of mRNA secondary structure, which would reduce the antisense-target hybridisation and antisense potency. Using this approach, development of effective antisense probes against proteins previously difficult to target, based on sequence difficulties at their initiation codon may now be possible. Therefore this high affinity targeting approach may re-invent antisense as a potent tool in cell biology.

With the probes αAS3 and βAS2 I have developed two effective isoform specific probes against the major PKB isoforms. Possible unique roles or isoform specific differences between these 2 PKB isoforms can be investigated. Also, the two probes can be effectively used singularly or in tandem to assess general cellular roles of PKB. The specific targeting of different isoforms of the same protein is a unique feature of antisense as most other forms of affecting the cellular levels/function of a protein usually are not isoform specific. Therefore this antisense strategy could enhance the potential for investigating possible isoform specific roles of multiple isoform proteins such as PKC or PI3K. The developed PKB antisense probes do not target the initiation codon but are effective against other regions of the mRNA. This should also aid isoform specific design, as it will be possible to target different mRNA regions between the different isoforms particularly in areas with low homology.

The basic design brief and transfection protocol for the probe βAS2 was very similar to that used in developing the PKBα probe αAS. Therefore, similar rules apply for the rationale behind the development of the PKBβ antisense strategy. For example, the PKBβ probes were synthesised as RNase-H stimulating phosphorothioate oligonucleotides. Also lipofectin^{TM} ($8\mu gml^{-1}$) was used for the first 8 hours to assist transfection of these oligonucleotides into cells. The probe βAS2 was transfected using, the heat inactivated serum media for most of the transfections in the fibroblasts, or 0.25% BSA supplemented serum free media in the adipocytes. The various merits and drawbacks of the basis of this antisense transfection will not be further discussed here as they are given a thorough treatment in Chapter 3.2.

In order to be used to its full potential the PKBβ probe, transfection conditions of βAS2 were optimised for maximum potency in the 3T3-L1 cells. Concentration dependent experiments for this probe in the 3T3-L1 fibroblasts confirmed βAS2 to be an effective antisense agent over a range of concentrations and

behave in a dose-responsive fashion, yielding an IC_{50} 2μM. This probe was found to give a maximal antisense effect at 8μM in a 96-hour transfection period with this optimum level causing a 90% depletion of PKBβ protein levels.

Use of the βAS2 probe at 8μM over a 96-hour transfection time was found to be a very effective antisense protocol, constantly yielding a significant depletion in PKBβ protein to <10% of the levels seen in control cells. Therefore, a time course study for this probe was not carried out in the PKBβ antisense development experiments. The reason for not further characterising the most effective transfection time for this probe was two fold; firstly the >90% depletion of PKBβ protein achieved with a 96 hour transfection period and 8μM βAS2 was proving to be a very effective antisense combination. Secondly, as a 96 hour transfection protocol was an effective transfection time for both the βAS2 and αAS3 probe, having the same optimum transfection time for both probes would be useful when comparing the two probes in a single experimental batch This would also be advantageous in experiments using both probes in tandem to investigate the combined roles of the α and β isoforms.

Suitable control oligonucleotides were designed to test out the specificity of the potential PKBβ antisense probe βAS2. Therefore sense, random and 4 mismatch (base changes) probes were developed and used to test out the antisense potency and specificity of βAS2 in the 3T3-L1 cells. These βAS2 control oligonucleotides were found to have little effect on the PKBβ protein levels when compared to control levels (lipofectin™ only/untreated) or the massive depletion seen with the βAS2 antisense probe. Therefore, only the antisense probe βAS2 successfully depleted PKBβ suggesting only this oligonucleotide could act as a PKBβ antisense probe

It is interesting to note that the βAS2 sense control probe did consistently give slightly lower levels of PKBβ protein at around 75% of the levels seen with the other control oligonucleotides and the lipofectin™/untreated cells. This phenomenon is not unusual in antisense techniques and involves the binding of the sense probe to the complementary strand of the PKBβ DNA in the nucleus, possibly having some inhibitory effect on PKBβ mRNA transcription and ultimately PKBβ protein translation. However, that said, it is clear that any effect on the PKBβ protein levels by the sense probe is very small and fairly insignificant compared to that seen with the βAS2 antisense probe. Therefore, this will not adversely effect the use of these control probes or the interpretation of the βAS2 antisense results.

Control blots against various other cellular proteins including; MAPK and p70S6K showed that the βAS2 probe was specific for PKBβ as this probe and its control oligonucleotides did not alter the expression of any of these proteins compared to the lipofectin™ only/untreated cells. Important in confirming the β isoform specificity was the fact that the βAS2 probe and its controls showed similar protein levels of PKBα to control treatments, indicating the unique targeting of only PKBβ by the βAS2 probe. The βAS2 probe also did not affect the total cellular protein or cellular function suggesting it was acting solely against PKBβ. For example, growth factor induced phosphorylation and activation of MAPK still occurred in antisense treated cells suggesting cellular ATP levels were maintained.

Therefore, it is clear that in the fibroblasts, an effective PKBβ antisense probe had been developed and the conditions for its use optimised. Also control oligonucleotide and control Western blots had revealed that the probe βAS2 acts specifically against PKBβ yielding a >90% depletion in PKBβ protein levels.

Having confirmed the effectiveness of this PKBβ antisense probe in 3T3-L1 fibroblasts it was then tested in the 3T3-L1 adipocytes. The PKBβ probe, βAS2 was found to be a very effective antisense agent in the 3T3-L1 adipocytes depleting the PKBβ isoform protein levels by over 90%. These adipocyte transfections were initially performed using the same transfection time and antisense concentration conditions as in the fibroblasts, however since the adipocytes are a more stable cell the transfections were carried out in serum free media throughout. As with the PKBα probe, the β isoform specific probe βAS2 was found to most effective in the 3T3-L1 adipocytes when used at the same optimum conditions (8μM/96 hours) as in the fibroblasts. Therefore βAS2 is not only an effective PKBβ antisense in the fibroblasts but also the adipocytes. As the conditions for use of βAS2 was similar between these 2 cell lines it would be interesting to investigate the various uptake and distribution parameters of this probe between these cell lines to see if there is any variation between them. The ability to be able to use the PKB antisense probes effectively in both cell lines could be very useful in investigating the involvement of PKB in cell specific functions or responses to different growth factors. For example, the adipocytes are a more insulin responsive cell line and so will be useful for investigating the role of PKB in insulin signalling. The fibroblasts conversely, are more responsive to other factors, for example, EGF enabling the study of the roles of PKB in these signalling pathways.

It is interesting to note that double the concentration of βAS2 (8μM) is required to give a similar depletion of PKBβ compared to the depletion of PKBα protein obtained when treated with 4μM of αAS3. The reasons for this difference are unclear but are likely be to be due to different binding and accessibility factors between these two probes and the mRNA sequences they target. Research into this

possibly using fluorescent labelled antisense probes would perhaps show whether any differences in the uptake or processing of the 2 probes or in the 2 cell lines exists. A greater understanding of these parameters may also be useful in the design of future antisense agents.

It would also be very interesting to test the effectiveness of the PKBβ antisense probe; βAS2 in other cell lines including different mouse cells and cell lines from different species. Different cell lines could then be selected to act as models of key processes PKBβ may be involved in or investigated to analyse the response of PKBβ to different growth factors. These studies could help to provide an important insight into the regulation and roles of PKB in response to different factors and across a wide spectrum of cell types or species.

The sequence targeted by the βAS2 antisense probe shows major identity to the same regions in the published rat PKBβ sequence with only one base difference between the two. Therefore, this probe with one base change should be able to be used in various rat cell lines to show a cross species responsiveness of this probe. Again, with this probe it will be necessary to fully characterise and optimise this probe in new cells as well as periodically re-characterising the probe's use in mouse cell lines to account for possible transfection variations.

The βAS2 probe is not suitable for use in human cell lines as the region of mouse PKBβ mRNA targeted by this probe is different in humans having 5 base differences. Therefore, in order to target PKBβ in human cell lines a different probe would need to be developed. This marked difference between the sequences of mouse and human PKBβ mRNA may make it difficult to target other mammalian sequences if they are more similar to the human than the mouse and rat mRNA sequences.

Although other areas of the PKBβ mRNA for example the initiation codon were not tested as potential antisense targets, the PKBβ sequence clearly contains many other possible regions which antisense probes could be effectively targeted against. The area around the initiation codon in PKBβ does not contain any secondary structure and is far more GC rich than the similar region in the α isoform PKB, which was successfully targeted by αAS1 Therefore, this mRNA region in PKBβ could potentially be successfully targeted by an antisense strategy. This may be worth investigating further in an attempt to generate a second effective antisense against PKBβ possibly if targeting PKBβ levels in other species was required.

The multi-isoform probe αAS2 was also tested for its potential to knock out PKBβ protein. This probe is directed against the mRNA region which translates to the middle kinase domain of the PKBα protein and is the region of greatest homology between the α, β and γ isoforms. This region, and hence the αAS2 probe is potentially interesting, due to the possibility it could act against all 3 known PKB isoforms. Therefore, this probe could possibly act as a multi-isoform antisense probe and lead to total PKB protein knockout.

Tests on PKBβ protein depletion using this probe in 3T3-L1 fibroblasts did not produce a significant decrease in PKBβ protein with a <30% depletion in PKBβ achieved in these cells using a 5μM/96 hour transfection protocol. Under these conditions αAS2 was not an effective PKBβ antisense probe. When used against the α isoform using the same conditions this probe was a more successful antisense agent, depleting PKBα by around 60%. However, these levels of depletion were far less than those obtain with the probes αAS1 or αAS3.

This probe did have some antisense effect on both the α and β isoforms so it may be possible to develop this probe as a multi isoform probe. In theory this probe could bind and potentially have an antisense effect on all three PKB isoforms. Therefore, it may be that a higher concentration or longer transfection time would be required to elicit a potent antisense effect against all 3 isoforms. This probe could be worth investigating in the future to try to develop a total PKB antisense probe. Alternatively, other homologous sequences in the PKB isoforms which are GC rich could be targeted for antisense attack in the hopes of generating a potent multi-isoform antisense. Development of a single antisense probe against all 3 known PKB isoforms may be useful in establishing the general non-isoform specific roles PKB. However, targeting the mRNA region the probe aAS2 is directed against may be problematic, since this region translates to the protein kinase domain and is highly conserved in other kinases including PKC isoforms and SGK.

In order to possess isoform specific PKB antisense probes against all 3 PKB isoforms the development of an effective PKBγ antisense probe will need to be completed. A PKBγ specific antisense probe will also be useful in identifying the individual and collective roles of PKB isoforms. The publication of the mouse PKBγ mRNA sequence has enabled the design of potential PKBγ antisense probes. This sequence has already been analysed using the find-probe program and potential probes have been selected for further analysis. The mouse PKBγ mRNA sequence contains many more regions with a high degree of secondary structure (i.e. hairpin loops) than the other two PKB isoforms. Therefore, targeting this sequence with antisense probes may be more difficult to achieve. As with the α and β isoform probes,

PKBγ probes targeting the 3-translated region look to be the most promising, based on their high theoretical mRNA binding affinity (i.e. GC rich regions). Therefore 2 potential antisense probes directed against the 3`translated mRNA region of PKBγ have been designed. These selected sequences have been analysed for self-complementation (oligo programme) and for similarities to other mRNA sequences (mismatch database). These two probes target the apparently highly antisense sensitive 3`translated region previously targeted but also do so with a high theoretical antisense potency/affinity and are specific for PKBγ based on computer models. The potential probes have subsequently been synthesised as phosphorothioate oligonucleotides and are currently undergoing testing.

Despite being the most recently identified PKB isoform and having a more limited tissue distribution several potentially important features/roles for the γ isoform have already be postulated. For example, PKBγ has been found to be over expressed in several cancers (i.e. prostrate cancer) and to possibly act as a survival factor in these cells (reviewed in Datta *et al.*1999). Also in several cell lines, PKBγ has been found to be particularly sensitive to stimulation by certain growth factors for example; this isoform is highly activated by insulin in certain cells. Therefore PKBγ may be important in the functioning of PKB in cell lines which express this isoform. Also there is some evidence that PKBγ is expressed at low levels in both the 3T3-L1 fibroblasts and adipocytes. Therefore, a probe against this isoform is essential in elucidating the roles PKB plays in these cells.

The main stumbling block to testing the effectiveness of any potential PKBγ antisense probes is the lack of a PKBγ antibody which is suitable for western blotting. Therefore, it may be necessary to analyse the possible effects of the potential PKBγ antisense probes on PKBγ mRNA levels instead of looking at changes in the protein expression. This could perhaps be done using a quantitative RT-PCR protocol to directly analyse any changes in the mRNA levels. Therefore, by using a suitable mRNA level analysis technique or by obtaining an appropriate PKBγ antibody to look at the protein levels it is hoped that these potential PKBγ antisense probes will soon be tested for their antisense effectiveness in the 3T3-L1 cells.

Hopefully these experiments will lead to the generation of a highly potent and specific PKBγ antisense agent. This would yield isoform specific antisense probes against all 3 PKB, enabling the thorough testing of the individual roles of the PKBγ isoform and also the general function of PKB.

It will be necessary to use these probes in combination or a multi-isoform probe to remove all three isoforms of PKB. Also, use of 2 of the probes in tandem, leaving only one specified PKB isoform to be expressed will also help in the assignment of PKB isoform specific and general functions as a component of cell signalling. Computer analysis of the potential probes shows that they should not hybridise to each other so they should be able to be used together in transfections. However, it will be necessary to test out the antisense potency of the individual probes when used together on the cells, to confirm each probe works effectively at the established optimum conditions and alterations are not required for tandem/multiple use.

In summary, βAS2 an effective antisense probe against PKBβ has been designed, developed and tested in 3T3-L1 fibroblasts and adipocytes and can now be used to investigate the cellular roles of this isoform of PKB. As with the previously developed PKBα antisense probes, my PKBβ specific probe, βAS2, is the first known direct effector/inhibitor of PKBβ endogenous protein expression anyone has developed. Therefore, this antisense probe should be of great use and importance in the establishment of the cellular functioning of PKB.

Possible antisense probes against the third PKB isoform, PKBγ have also been designed but are waiting testing and characterisation. When developed, this PKBγ antisense probe would also be the first expression level/functionality inhibitor to specifically target this isoform. It is also not only interesting but important to realise that both the successfully designed PKBβ probe βAS2, and the theoretical PKBγ target the 3'translated region of their respective mRNA sequences originally so successfully targeted by the PKBα antisense. These findings further emphasise the great potential of this region as an antisense "hot spot" and as a region which is an ideal candidate for targeting with future antisense probes against other important cellular proteins. Having successfully established the PKBα and PKBβ specific antisense probes, and with the specific PKBγ probe in the pipeline, next these probes need to be used to test the cellular roles of PKB. Therefore, these probes will be tested in a variety of cells and their effect on the downstream functioning of PKB in response to a variety of stimuli analysed.

With the development of these PKB antisense probes I have developed the first agents to direct target cellular levels of PKB. These probes will therefore be important in elucidating the roles of individual isoforms of PKB and also the general cellular functioning of PKB. These antisense probes act on the endogenous levels of PKB and so will not be affected by the possible problems associated with PKB overexpression studies. These include, the possible interference of PKB mutants with upstream factors such as PDK-1, which may affect PDK-1 function. Also overexpressed PKB may act on non-

physiological substrates or bind to substrates usually acted on by other kinases, for example, SGK, which shares similar putative substrate specificity to PKB. The targeting of individual PKB isoforms will help to unravel any possible isoform specific roles of PKB. These probes will also help to decipher the position PKB occupies relative to other signalling proteins and establish the cellular importance of this protein. This will aid the knowledge we have of cell signalling and the position of PKB in the complex process, and will hopefully be helpful in understanding diseases in which this pathway has been implicated.

Chapter 5 - Using the PKB Antisense Probes to Assess the Role of PKB in Key Cellular Processes

5.1 - General Introduction

Having developed effective antisense probes against both the α and β isoforms of PKB and optimised their conditions of use in 3T3-L1 fibroblasts and adipocytes it was now possible to use these tools to investigate the role of PKB in these cells. PKB has many proposed cellular roles in metabolism, cell survival, cell growth and differentiation. The aim was to use the antisense probes to test and elucidate the function of PKB in these fields.

As it had been shown that the probes were effective in both 3T3-L1 fibroblasts and 3T3-L1 adipocytes this gave a choice of cell types in which to study the possible roles of PKB. 3T3-L1 adipocytes are a model cell line in insulin signalling, so are very good for use in the study of metabolic events which may involve PKB, for example, glucose uptake, glycogen synthesis (and glycogen synthase activity), lipid synthesis and protein synthesis. During the differentiation of 3T3-L1 fibroblasts into adipocytes an increased expression of insulin receptor is observed. This increased level of insulin receptors and hence insulin sensitivity remains high in the 3T3-L1 adipocytes. 3T3-L1 fibroblasts on the other hand, may prove better models for investigating the roles of other growth factors (i.e. EGF) in the PKB pathway. If necessary certain roles of PKB could also be studied in other cell types if they were deemed more suitable models for these possible actions. The roles of PKB in signalling in cells for other species could also be analysed

5.2 - The Possible Roles of PKB in Cellular Differentiation

5.2.1- Introduction

The differentiation of cells from one type to another type is a very complex physiological process, which is necessary for the development of different cell types and hence is essential in development and maintenance of an individual organism. Identifying the numerous factors that cause a stem cell to undergo terminal differentiation into a specialised cell is crucial for gaining an understanding of

the whole process of development. This should help in our knowledge of not only which pathways bring about such changes, but also how such processes can be de-regulated leading to developmental abnormalities.

Since differentiation plays a crucial role in the maintenance of an organism, insights into this process may help give a clearer picture of the homeostasis of an individual. Aberrant control of differentiation has also been linked to the development and progression of a wide variety of cancers and therfore an understanding of what signals are related to the control of differentiation may help to decipher some of the pathways involved in cancer.

Cell signalling has a critical role to play in regulating differentiation. Many external stimuli, for example, growth factors and hormones act via membrane bound receptors to direct a cell towards a differentiated phenotype. These signalling pathways frequently involve receptor tyrosine kinase pathways and hence downstream protein kinases and phosphatases have key functions. Key roles for PI3K in the differentiation of a variety of cell lines have been identified based on a variety of experiments using PI3K active/inactive mutants and the PI3K inhibitors wortmannin and LY294002. For example, LY294002 completely prevented the differentiation of muscle myoblasts into myotubes, a process also referred to as myogenesis (Kaliman *et al.* 1996).

A role for PKB in the cellular differentiation of certain cells has been proposed, based on findings from several experiments. An increased level of expression of PKBβ has been observed during the differentiation of Sol8 muscle cell myoblasts into myotubes suggesting a possible role for this isoform of PKB in this differentiation process (Calera *et al.* 1998). This differentiation process can be induced by cell confluence, serum withdrawal, or the action of insulin or IGF-1 and is characterised by the increased expression of the protein markers, myosin and myogenin. With all these differentiating conditions, the levels and activity of PKBβ was found to increase dramatically and correlate with the increased expression of the muscle markers (Calera *et al.* 1998).

The levels of the PKBα isoform, however, were found to remain at a similar low level during differentiation, although a slight increase in activity was observed with the onset of differentiation/myogenesis. Interestingly, the PI3K inhibitor, LY294002 was found to prevent the increased expression of PKBβ and myosin/myogenin and inhibit myogenesis (Calera *et al.* 1998).

Therefore, it is apparent, that in Sol8 myoblasts, changes in the levels and activity of the PKBβ pathway are linked to the differentiation of these cells into myotubes, possibly via increased expression of key muscle proteins.

A role for PKB in the differentiation of another type of muscle cell precursor into a committed or terminally differentiated muscle cell has also recently been proposed. C2C12 myoblasts that normally proliferate and are mononucleated can be induced to differentiate by confluence/low serum and fuse together forming post-mitotic, elongated and multinucleated myotubes. If during this differentiation process, insulin or IGF-1 is also present, a hypertrophic effect is exerted resulting in the generation of thicker myotubes (Rommel *et al.* 1999). Transfection of the undifferentiated myoblasts with a constitutively active PKBα mutant, resulted in the spontaneous differentiation of these cells into thick myotubes, similar to those obtained with insulin or IGF-1 treatment. This mutant also increased the expression of the myotube markers, myogenin and p21CIP in a similar fashion to that seen with insulin and IGF-1 (Rommel *et al.* 1999). Interestingly, this active PKBα mutant was also found to inhibit the activity of the Raf/Erk pathway, which had been previously shown to exert a negative effect on muscle hypertrophy (Rommel *et al.* 1999). Therefore, these experiments suggest a role for PKBα in the differentiation of C2C12 myoblasts into myotubes, with one possible function of PKB in this process being inhibition of the anti-differentiation Raf pathway.

PKB expression has also been found to be low in the multipotent fibroblast cell line 10T1/2, but to be dramatically increased when these cells are induce to differentiate into myocytes by the action of *Myo*D, again suggesting a role for PKB in cell differentiation (Altomare *et al.* 1995). Differentiation of pluripotent P19 embryonal carcinoma cells by the action of retinoic acid also caused a dramatic increase in PKB levels after day 3, which subsequently remains high in the terminally differentiated P19 cells. This further indicates a dramatic change in PKB expression during differentiation and shows a likely role for PKB in this process (Coffer *et al.* 1998).

Overexpression of a constitutively active PKBα mutant in 3T3-L1 fibroblasts was found to bring about spontaneous differentiation of these cells into adipocytes. Conversely, the use of a dominant negative mutant of PKB prevented this differentiation occurring in the presence of the differentiation cocktail (Kohn *et al.* 1996, Magun *et al.* 1996). Therefore, it is likely that there is a

role for PKB in this differentiation process. However, since the current evidence for such a role is based on overexpression studies and so uses non-endogenous protein levels further study is required to confirm this likely role (Kohn *et al.* 1996, Magun *et al.* 1996).

In summary, it is likely that PKB has roles to play in the differentiation of some cell types in some species and so further study of these roles is warranted. Also it appears apparent that isoform specific functional differences may exist when considering the roles of PKB in cell differentiation, especially as the expression profiles of these isoforms appears to change specifically and dramatically when may cell lines are induced to differentiate. However, as yet no studies have looked at the effects of alterations of the endogenous PKB levels would have on cell differentiation or the differentiated state. Therefore, it is important to investigate this complex cellular process using the isoform specific antisense probes I have designed and developed, in order to establish the role of PKB in cell differentiation and if any isoform specific differences in the regulation of this process by PKB exist.

5.2.2Results:

5.2.2.1 Possible roles of PKBα in the Differentiation of 3T3-L1 Fibroblasts into Adipocytes

3T3-L1 fibroblasts can be induced to differentiate into adipocytes, by the addition of a cocktail of factors including insulin over an eight day protocol (see methods 2.2.4) during which time an accumulation of fat droplets and other morphological changes including a rounding up of cells can be clearly seen. These major physiological alterations in cell appearance and hence function, can be viewed under the microscope and recorded by photography, this makes it easy to distinguish between cells which have undergone differentiation to adipocytes and those which remain in the undifferentiated fibroblast state.

It was therefore decided to transfect the fibroblasts with the αAS3, antisense using the optimised conditions for maximal PKBα depletion (4μM/96 hour) and then try to differentiate these cells into adipocytes using the standard differentiation protocol. In these experiments, after the initial 96-hour antisense treatment to deplete PKBα, the differentiation of the cells was attempted in the

continued presence of 4μM of αAS3 to maintain the depletion of PKBα. The differentiation of the 3T3-L1 fibroblasts was performed using the standard cocktail of factors over the eight-day protocol. The other difference from the standard differentiation protocol being that heat inactivated serum was used to prevent the possible effects of serum factors on the antisense.

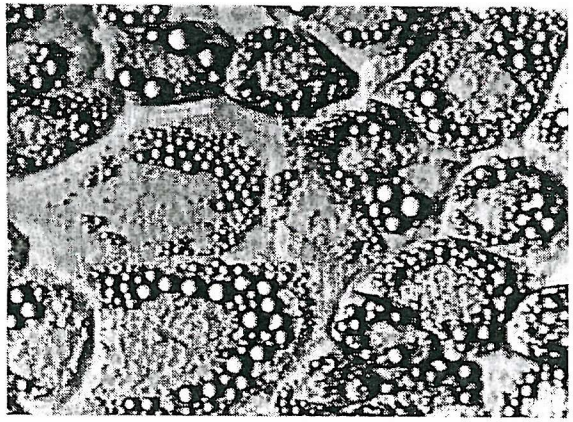
In the initial experiment, the differentiation of 3T3-L1 fibroblasts into adipocytes was compared between cells treated with the αAS3 antisense probe, treated only with lipofectin™ or left untreated prior to differentiation. In these preliminary studies, it was found that the use of the αAS3 antisense prevented the differentiation of the fibroblasts into adipocytes whereas fibroblasts treated with lipofectin™ only or not treated prior to attempting differentiation could be converted into adipocytes (data not shown). This indicated a possible role for PKBα in the differentiation of 3T3-L1 fibroblasts into adipocytes.

The next step was to further test the role of PKBα in this differentiation process. This was done by comparing the effects that pre-treatment of the 3T3-L1 fibroblasts with αAS3 has on cellular differentiation, to the effects seen in fibroblasts treated with the previously designed PKBα control oligonucleotide, before attempting the differentiation of these cells. 3T3-L1 fibroblasts which were left untreated, treated with lipofectin™ only, or treated with the control oligonucleotides (sense, random, 4mismatch) could still be induced to differentiate into adipocytes as indicted by the rounding up of these cells and visible lipid droplet accumulation (see figure 5.1). However, αAS3 treated fibroblasts could not been differentiated into adipocytes and so retain the characteristic thread like appearance and general morphology of the fibroblast cell (figure 5 1). Therefore, it is clear that the effects αAS3 has on 3T3-L1 fibroblast differentiation are as a direct result of its antisense function and not any non-specific events and thus indicate a role for PKBα in the differentiation process.

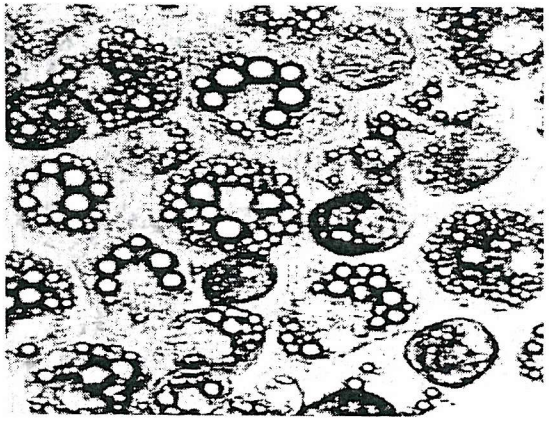
From these studies, it is apparent that PKBα has a critical role in the differentiation of 3T3-L1 fibroblasts into adipocytes, as depletion of the protein levels of this isoform by around 90% prevents differentiation in the presence of differentiation factors. However, control treatments including other oligonucleotides were without any noticeable effect on this complex cellular process.

Figure 5.1 - αAS3 treatment of 3T3-L1 fibroblasts prevents their factor induced differentiation into adipocytes. 3T3-L1 fibroblasts (~95% confluent) were transfected as described in section 2.3.1 with 4μM of αAS2 or the matched control oligonucleotides. After 96 hours these cells were treated with differentiation agents and photographed 8 days post-differentiation. **A,** No ODN/lipofectin; **B,** lipofectin/no ODN; **C,** αAS3; **D,** Sense; **E,** Mismatch; **F,** Random.

Control

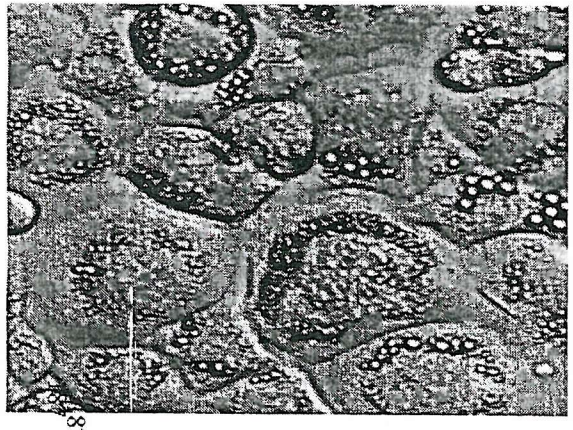


Lipofectin only

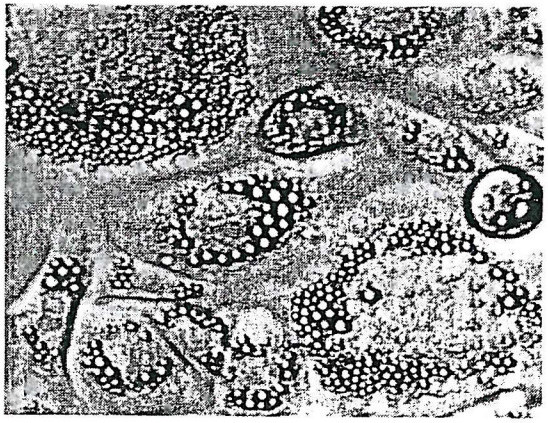


αAS3 oligo

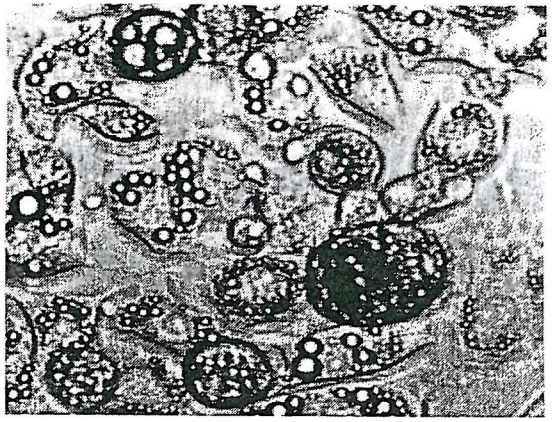
Sense oligo



Mismatch Oligo



Random Oligo



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5.2.3 Discussion of the Roles of PKB in Differentiation

The results obtained in these experiments have shown a crucial function for PKBα in the differentiation of 3T3-L1 fibroblasts into adipocytes. The use of the αAS3 probe completely inhibited this differentiation process, when used at its optimal concentration of 4μM for 96 hours before the differentiation was commenced and then subsequently during the differentiation. The genuine antisense effect of this probe and hence the specific role of PKBα in this process was confirmed by the fact that matched control oligonucleotides did not affect differentiation.

My results show that endogenous PKBα is required for the differentiation of 3T3-L1 fibroblasts into adipocytes. This indicates that the other isoforms of PKB cannot substitute for the role of PKBα in this differentiation process. These results also show key functions of PKB can be successfully determined by the depletion of one isoform of PKB. The apparent role for PKBa in this differentiation also correlate with previous overexpression studies, suggesting PKB could be involved in this process (Magun *et al.* 1996, Kohn *et al.* 1996). Subsequently, insulin has been shown to stimulate adipogenesis by the generation of $PI3,4P_2$ and hence activation of PKB, emphasising a likely role for PKBα in this process (Gagnon *et al.* 1999).

However, interestingly, PKBα protein levels have been proposed to decrease during the differentiation process, with a simultaneous increase in the levels of the other major PKB isoform PKBβ observed (Hill *et al.* 1999). It is suggested, that in the 3T3-L1 fibroblast cells, PKBα is the predominant isoform, with the PKBβ isoform less strongly expressed, whilst in the adipocyte cells generated by differentiation the situation is reversed with the β isoform predominating (Hill *et al.* 1999). Therefore, it possible that observed role PKBα has in this differentiation could occur early on in the commitment of these fibroblasts to change into the adipocytes. This may be further emphasised by the fact that PKBα is activated quickly by the differentiation factors; insulin, IBMX and dexamethazone that are present at the start of the differentiation protocol. PKBα may therefore possibly signal via a variety of downstream factors to aid in the commencement of differentiation, possibly via its proposed actions on transcription and translation (Gagnon *et al.* 1999).

The differentiation of 3T3-L1 fibroblasts into adipocyte cells is a very complex process and is likely to involve a series of factors. The involvement of the Ras/Raf signalling cascade in this process has long been known. Some of the roles of this pathway in cell differentiation are independent of the downstream Erk/Rsk kinases. However, it has been shown using the EAS1 antisense probe, that the Erk1/2 MAPK isoforms play a critical role in this differentiation as depletion of these proteins by the antisense probe resulted in an inhibition of differentiation (Sale *et al.* 1995). Differentiation of 3T3-L1 fibroblasts into adipocytes therefore requires signalling by multiple pathways. However, as yet despite the apparent involvement of these signalling proteins and now PKBα in the differentiation pathway, it is unclear exactly how these factors exert their key effects on the process and act together to bring about the differentiation.

It would be useful to investigate key markers of the differentiation process and then analyse the effects of the PKBα antisense of these factors. This could therefore establish the roles of PKBα in differentiation and show what altering the levels of established differentiation regulators has on these individual markers and try see which regulator may act on which differentiation marker. Adipocytes are a major storage cell line and are highly responsive to insulin, which has been shown to activate a variety of key effects including glucose uptake, glycogen synthesis and lipogenesis. Therefore, possible targets for PKBα in the differentiation process could be in the stimulation of the expression of key metabolic proteins, which are critical in establishing the adipocytic phenotype.

How PKBα performs its obviously important role in a critical cell process is unclear. It is tempting to speculate that PKB may act to inhibit GSK-3 activity or reduce its levels since a decrease in GSK3β during this differentiation has been previously observed (Benjamin *et al.*1994). Removal or inhibition of GSK3 would cause an upregulation of glycogen synthase and would be indicative of the conversion of the fibroblasts into adipocytes, which are storage cells (reviewed in Lawrence *et al.* 1997). Adipocytes are mainly characterised by their accumulation of lipid droplets, so it may be that PKBα has a role to play in increasing lipid synthesis. One such target could be the glucose transporters GLUT1 and GLUT4, which have been previously proposed to be acted on by PKB. For example, PKB has been previously shown to increase the expression of GLUT1 and to increase the translocation of GLUT4 to the plasma membrane, both events which have been found to be key in differentiation (Kohn *et al.* 1996, Tanti *et al.* 1997).

Another protein proposed to be involved in differentiation is p70S6K, which has also been suggested as a possible downstream target of PKB, perhaps via mTOR (Burgering *et al.*1995). The role of p70S6K in differentiation is also currently unknown, but would likely to be involved in regulating the translation of key differentiation proteins. The potential involvement of PKB in p70S6K activity is also controversial, and so therefore merits further study using the newly designed antisense probes. Before we can establish how PKBα affects cell differentiation, further studies on the general cellular roles of PKB, the processes it acts on and also what cellular events occur during this differentiation will be required. This should give a clearer picture of the targets of PKB and how these can bring about or enhance fibroblast differentiation into adipocytes.

Having established a role for PKBα in this differentiation, it is necessary to determine the role PKBβ plays in the differentiation of 3T3-L1 fibroblasts into adipocytes. The guidelines used for the αAS3 probe can be followed with βAS2 used at its optimum conditions prior to the differentiation. It would be interesting to examine the roles of PKBβ in differentiation since its expression has been found to be dramatically increased during the conversion of the fibroblasts to the adipocytes (Hill *et al.*1999). In sub-confluent 3T3-L1 fibroblasts (~ 50%), PKBβ protein levels are apparently undetectable, however once, confluent PKBβ protein appears to be expressed in the fibroblasts (Hill *et al.*1999). Upon addition of the differentiation media in this study, PKBβ expression increased slowly at first and then rapidly from day 3 onwards, reaching maximum levels at day 8 which were subsequently maintained in the adipocytes (Hill *et al.*1999). Interestingly, in the work of Hill *et al.* increased PKBβ expression during differentiation was closely followed by an increase in GLUT4 expression, possibly indicating a link between these two key proteins during differentiation and subsequently in the adipocytes (Hill *et al.*1999).

These experiments using other PKB antisense probes should show which isoforms of PKB are involved in differentiation or if all of them have roles to play. Hopefully, it may also enable us to establish whether they all have the same role to play or act on different parts of the complex differentiation pathway.

Having looked at the roles of PKB in the differentiation of 3T3-L1 fibroblasts into adipocytes, it would then be interesting to look at whether PKB isoforms play important role in the differentiation of other cell types. For example, it would be of great interest to examine the potential isoform specific role of PKB in muscle myogenesis previously detailed or perhaps the role of PKB in the differentiation of neuronal cell lines a process which PKB has also been implicated in (Coffer *et al.* 1998). This could therefore include investigating any potential roles for PKB in the differentiation of L6 and Sol8 muscle, PC12 and embryonic neurone, and stem cells. These studies should identify whether the roles of PKB in cellular differentiation are widespread or confined to a particular cellular species type, and also give an indication as to the relative importance of each isoform of PKB in these essential, but complex, processes.

5.3 The Possible Roles of PKBα and PKBβ in the Activation of p70 S6 Kinase and p85 S6 Kinase

5.3.1 Introduction to p70 S6 Kinase and p85 S6 Kinase (S6K)

40S ribosomal protein kinases (S6K) were initially identified as ribosomal kinases that regulate multiple phosphorylation of the 40S ribosomal protein S6 in mitogen stimulated cells (Jeno *et al.* 1988, reviewed in Dufner *et al.* 1999). Subsequent cloning analysis and molecular study lead to the identification of two isoforms of S6K, of 56.2kDa and 59.2kDa which are produced from the same mRNA transcript, by using alternative translational start sites (Banjerjee *et al.* 1990). The two identified S6K proteins were found to contain 502 and 525 amino acids respectively, with the longer form containing an additional 23 residues at its amino terminus. The shorter form therefore starts, at M24 of the longer form with the proteins having sequence identity from this point onwards (reviewed in Dufner *et al.* 1999). The 56.2kDa isoform has been found to have a predominantly cytoplasmic location and is usually referred to as p70S6K based on its apparent molecular mass of 70kDa based on SDS-PAGE migration. The 59.2kDa S6K isoform, which is usually known as p85S6K, based on its SDS-PAGE migration, has been found to have a nuclear location, suggesting alternatively roles for these two isoforms based on their subcellular location.

Interestingly, the nuclear targeting of p85S6K has subsequently been found to be due additional 23 amino acids at this isoforms N-terminal. This region of p85S6K has been found to possess a polybasic nuclear localisation signal, which is generated by a run of 6 arginines immediately after the initiation methionine (reviewed in Dufner *et al.* 1999)

S6Ks are believed to act as a regulator of the translation of a class of mRNA transcripts that contain an oligopyrimidine tract at their transcriptional start site or 5'TOP (reviewed in Peterson *et al.* 2000, reviewed in Dufner *et al.* 1999). They regulate the translation of these mRNAs by the phosphorylation of S6 (reviewed in Dufner *et al.* 1999). Whilst this 5'TOP class of mRNAs, may only represent 100 to 200 genes they can account for up to 25% of the total mRNA in the cell and usually encode for many of the components of the protein synthesis apparatus including ribosomal proteins and translational elongation factors (reviewed in Pullen *et al.* 1997). The role of S6K in controlling 5'TOP translation was confirmed using dominant interfering mutants of S6K which were found to suppress S6K activity and inhibit the mitogen-induced translation of 5'TOP mRNAs (Jefferies *et al.* 1997). It is postulated that the nuclear p85S6K is responsible for the phosphorylation and regulation of the free, chromatin bound nuclear form of S6, however this theory still requires experimental evidence (reviewed in Dufner *et al.* 1999). The ribosomal S6 protein is a component of the small (40S) subunit of eukaryotic ribosomes. It has been found to contain 5 phosphorylation sites which can be acted on by S6K, namely S235, S236, S240, S244, S247, which when phosphorylated, increase the binding and translational activity of this protein (reviewed in Proud 1996)

In addition to these roles, S6K has also been implicated in the regulation of E2F in cell proliferation, with expression of wild type but not kinase dead S6K inducing E2F-induced transcriptional activity in T-lymphocytes (Brennan *et al.* 1999). Overexpression of S6K also enhanced insulin gene transcription in pancreatic β-cells suggesting S6K may act to regulate insulin promoter activity (Brennan *et al.* 1999). S6K activity has also been implicated in cellular survival pathways suggesting S6K may also act as a regulator of apoptosis (Brenneisen *et al.* 2000, reviewed in Dufner *et al.* 1999). In these, and possibly other ways S6K plays a key role in cellular growth control mechanisms, with its regulation of transcription and/or translation controlling, protein biosynthesis and critical cellular events (reviewed in Dufner *et al.* 1999).

Deletion of the S6K gene in mouse was found to result in a small mouse phenotype suggesting a role for S6K in cell size, growth and proliferation (Shima *et al.* 1998). However, in mouse embryo fibroblasts with this deletion, neither S6 phosphorylation, 5'TOP mRNA translation or cell growth was greatly impaired (Shima *et al.* 1998). This finding lead to the discovery of a new S6K isoform termed S6K2 which has 70% amino acid identity with the other S6Ks and was found to be up-regulated in the S6K deletion mice, possibly acting to compensate for this loss (Shima *et al.* 1998). Subsequently, this S6K2 has been shown to be a nuclear S6K family member, which shares homology with the other S6K family members in the catalytic domain but possess a carboxyl terminal nuclear localisation signal that assists the nuclear localisation of this kinase (Shima *et al.* 1998). Homologues of S6K have also been found in other species, for example, in *Drosophila,* loss of function alleles of *d*S6K revealed that S6K controls cell size, growth and proliferation in these flies (reviewed in Dufner *et al.* 1999)

Inhibition of S6K activity by neutralising antibodies has been shown to compromise the ability of cells to progress through the G1 phase of the cell cycle emphasising, the role for S6K in the control of cell growth (Lane *et al.* 1993). Treatment of cells with the immunosuppressant drug rapamycin; which acts on the potential S6K activator mTOR (discussed later), has been shown to inhibit the role of S6K in translation and cell growth (Kuo *et al.* 1992,Chung *et al.* 1992) Conversely, a rapamycin-resistant mutant of S6K blocked, the inhibitory effects of rapamycin, allowing 5'TOP mRNA translation and hence the cell cycle to proceed (Jefferies *et al.* 1997). The apparent importance of S6K in regulating protein synthesis and cell growth means that it is crucial to understand how S6K acts to bring about these effects and what factors act on S6K to bring about its activation (reviewed in Pullen *et al.* 1997).

The general structure of S6K is a highly acidic N-terminal domain, followed by a serine/threonine kinase catalytic domain and lastly a C-terminal auto-inhibitory/regulatory section. S6K has been found to consist of 4 distinct modular domains (reviewed in Pullen *et al.* 1997). Region I stretches from the N-terminal of the protein to the catalytic domain and is sensitive to rapamycin. Region II is the catalytic domain; this module contains an active site for mitogen-induced phosphorylation and the activating T-loop. Domain III runs from the catalytic domain towards the carboxyl tail and contains 3 sites of phosphorylation, with this region conserved in many serine/threonine kinases. The final domain module IV lies at the C-terminus and is the putative auto-inhibitory domain,

which contains 5 phosphorylation sites and has homology with a similar region of the S6K substrate S6 (reviewed in Proud 1996, reviewed in Pullen *et al.* 1997).

There are 8 known phosphorylation sites in the endogenous S6K enzyme and another 5 predicated phosphorylation sites have been proposed (reviewed in Pullen *et al.* 1997). The phosphorylation sites S411, S418, T421 S424 and S429 all contain a proline residue at +1, are all found in domain IV (p70S6K isoform) and are homologous to sites in the S6K substrate S6. In serum starved or quiescent cells these residues are hypophosphorylated, but when these cells are stimulated with mitogens or serum the domain IV phosphorylation sites become hyperphosphorylated which acts to assist S6K activation. Mutation of the residues S411, S418, T421 and S424 to alanine was shown to suppress mitogen stimulated S6K activation whereas mutation of these sites to aspartate to mimic phosphorylation increased basal S6K activity (Weng *et al.* 1998).

For full activation of S6K, phosphorylation of T229 in the T-loop and residues S371, T389 and S404 in module III is required (Weng *et al.* 1998). Phosphorylation of S371 within domain III, the linker region, has recently been implicated in regulating the maximal activation of S6K. This site has shown to be phosphorylated in response to mitogen stimulation and enhance S6K activation whilst mutation of this site III serine to an alanine, results in a reduced S6K activation by at least 20% (Weng *et al.* 1998). This indicates a key-activating role for this site. Site S404 which also lies within this linker region has been shown to play a modulatory role in S6K activation, with phosphorylation at this site assisting maximum S6K activation (reviewed in Dufner *et al.* 1999). However, as yet, the exact role of these two sites in the activation profile of S6K is unclear and requires further characterisation (reviewed in Dufner *et al.* 1999).

It is believed that phosphorylation of residue T389 in module III is the major event leading to S6K activation (Pearson *et al.* 1995). This residue along with residues T229 and S404 are dephosphorylated in response to rapamycin or wortmannin (PI3K inhibitor), which brings about S6K inactivation, but principally the inactivation target is dephosphorylation of T389 (Weng *et al.* 1995). Interestingly, the rapamycin-induced dephosphorylation of these S6K sites has been shown to occur in a hierarchical fashion of T389 > S404 > T229, with T389 dephosphorylation most closely paralleling loss of S6K kinase activity (reviewed in Dufner *et al.* 1999). Substitution of T389 to E389 suppresses the inhibitory effects of these drugs and also elevates T229

phosphorylation (Pearson *et al.* 1995). However, a T389A substitution inactivates the kinase and also blocks T229 phosphorylation (Pearson *et al.* 1995). These and other experiments suggest phosphorylation of T389 is the key event in S6K activation as phosphorylation of this site not only leads to S6K activation but also appears to modulate phosphorylation of the T229 site (reviewed in Dufner *et al.* 1999).

Mutation of the residue T229 have also been shown to affect S6K activity with mutations of this residue to alanine or glutamate ablating kinase activity and not potentiating T389 phosphorylation (Pearson *et al.* 1995, reviewed in Pullen *et al.* 1997). Thus, it appears the phosphorylation of T389 may regulate the phosphorylation of T229 in a co-ordinated fashion, which brings about modulations in S6K activity (Weng *et al.* 1998). Interestingly, the sites T229 and S404 have been found to have basal phosphorylation under serum starved conditions, whereas no phosphorylation is detectable on any other sites (Weng *et al.* 1998). However, in response to stimuli, i.e. insulin, S6K shows greatly increased phosphorylation at all sites, resulting in maximal S6K activity (Weng *et al.* 1998). Therefore, it is apparent that phosphorylation of many sites within different modules of the enzyme is important in regulating S6K activity.

Interestingly, the N-terminal domain of S6K also seems to be important in S6K activation. Deletion of this module has been to shown to impair kinase activity and dramatically reduce T389 phosphorylation (Dennis *et al.* 1996). The kinase activity of this S6K mutant could be rescued by deletion of the carboxyl terminal 104 amino acids, including the auto-inhibitory domain (Dennis *et al.* 1996). A carboxyl terminal deletion mutant was found to increase T229 phosphorylation in response to stimuli and hence increase S6K activity. These results suggest that the N-terminal domain of S6K is involved in facilitating the phosphorylation of the carboxyl terminal sites and hence in removing the auto-inhibitory effect of this domain when it is not phosphorylated. Therefore, it is clear that for maximum activity all sites on the enzyme need to be phosphorylated and all domains need to be present (reviewed in Dufner *et al.* 1999).

The current model for activation of S6K involves the hyperphosphorylation of the serine and threonine sites in module IV which is then believed to stabilise interactions of the enzyme with effector molecules and remove the inhibitory action of this domain on the catalytic domain of S6K (reviewed in Pullen *et al.* 1997). This stabilisation/rearrangement allows a mitogen-regulated

kinase to phosphorylate T389, which then facilitates the phosphorylation of the T-loop site T229 leading to full activation (reviewed in Dufner *et al.* 1999). It is therefore important to establish which kinases are involved in these phosphorylation events, and how these knit together to regulate S6K activity (reviewed in Pullen *et al.* 1997, reviewed in Proud 1996).

S6K is known to be activated by a variety of factors including, serum, insulin, platelet-derived growth factor (PDGF), phorbol esters (TPA), interleukin-2 (IL-2) and heat shock (45°C). Stimulation by these activation pathways results in the phosphorylation of multiple serine and threonine residues leading to activation of the kinase (Kim *et al.* 1999, Xu *et al.* 2000, reviewed in Dufner *et al.* 1999). To date, many kinases, including PDK-1, mTOR and PKC have been proposed to be involved in this activation with the evidence for some, better than others (Kumar et al. 2000, Isotani et al. 1999, Pullen *et al.* 1998, Burnett *et al.* 1998, Akimoto *et al.* 1998). It is most likely that multiple kinases and pathways are involved in activating S6K to the maximal level, with the roles and importance of each varying between the stimulation and cell type investigated.

Use of other cell signalling inhibitors has also added to our understanding of the complex process involved in S6K phosphorylation and activation. The PI3K inhibitor, wortmannin and LY29304 have been shown to inhibit S6K activation in a variety of cells suggesting a role for the PI3K pathway (Weng *et al.* 1995 & 1998). Also active mutants of PI3K have been shown to increase S6K activity. However, not all the data from mutant studies is consistent with PI3K activation being a requirement for S6K activation in some cells, so the situation may not as clear-cut, with possible PI3K-dependent and independent pathways involved (reviewed in Dufner *et al.* 1999).

One of the major mechanisms by which S6K is activated is via the PI3K related kinase mTOR (mammalian target of rapamycin) (reviewed in Thomas *et al.* 1997). This protein has been implicated in S6K activation by the action of the immunosuppressant compound rapamycin which is also a potent inhibitor of S6K activation. It is likely the mTOR lies upstream of S6K, and mTOR inhibition by rapamycin is the point where the activation of S6K is sensitive to the inhibitory effects of rapamycin (reviewed in Thomas *et al.* 1997). An mTOR mutant, which is resistant to rapamycin, has also been found to protect S6K activity in the presence of this inhibitor, further indicating a role for mTOR as an upstream regulator of S6K (Dennis *et al.* 1996). However it is still unclear as to whether this is a direct action or via an as yet unidentified effector molecule

(reviewed in Thomas *et al.* 1997). The possible mechanisms by which S6K may be regulated by the action of mTOR are discussed at the end of this chapter (see section 5.3.3).

A role for PKB in S6K activation has been implied based on a series of experiments. Initially PI3K pathway studies using the inhibitor wortmannin suggest that as PKB is a downstream component of this pathway it may also signal to S6K (reviewed in Kandel *et al.* 1999). Secondly, PKB has been proposed to be an upstream activator of mTOR, which is known to be upstream of S6K, suggesting a possible PI3K/PKB/mTOR/S6K pathway (Scott *et al.* 1998). However, as will be discussed later, the possible physiological importance of PKB in the activation of mTOR and the exact role of mTOR in S6K activation remain unclear and needs further study. Experiments using PKB mutants have also suggested a possible role for PKB in S6K activation. For example a gag tagged PKB which is membrane localised and constitutively active was found to activate p70S6K in a variety of cells although, no direct phosphorylation of P70S6K by PKB has been shown (Reif *et al.* 1997). A dominant negative PKBα mutant was also found to inhibit p70S6K activation in response to heat shock and PDGF stimulation in CHO cells (Kuroda *et al.* 1998). However, as shall be seen in the discussion at the end of this section these results are somewhat controversial and therefore it is important to establish what if any roles the PKB isoforms play in the growth factor induced activation of the S6K isoforms p70S6K and p85S6K.

With this in mind, my antisense oligonucleotide approach was employed to investigate the possible role of PKBα and PKBβ in the activation of p70S6K and p85S6K in 3T3-L1 fibroblasts stimulated with a variety of growth factors and external stimuli.

5.3.2 Results

5.3.2.1 Optimisation of a Western Blot Gel Shift for Analysis of S6K Activation

In order to analyse the effects that depleting PKBα and PKBβ with my antisense probes has, on the activation of S6K, a suitable technique for determining S6K activation needed to be established. S6K activation in response to external stimuli, is directly attributable to hyperphosphorylation on a series of key serine and threonine residues (Weng *et al.* 1998), therefore, it was felt that a method

of monitoring the phosphorylation state of S6K would be a suitable measure of S6K activation. Using an S6K specific antibody, obtained from UBI, clearly detectable protein levels of both the p70S6K and p85S6K S6K isoforms were observed in 3T3-L1 fibroblasts when crude cell lysate was run on a 10% SDS-PAGE gel and visualised by Western blotting (data not shown).

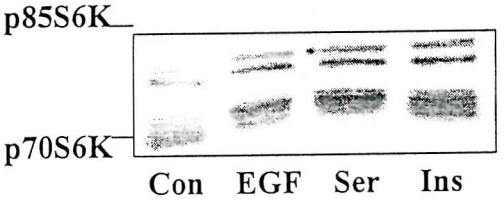
Subsequent investigation of the SDS-PAGE/Western blot profile of the S6K isoforms revealed it was possible to detect changes in the migration of the S6K proteins in response to growth factor stimulation of the fibroblasts. A slower migrating band of either S6K isoform was clearly observed in growth factor stimulated cell, with this retarded or shifted band acting as a clear marker of the increased phosphorylation state and hence activation of S6K in these stimulated cells (previously shown by, Weng *et al.* 1998). Further analysis of this S6K electrophoretic mobility variation established that using a longer (10 x 8cm), lower percentage acrylamide (7.5%) and lower bis-acrylamide (0.09%) polyacrylamide gel than normally used (see section 2.6), provided the optimum separation of the inactivated and activated forms of S6K (data not shown). Therefore, it was decided that p70S6K and p85S6K activation could be effectively monitored by SDS-PAGE/Western blotting analysis with a 10cm by 8cm, 7.5% acrylamide / 0.09% bis-acrylamide SDS-PAGE gel giving the maximum resolution of bands and so these conditions subsequently employed (data not shown).

Stimulation of 3T3-L1 fibroblasts, serum starved for 24 hours, with a variety of external growth factors was found to induce the phosphorylation and activation of both p70S6K and p85S6K as clearly indicated by the observed band shift (see figure 5.2). These factors were all used in accordance with their time/concentration levels previously found to give the optimum receptor and or downstream target (i.e. Erk1/2) activation and compared to untreated cells (see figure 5.2 legend). Factors including serum, PDGF, EGF, insulin and the phosphatase inhibitor, sodium orthovanadate, were all shown to band-shift and hence activate S6K. The growth factor-induced phosphorylation of S6K, and hence its band shift activation profile, was also found to prevented by pre-treatment of the fibroblasts with the PI3K inhibitor, wortmannin (see Figure 5.2).

This not only indicates that S6K activation in 3T3-L1 fibroblasts is PI3K-regulated, but also that this Western blot assay system can be used as a suitable technique for analysing the activation of the 2 S6K isoforms; p70S6K and p85S6K (see figure 5.2a).

SGK Activation Profiles in 373-L1 Fibroblasts

A. Control, EGF, Serum or Insulin Treatment



B. Wortmannin/Serum, Serum, Vanadate or PDGF Treatment

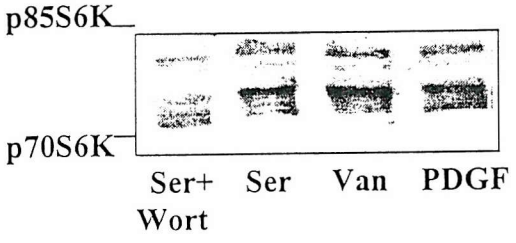


Figure 5.2- Agonist treatment of 3T3-L1 fibroblasts results in a change in the electrophoretic mobility of p70S6K and p85S6K which can be attenuated by prior treatment with wortmannin. 3T3-L1 cells (~95 confluent) were serum starved for 24 hours prior to treatment with agonist. Where necessary cells were incubated with 100nM wortmannin for 40 minutes prior to agonist treatment. **A,** Representative band-shift Western blot for p70S6K/p85S6K: Lane 1, Untreated Control; Lane 2, EGF (100ngml-1/6mins); Lane 3, FBS (20%$^{v}/_{v}$ /10mins); Lane 4, Insulin (100nM/10mins). **B,** Band-shift Western blot for p70S6K/p85S6K: Lane 1, Wortmannin (100nM/40mins) + FBS (20%$^{v}/_{v}$/10mins); Lane 2, FBS (20%$^{v}/_{v}$/10mins); Lane 3, Vanadate (10mM/40mins); Lane 4, PDGF (20ngml^{-1}/6mins).

5.3.2.2 Use of the PKBα and PKBβ antisense probes to analyse the possible role of PKB in S6K Activation

Having established a suitable method for analysing S6K activation in 3T3-L1 fibroblasts and shown that a variety of growth factors can activate these proteins, it was now important to examine the possible roles of PKBα and PKBβ in this activation profile. Following optimal condition antisense treatment of the fibroblasts with either, the PKBα probe αAS3 (4μM/96 hours) or the PKBβ probe βAS2 (8μM/96 hours), the cells were either left unstimulated or stimulated with serum, PDGF or vanadate and the resulting cell extracts analysed using the established S6K SDS-PAGE/Western blot protocol. It should also be noted that in these experiments, the final 24 hours of the 96-hour transfection protocol is performed in serum free (0.25% ($^{w}/_{v}$) BSA) conditions in order to generate the serum starved/basal state of S6K prior to the stimulation experiments.

In 3T3-L1 fibroblasts stimulated for 10 minutes with 25%($^{v}/_{v}$) serum, prior treatment with either the αAS3 or βAS2 probes did not affect the serum-induced phosphorylation shift and activation of either p70S6K or p85S6K to any significant level (see figure 5.3a). Both these antisense treatments were found to deplete their respective PKB isoforms by over 90%, yet despite the removal of either PKBα or PKBβ activity, S6K activation followed the same pattern observed in serum-stimulated control cells (no treatment or lipofectin™ only). This suggests that the specific removal of either PKBα or PKBβ activity from 3T3-L1 fibroblasts is not sufficient to prevent S6K activation in response to serum stimulation.

The possible involvement of the PKBα and PKBβ isoforms in activating S6K in serum stimulated 3T3-L1 fibroblasts was further investigated by comparing the serum induced activation of S6K in response to prior treatment with the PKBα or PKBβ antisense probes, or their corresponding control oligonucleotides. Prior treatment of 3T3-L1 fibroblasts with either αAS3 or its matched oligonucleotide controls did not affect the serum induced activation of either S6K isoform, further suggesting that PKBα does not have an isoform specific effect on S6K activation (see figure 5.3b). A similar situation was found when 3T3-L1 fibroblasts were pre-treated with the βAS2 or its matched oligonucleotide controls before serum stimulation, with the activation profiles of p70S6K and p85S6K, in response to serum, being very similar in the all oligonucleotide treated and control cells. Therefore, as was the case with the α isoform, these results indicate that PKBβ does not have

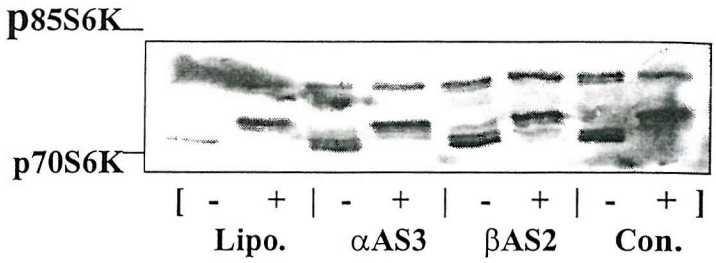
an isoform specific role in the activation of S6K (see figure 5.3c).

As 3T3-L1 fibroblasts are known to contain both the α and β isoforms of PKB, the next task was to see if the growth factor-induced phosphorylation and activation of S6K could still be achieved if neither PKB isoform is present. Therefore, the two PKB antisense probes αAS3 and βAS2 were used at their optimum conditions in tandem in the 3T3-L1 fibroblasts prior to serum stimulation. The use of these two probes in tandem was found to significantly deplete both PKB isoforms as expected but did not affect the serum-induced phosphorylation or activation of S6K as indicated by band shift (see figure 5.3d). Therefore, as seen previously when these antisense probes were used separately, when they are used in tandem to deplete both PKBα and PKBβ, no change in the serum induced activation of p70S6K or p85S6K is observed. This suggests that neither PKBα nor PKBβ have a role to play in the serum-induced activation of p70S6K or p85S6K in 3T3-L1 fibroblasts.

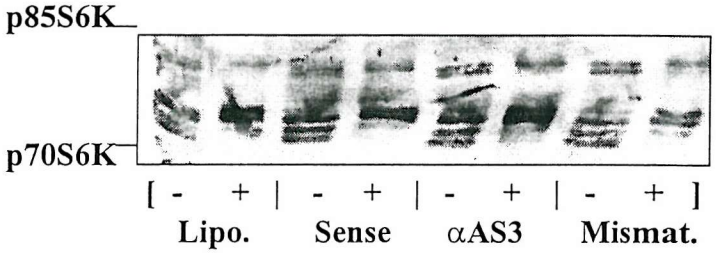
Having established using the designed antisense probes that neither PKBα or PKBβ has a role in serum stimulation of S6K, I next looked at the possible roles of these two isoforms in vanadate and PDGF induced S6K activation in 3T3-L1 fibroblasts. Similar initial experiments to those used in analysing the role of PKB in serum-induced S6K activation, revealed that depletion of either PKBα or PKBβ individually is not sufficient to prevent the activation of S6K by vanadate or PDGF (data not shown). This was further evidenced in experiments comparing the activation of the S6K isoforms, in response to PDGF and vanadate, in cells treated with either, one of the antisense probes or their matched the controls. This again showed no effect on p70S6K or p85S6K phosphorylation or activation (data not shown)

As with the serum stimulation experiments, the 2 antisense probes were then used in tandem on the 3T3-L1 fibroblast cells prior to stimulation with either vanadate or PDGF. These results again showed that depletion of both PKBα and PKBβ isoforms together did not prevent S6K activation in response to either vanadate or PDGF, suggesting that neither isoform is involved in the phosphorylation or activation of p70S6K or p85S6K by these factors in 3T3-L1 fibroblasts (see figure 5.3e). Therefore, these results together suggest that in 3T3-L1 fibroblasts nether PKBα or PKBβ is involved in the growth factor phosphorylation or activation of either p70S6K or p85S6K.

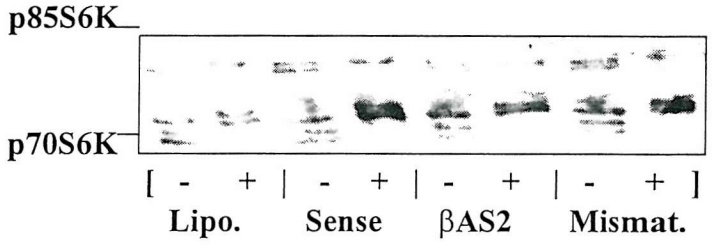
A. αAS3 or βAS2 Treatment +/ - Serum



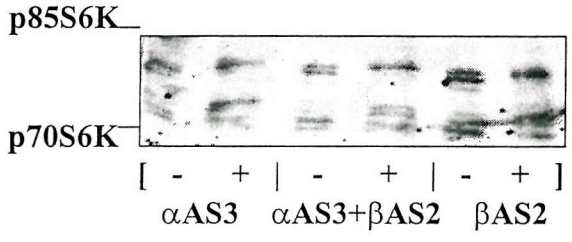
B. **αAS3 Treatment +/ - Serum**



C. **βAS2 Treatment +/ - Serum**



D. αAS3 and/or βAS2 +/ - Serum



E. αAS3 and/or βAS2 +/ - Vanadate or PDGF

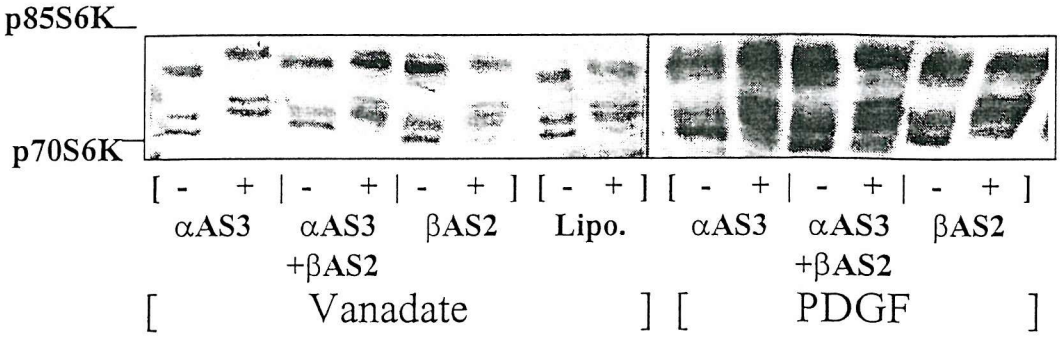


Figure 5.3 αAS3 and βAS2 treatment of 3T3-L1 fibroblasts does not affect p70S6K/p85S6K phosphorylation/activation as assessed by electrophoretic mobility shift assay. 3T3-L1 fibroblasts (95%) confluent were transfected for 96 hours with αAS3 (4μM), βAS2 (8μM) or both antisense probes in tandem as described in section 2.3.1. Subsequently the cells were incubated with various agonists and the results analysed by band-shift Western blotting. **A,** Anti-S6K Western blot indicating that FBS (20%$^{v}/_{v}$/10mins) induced activation of p70S6K/p85S6K is not affected by prior αAS3 or βAS3 treatment. **B,** Anti-S6K Western blot showing that FBS (20%$^{v}/_{v}$/10mins) activation of p70S6K/p85S6K is not altered by prior treatment with αAS3 or its matched control oligonucleotides. **C,** Anti-S6K Western blot showing that FBS (20%$^{v}/_{v}$/10mins) induced activation of p70S6K/p85S6K is not altered by prior treatment with βAS2 or its matched control oligonucleotides. **D,** Representative anti-S6K Western blot indicating that p70S6k/p85S6K activation by FBS (20%$^{v}/_{v}$/10mins) is not affected by prior treatment of cells with αAS3 and βAS2 in tandem. **E,** Representative Anti-S6K Western blots indicating that p70S6K/p85S6K activation by Vanadate (10mM/40mins) or PDGF (20ng.ml^{-1}/6mins) is not altered by prior treatment with αAS3 and/or βAS2.

5.3.3 Discussion

5.3.3.1 Development of a Suitable Assay for the analysis of S6K Phosphorylation and Activation

The development of a SDS-PAGE/Western blot protocol for the phosphorylation profile and hence activation, of S6K enables the study of S6K activation in response to a variety of growth factors and allows analysis of the role PKB may play in this pathway. This assay system was developed for use with the UBI S6K antibody with the optimum SDS-PAGE conditions for maximum resolution of individual phosphorylation states of S6K established using a 10cm by 8cm 7.5% acrylaminde/0.09% bis-acrylamide SDS-polyacrylamide gel. This gel system clearly showed slower migrating bands for both the p70S6K and p85S6K with a variety of growth factor stimulations indicating their hyperphosphorylation state and hence, activation, in response to these stimuli. These slower migrating or activation bands were not seen in growth factor-stimulated cells, which had been previously treated with the PI3K inhibitor wortmannin, which is known to also inhibit S6K activation. These results indicated that the electrophoretic mobility shift in p70S6K and p85S6K observed with growth factor stimulation can be directly attributable to the activation of these isoforms and hence can be used as method of analysing S6K activation and the roles PKB may play in this.

Interestingly, with this Western blot assay system multiple closely spaced immunoreactive bands are often observed creating a ladder like array of S6K proteins. This array of differently migrating proteins is assumed to represent the differing degrees of phosphorylation of S6K. Some laddering of S6K protein was actually observed in unstimulated cells, which would be indicative of the basal hypophosphorylated state of S6K. However, with growth factor treatment the migration pattern showed a clear difference to this basal state with the appearance of a more slowly migrating band only seen with stimulation and therefore representing maximum phosphorylation and hence activation of S6K.

The array of slower migrating S6K bands, indicating different phosphorylation levels in these experiments are in accordance with the previous findings of Weng *et al.* (Weng *et al.* 1998). This group found an array of p70S6K bands in S6K blots particularly after stimulation and showed

these to represent different degrees of phosphorylation. They also found that the progressively slower mobility observed, is not primarily due to increased negative charge generated by multiple phosphorylations but by altered conformation caused by phosphorylation at specific sites particularly T389 and T229 (Weng *et al.* 1998). These two sites were shown to have a disproportionate affect on S6K mobility which reflects the importance of these sites in S6K activation. Further studies by this group revealed that only the most slowly migrating band representing fully phosphorylated S6K possessed any S6K activity (Weng *et al.* 1998).

These experiments, therefore, agree with my findings that the slowest band of either S6K isoform, which appears only in stimulated cells, should be used as a measure of S6K activation. Thus, it is clear that this form of assay can be used effectively to monitor S6K activation, as long as only the appearance/disappearance of this band is analysed. Hence it is clear that with this SDS-PAGE system I have developed an effective method for studying the activation profile of p70S6K and p85S6K

5.3.3.2 Possible Roles of PKBα and PKBβ in S6K Activation

The two designed PKB antisense probes directed against the α (αAS3) and β (βAS2) isoforms were used to analyse the potential roles of PKBα/β in S6K activation by a variety of factors previous shown to activate all the PKB and S6K isoforms. Transfection of 3T3-L1 fibroblasts with either antisense probe individually, was found not to prevent S6K activation in response to serum, PDGF or vanadate stimulation. This suggested that depletion of either the α or β isoform independently, was not sufficient to prevent the activation of S6K. These findings were also confirmed using various matched control oligonucleotides for each isoform further indicating neither the α or β isoform is solely responsible for S6K activation.

Having established that removal of these isoforms individually, does not affect S6K activation, the two PKB antisense probes were used in tandem to deplete both PKBα and PKBβ. These results showed that removal of both these PKB isoforms simultaneously did not prevent the band-shift of S6K in response to serum, PDGF and vanadate suggesting the neither PKBα nor PKBβ is involved in growth factor activation of S6K in 3T3-L1 fibroblasts.

These results still need to be treated with some degree of caution, as it is possible the small amount of PKBα or PKBβ remaining following the respective antisense treatments is sufficient to still activate S6K. However, as these two antisense treatments deplete their respective PKB isoforms by well over 90% and this level of depletion of PKBα has already been shown to inhibit differentiation, is seems unlikely that low levels of these PKB isoforms contribute to S6K activation.

It would be interesting to further investigate any roles PKBα and PKBβ has in S6K activation by performing a kinase assay for S6K activity in response to a variety of growth factors and the antisense treatment. This could perhaps be performed by immunoprecipitating S6K from cells treated with various stimuli/antisense and analysing the effects of these on overall S6K kinase activity towards a specific substrate. This would hopefully rule out any possible roles for PKBα and PKBβ in modulating S6K activity, which could not be detected by the Western blot analysis.

The presence of PKBγ in 3T3-L1 fibroblasts also need to be considered, in analysing the roles of PKB in S6K activation. It could be that the presence of PKBγ in the PKBα/β depleted cells is sufficient to perform the role of PKB in S6K activation in a non isoform-specific fashion. This could indicate that the presence of any PKB isoform in adequate levels, is sufficient to mediate growth factor activation of S6K and hence that PKB generally signals to S6K rather than in an isoform specific manner. Alternatively, PKBγ, may be the only PKB isoform that can mediate growth factor signals to S6K and so activate S6K in an isoform-specific way. It is therefore important, to assess the role of PKBγ individually and PKB generally by using the PKBγ specific and multiple PKB isoform antisense probes currently under development.

Therefore, these experiments raise questions about the possible involvement of PKB in the activation of S6K. It has been found that neither the PKBα nor PKBβ isoforms act in an isoform-specific manner towards S6K and that removal of both of these does not prevent S6K activation by a variety of growth factors. Whilst the possible role of PKBγ still needs to be addressed the lower general expression of this isoform suggest that it also may not have a major role to play in growth factor induced S6K activation. Therefore, these experiments point towards a PKB independent activation mechanism for S6K in 3T3-L1 fibroblasts.

It would also be interesting to analyse the effects of the various PKB antisense probes on the activation of S6K in other cell lines and in response to other stimuli, including not only other growth factors, but also events such as heat shock, oxidative stress or osmotic shock. These experiments would enable the study of the possible role PKB has in S6K activation in other situations and hopefully clarify what, if any, roles this protein has in the S6K pathway. This would hopefully establish whether the lack of roles for PKBα of PKBβ in S6K activation observed in 3T3-L1 fibroblasts is a cell type or stimulation factor-specific event or whether S6K activation generally occurs via of PKB independent route

The role of PKB in the activation of S6K is very controversial, with most recent evidence suggesting that PKB and S6K lie on separate/parallel pathways downstream of PI3K/PDK1 (reviewed in Vanhaesebroeck *et al.* 2000, reviewed in Dufnar *et al.* 1999). This would therefore be in agreement with my findings in the 3T3-L1 fibroblasts that show that PKBα and PKBβ do not appear to activate S6K. The current arguments for and against a role for PKB in S6K activation are therefore discussed below.

Initial studies into the possible role of PKB in S6K activation showed that a gag tagged PKB which is therefore membrane localised and constitutively active, activates S6K in a variety of cells, although no direct phosphorylation of S6K by PKB has been shown (Reif *et al.* 1997). Secondly, a dominant negative PKBα mutant was found to inhibit S6K activation in response to heat shock and PDGF stimulation in CHO cells (Kuroda *et al.* 1998). The protein mTOR is the target site for the inhibitory action of rapamycin, which is also a known inhibitor of S6K activation. Therefore, a role for mTOR in S6K activation is indicated and has subsequently been shown using rapamycin resistant-mTOR mutants, which still activate S6K even in the presence of rapamycin (Von Manteuffel *et al.* 1997). Since PKB has been shown to directly phosphorylate and activate mTOR *in vitro* and in overexpression studies, this suggests that PKB may signal to S6K via its activation of mTOR. (Scott *et al.* 1998)

However, recently some of these initial findings have been questioned by new experimental evidence. The overexpression of dominant negative mutants of PKB has also been shown to affect the function of the upstream PKB activator PDK1 by binding endogenous PDK1 and preventing its action on other downstream targets (reviewed in Vanhaesebroeck *et al.* 2000). This could be of key

importance in the findings that dominant negative PKBα mutants inhibit S6K activation, since as will be discussed later, PDK1 has also been proposed to directly phosphorylate and activate S6K (Pullen *et al.* 1998).

Interestingly, it has been found recently that whilst a membrane targeted PKB mutant, which is therefore constitutively active caused S6K activation, a double aspartate substituted active mutant (E308T/E473S) did not activate S6K (Dufner *et al.* 1999b). However, the membrane targeted active mutant did not act on the other potential PKB targets GSK-3 and 4E-BP1, whilst the cytosolic active mutant acted on both these likely downstream targets (Dufner *et al.* 1999b). This suggests that the cellular localisation of PKB may be important in identifying its downstream proteins targets. Since endogenous PKB has been shown to be predominantly cytosolic when activated, its possible role in the activation of S6K, is therefore, called into question. PKBα has also been shown not to directly phosphorylate p70S6K either *in vitro* or *in vivo* (Alessi *et al.* 1998, Pullen *et al.* 1998).

The role of PKB in activating mTOR is also not clear, since experiments indicating a direct link between these two proteins have only been shown *in vitro* or in overexpression studies, which often are not true representations of substrate specificity (Scott *et al.* 1998). The role of mTOR in S6K activation is also unclear (reviewed in Dufnar *et al.* 1999). *In vitro* mTOR was found to phosphorylate T389 of a bacterially expressed S6K, but not to act on the mammalian form (Burnett *et al.* 1998). mTOR has also previously been show to directly phosphorylate 4E-BP1 at sites in a S/T-P motif, however, the T389 and other sites in S6K do not possess this motif, suggesting mTOR may not act directly towards this site (Burnett *et al.* 1998). The T389 phosphorylation site which has been found to be key in activating S6K is still phosphorylated in a rapamycin-resistant S6K mutant in the presence of rapamycin, whilst mTOR activity is completely inhibited (Dennis *et al.* 1996). Current evidence now points towards the regulatory role of mTOR in S6K activation to occur via the inhibition of an S6K phosphatase, possibly PP2A and so acts to prevent inactivation/inhibition of the S6Ks, rather than directly activating these proteins (Peterson *et al.* 1999, reviewed in Dufnar *et al.* 1999).

Further lines of evidence against a role for PKB dependent routes for S6K activation are also mounting. Studies in Balb/c-3T3 cells showed that calcium depletion did not affect growth factor-induced PKB activity, but completely abolished S6K activity. Likewise, whilst increasing intracellular calcium increased S6K activity, there was no increase in PKB activity (Conus *et al.* 1998). The phorbol ester TPA was also found to activate S6K via a PI3K/PKB independent route, probably involving various PKC isoforms and possibly Erk 1/2 activation (Herbert *et al.* 2000). In Swiss 3T3 cells, oxidative stress has been shown lead to the generation of $PI3,4P_2$ and subsequent activation of PKB, however, no activation of S6K is observed (Van der Kaay et al. 1999). Conversely, in response to osmotic stress, the lipids $PI3,4,5P_3$ and $PI3,4P_2$ are generated and S6K activation observed, however, no activation of PKB is seen due to the simultaneous activation of a PKB inhibition pathway in response to this stress (Van der Kaay et al. 1999).

In 32D myeloid progenitor cells overexpressing IRS-1 or IRS-2, insulin activates PKB, S6K and leads to the phosphorylation of BAD (Uchida *et al.* 2000). However, in cells expressing IRS-4, insulin has been shown to only activate PKB and phosphorylate BAD, with no observed activation of S6K, indicating PKB and S6K may lie on distinct pathways (Uchida *et al.* 2000). In L6 myotubes, it appears that insulin induced activation of S6K occurs by two mechanisms with two phases of activation. In the initial activation phase, a role for PI3K and perhaps PKB has been proposed, but in the second or late phase activation appears to be independent of the PI3K/PKB pathway. Interestingly, recently a dominant negative double alanine PKB mutant (A308T/A473S) was shown to inhibit S6K activation by less than 5% in response PDGF treatment again indicating PKB is not a major factor in S6K activation (Ballou *et al.* 2000).

Therefore, it is apparent that PKB is not involved in S6K activation in many cell types and in response to a variety of stimuli. This is in direct agreement with my findings in 3T3-L1 fibroblasts which show that PKBα and PKBβ are not involved in S6K activation in these cells. It is possible that any involvement of PKB in S6K activation, may be confined to certain cell types or certain stimuli, which is why it is important to study this further using the various designed antisense probes (Kim *et al* 1999).

The question therefore remains, as to what is the major pathway of S6K activation and what proteins are involved in this activation. The involvement of PI3K in S6K activation has been indicated in studies using the PI3K inhibitors, LY29004 and wortmannin, as well as experiments using various PI3K mutants (reviewed in Dufner *et al.* 1999). The discovery of the upstream PKB activator, PDK1, has indicated a new route for S6K activation (Alessi *et al.* 1998a & b). PDK1 not only directly activates PKB, but also directly phosphorylates and activates other downstream proteins including SGK, PKC and PRK (reviewed in Peterson *et al.* 1999b). S6K has been found to belong to the AGC family of protein kinases that PDK1 acts on (reviewed in Peterson *et al.* 1999b). This raised the possibility that PDK1 could phosphorylate S6K on the homologous site to the T308 site PDK1 acts on in PKB. Studies have subsequently revealed that PDK1 can directly phosphorylate this site, T229 in S6K both *in vitro* and *in vivo*, with this phosphorylation resulting in the activation of S6K (Pullen *et al.* 1998, Alessi *et al.* 1998b). Therefore, this suggested that PDK1 could directly phosphorylate and activate S6K. An inactive PDK1 mutant has also been shown to block S6K activation further enhancing the claim that PDK1 signals to S6K (Alessi *et al.* 1998b).

The phosphorylation on T229 of S6K by has been found to be dependent on the phosphorylation of the T389 site and so therefore the identity of the kinase that acts on this site is sought (reviewed in Dufner *et al.* 1999). Although mTOR can phosphorylate this site, the data surrounding this role is somewhat controversial, as I have already discussed. Interestingly, the T389 site has been identified to be the same as the PDK-2 in other ACG kinases, for example S473 in PKBα suggesting a similar model of activation for this family of kinases (reviewed in Peterson *et al.* 1999b). PDK1 has been recently shown to also phosphorylate this site in S6K, with this PDK1 directed phosphorylation of T389, corresponding to S6K activation (Balendran *et al.* 1999). Therefore, it appears that PDK1 may be able to act against the PDK1 (T229) and PDK2 (T389) sites in the S6K activation, a situation which is similar to the proposed dual role for this kinase in PKB activation (Balendran *et al.* 1999). Curiously, however, with PDK1 dual phosphorylation/activation of PKB the PIF fragment is required, whereas the presence of this fragment actually prevents PDK1 from phosphorylating either site of S6K and hence activating this protein (Balendran *et al.* 1999). Therefore it is clear that whilst PDK1 plays an important role in S6K activation the exact function of this kinase in the activation profile of S6K needs to be resolved.

Other possible pathways involved in S6K activation include PKC, Erk 1/2 and rac-1, however, the importance of these proteins in the physiological activation of S6K is unclear (reviewed in Dufner *et al.* 1999). Other possible activators of S6K, may have yet to be identified particularly kinases which act on the carboxyl terminal auto-inhibitory domain phosphorylation sites. Therefore, it is important to try to identify the possible proteins involved in S6K activation and the mechanisms and importance of each of these. The results presented in this chapter and current experimental evidence, suggest that PKB does not have an important role to play in S6K activation. Thus further study is required not only to establish what if any roles the PKB isoforms may have in the S6K pathway in different cells, but also to establish exactly how S6K is regulated. It would also be interesting to investigate events downstream of S6K to establish, what if any, isoform specific roles the p70S6K and p85S6K forms have, and exactly what functions S6K mediates. Therefore, the S6K pathway represents a major cell signalling pathway that requires a great deal of further study.

Chapter 6: Development of a Suitable Kinase Assay for Measuring GSK-3 Activity

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6.1 Glycogen Synthase Kinase-3 (GSK-3)

6.1.1 Introduction to GSK-3

One of the most widely investigated potential PKB substrates is glycogen synthase kinase-3 (GSK-3) which was originally identified as a serine/threonine kinase that phosphorylates and inactivates glycogen synthase but is itself serine phosphorylated and inhibited in response to insulin stimulation (Cohen *et al.* 1982, Cohen *et al.*1985). Since this initial work, GSK-3 has been found to be a much more complex protein with many potential roles in metabolic control, including the negative regulation of glycogen synthase. Overexpressed GSK-3 has also been shown to phosphorylate many other substrates in mammalian cells including, eukaryotic initiation factor 2B (eIF2B), the microtubule associated phosphoprotein tau, β-canetin, IRS1/2 and transcription factors such as c-Jun, l-*myc* and CREB (Plyte *et al.* 1992). Therefore, GSK-3 appears to play a critical role in the regulation of a variety of cellular processes including transcription, glycogen synthesis, protein synthesis and possibly apoptosis. Hence a greater understanding of this protein's cellular functions, how it is regulated and what role PKB plays in its regulation is essential in unravelling the complex signalling pathways involving GSK-3.

GSK-3 is a fairly low abundance protein which was originally purified as a 51 kDa protein in 1982. However, when the cDNA library from rat brain was screened it revealed that there were two distinct isoforms of GSK-3 derived from different genes. The first was found to be the 51 kDa protein and was called GSK-3α. The second cDNA region encoded a 47 kDa protein which has a central 30KDa region homologous to other protein kinases. This protein was found to be 98% homologous to GSK-3α and so hence was termed GSK-3β. Despite the close structural and substrate homology between the two isoforms, outside the catalytic domain these proteins were found to be only distantly related. GSK-3α comprises 483 amino acids, whist GSK-3β, the smaller isoform, has only 419, with this difference mainly due to an extra 40 amino acids N-terminal of the

catalytic domain of GSK-3α. The presence of two differing isoforms was subsequently proved using polyclonal antibodies raised against each isoform (Woodgett 1990).

GSK-3 appears to be a fairly ubiquitous protein based on zoo blot analysis of genomic DNA, with vertebrates having 98% amino acid homology in the catalytic domain and generally a greater than 65% identity in this domain (Plyte *et al.* 1992). To date, GSK-3α and GSK-3β proteins have been identified and cloned in rat, mouse and human cell lines. A GSK-3β homologue, the zeste-white/shaggy gene product, has been identified in *Drosophila* and has been found to be involved in embryogenesis and segmentation (Ruel *et al.* 1993). GSK-3 homologues have also been identified in *S. cereviase* with the GSK-3β homologues MCK1 and MDDS playing roles in meiosis and chromosomal aggregation (Puziss *et al.* 1994, Perrimon *et al.* 1989). A GSK-3 homologue identified in the slime mould *Dictyosidium* has been shown to play similar roles in cell fate regulation, whilst homologues in *Xenopus* and S. *pombe* also have developmental / differentiation controlling roles (Harwood *et al.* 1995, Plyte *et al.* 1996, He *et al.* 1995). Thus GSK-3 is highly conserved throughout evolution and so is likely to play fundamental roles in ubiquitous cellular processes.

In most cells analysed, the β isoform has been shown to be the more predominant isoform and to exhibit a generally higher relative activity towards most GSK-3 substrates. GSK-3 is believed to be mainly a cytoplasmic enzyme although a significant amount is believed to be associated with membranes, the nucleus and the mitochondria. Both isoforms are also believed to be independently expressed in certain cell types, but as a general rule at least one isoform always seems to be present.

6.1.2 Possible Roles for GSK-3 and Potential GSK-3 Substrates

Many potential roles for GSK-3 in regulating a variety of cellular processes have been proposed. For example, overexpression of GSK-3 has been shown to inhibit basal and insulin stimulated glucose transport by 30-40% in 3T3-L1 cells (Summers *et al.* 1999). Conversely, the GSK-3 inhibitor lithium has been found to increase basal levels of glucose transport by up to 3-fold and to act synergistically with sub maximal insulin concentrations to stimulate glucose uptake by 10-15

fold (Orena *et al.* 2000). The same experiments also showed GSK-3 to negatively regulate or inhibit glycogen synthesis with insulin and/or lithium, again acting to remove/relieve the GSK-3 mediated inhibition of glycogen synthesis (Orena *et al.* 2000). GSK-3 was also found to inhibit glycogen synthesis in a variety of other cells including L6 myotubes, A431 cells, myoblasts and epididymal fat cells (Ukei *et al.* 1998, Saito *et al.* 1994, Welsh *et al.* 1994, Hurel *et al.* 1996, Moule *et al.* 1997. Growth factor treatment of these cells acts to relieve this inhibition. Interestingly, GSK-3 activity has also been found to be increased in skeletal muscle in patients suffering form type 2 diabetes, indicating that GSK-3 may have a role in this insulin resistance linked disorder (Nikoulina *et al.* 2000).

GSK-3 has also been proposed to inhibit protein synthesis at the level of translation initiation probably via direct phosphorylation and inhibition of initiation factors (Welsh *et al.* 1993 & 1997). A role for GSK-3 in the negative regulation or inhibition of transcription has also been suggested based on GSK-3 direct action on transcription factors and its inhibition of transcriptional activation (de Groot *et al.* 1993, Nikolakaki *et al.* 1993). Recently GSK-3 has also been shown to have possible apoptotic inducing potential suggesting it may act in opposition to growth factor mediated cell survival pathways. Overexpression of GSK-3 in Rat1 fibroblasts and PC12 cells was shown to induce apoptosis whilst conversely overexpression of dominant negative GSK-3, was shown to prevent apoptosis induced by serum withdrawal or PI3K inhibitors (Hetman *et al.* 2000).

GSK-3 is predominantly a cytosolic, enzyme but also been found in membranes and the nucleus which may explain how GSK-3 is able to act on such a wide variety of substrates including those contained within the nucleus. GSK-3 appears to have important roles in the regulation of glucose metabolism, transcription and translation as well as in functions and apoptosis. Therefore, GSK-3 is likely to be involved in controlling many important cellular processes and hence identification of its possible direct cellular substrates is important in unravelling its exact cellular roles (reviewed in Plyte *et al.* 1992, Welsh *et al.* 1996).

GSK-3 has been shown to phosphorylate many substrates that can be split into two distinct groups. The first group of substrates has been found to require prior phosphorylation by a different kinase before GSK-3 can phosphorylate them (reviewed in Plyte *et al.* 1992). Substrates within this group usually require phosphorylation on serine or threonine residues 4 places C-terminal of the site

GSK-3 acts on. Substrates in this group include glycogen synthase which is previously phosphorylated by casein kinase 2, and the G-subunit of PP1 after prior phosphorylation by PKA (reviewed in Plyte *et al.* 1992). The substrates within this group appear to have a conserved structural motif of S-X-X-X-S(P)- for GSK-3 to be active towards them, with other members of this group including CREB protein and ATP citrate lyase (Benjamin *et al.* 1994, reviewed in Plyte *et al.* 1992). The other classified group of substrates are those which do not have a requirement for a prior phosphorylation, with members of this group including the transcription factors c-Jun and l-*myc* and the microtubule associated protein tau (reviewed in Plyte *et al.* 1992).

6.1.2.1 Glycogen Synthase (GS):

The action of GSK-3 on GS is probably the best known and best understood of all the actions of GSK-3. GSK-3 phosphorylates GS at four serine residues in the sequence RPASSRPPSPSLSRHSSPHQSEDEE with this occurring after prior phosphorylation of GS by casein kinase-2 (reviewed in Plyte *et al.* 1992, Cohen *et al.* 1982). The phosphorylation of GS by GSK-3 inhibits GS and thus prevents the conversion of glucose to glycogen (Eldar-Finkelman *et al.* 1995, reviewed in Plyte *et al.* 1992). In order to activate GS and hence synthesise glycogen, GS needs to be dephosphorylated and further inhibitory phosphorylation needs to be prevented. It is believed that insulin (a GS activator) acts by inhibiting GSK-3 and by dephosphorylating GS, using a specific protein phosphatase (reviewed in Krebs 1993). By these 2 mechanisms it is proposed that insulin activates GS and stimulates glycogen synthesis (reviewed in Plyte *et al.* 1992, Cohen *et al.* 1982, Lawrence *et al.* 1997). It is also believed that the relative contribution each of these two mechanisms plays in GS activation depends on various factors including the cell type and stimulus used (Fiol *et al.* 1988, Hemmings *et al.* 1982, Van Lint *et al.* 1993).

6.1.2.2 Insulin Receptor Substrate

Recent studies have shown that GSK-3 can also direct phosphorylate IRS1/2 at key serine residues and thereby act to modulate the IRS signalling pathway (Eldar-Finkelman *et al.* 1997). Phosphorylation at these sites by overexpressed GSK-3 has been shown to make IRS1 a much poorer substrate for the insulin receptor tyrosine kinase activity, and hence lead to a decreased phosphorylation on IRS-1 in response to insulin signalling (Eldar-Finkelman *et al.* 1997).

Overexpression of GSK-3 was shown to negatively regulate tyrosine phosphorylation of IRS-1 and thereby act to inhibit the insulin signalling pathway through this protein (Eldar-Finkelman *et al.* 1997). In this way, GSK-3 may act to prevent its own down regulation by insulin stimulated pathways in a negative feedback loop directed towards IRS proteins. This possible route of GSK-3 action has been proposed as the mechanism by which GSK-3 acts to inhibit glucose uptake and perhaps also acts in the regulation of other cellular functions including survival/apoptosis.

6.1.2.3 Microtubule Protein Tau

GSK-3β has been found to be identical in function and amino acid composition to tau protein kinase 1, one of two tau protein kinases presently known (Wagner *et al.* 1996). Tau is a neuronal microtubule associated phosphoprotein that is found mainly in axons. Six tau isoforms have so far been identified in the brain with these expressed in a developmental and cell type specific fashion (Imahori *et al.* 1997, Wagner *et al.* 1996, Lovestone *et al* 1996). Tau is found to bind to microtubules and to be involved in microtubule assembly. Hence it is important in the formation and maintenance of axons by microtubule array assembly and also promotes tubulin polymerisation. Tau has been found to be phosphorylated on various serine and threonine residues throughout its length, with many of these being sites for GSK-3β phosphorylation (Takashima *et al.* 1993). The exact role of this phosphorylation is unclear but it is likely to be regulation of tau roles in microtubule association and organisation. For example tau has been found to be heavily phosphorylated on the GSK-3β target sites in the foetal brain with this event linked to developmental control (Lovestone *et al.* 1996, Takashima *et al.* 1993, Ishiguro *et al.* 1993).

Tau is also hyperphosphorylated in the brains of Alzheimer's disease (AD) sufferers with this leading to the formation of paired helical filaments (PHF) which are so characteristic of AD (Wagner *et al.* 1996). PHFs act to destabilise microtubules leading to neuritic dystrophy and axonal transport problems ultimately resulting in cell death. In fact, the rate of clinical decline in AD has been found to be proportional to the level of PHFs development (Wagner *et al.* 1996, Imahori *et al.* 1997). The sites at which tau is hyperphosphorylated in AD have been identified as proline directed serines and threonines and have since been shown to be the sites at which GSK-3β phosphorylates Tau (S202, S235, S396 and S404). The phosphorylation of tau at these sites has been found to reduce tau affinity for microtubules and reduce its ability to promote microtubule

assembly (Takashima *et al.* 1993, Irving *et al.* 1997). Thus GSK-3β hyperphosphorylation of tau appears to lead to the generation of abnormal tau which may be involved in AD pathology (Imahori *et al.* 1997).

Interestingly β–amyloid, which is a characteristic protein in AD pathology appears also to be an activator of GSK-3β (Takashima *et al.* 1993). β-amyloid is believed to interact with neurones and increase the activity of GSK-3β, leading to the generation of paired helical filaments (consisting of hyperphosphorylated tau) and ultimately neurotoxicity or cell death (Takashima *et al.* 1993). The exact process by which β-amyloid leads to an increase in the activity of GSK-3β is still unclear with postulated ideas including ones involving calcium channels or a direct binding activation (Takashima *et al.* 1993).

6.1.2.4 Other Neuronal Substrates/Roles for GSK-3

Recently it has been proposed that GSK-3 may act via a different mechanism in its apparent role in the pathology of AD. GSK-3β has been found be present in the mitochondria and to phosphorylate and inhibit mitochondrial pyruvate dehydrogenase (PDH), the enzyme responsible for the conversion of pyruvate to acetyl CoA and so ultimately responsible for acetylcholine production in neurones (Hoshi *et al.* 1996). Prolonged inactivation of PDH would lead to mitochondrial dysfunction due to lack of acetyl CoA and eventually to cell death due to an energy deficiency. In neurones, the lack of acetylcholine would also lead to problems related to nervous signal transmission. PDH activity has been found to be decreased in AD brains with this leading to impaired acetylcholine production and disturbed glucose metabolism (Hoshi *et al.* 1996 & 1997, Imahori *et al.* 1997). β-amyloid treatment of cells has also been shown to decrease PDH activity due to a two fold increase in GSK-3β mediated PDH phosphorylation (Hoshi *et al.* 1996&1997). Therefore, it seems apparent that abnormally active GSK-3 may have a role in the pathology of AD and possibly other neurological disorders via its destabilising and inhibitory actions on tau and PDH (Hoshi *et al.*1996 & 1997).

GSK-3 is also involved in the phosphorylation of neurofilaments, which are involved in regulating axonal calibre and so are direct determinants of transmission speed (Guidato *et al.* 1996). GSK-3 has been found to phosphorylate the neurofilament NF-H on its side arm carboxyl terminal, with

this phosphorylation causing an increase in spacing and so altering axonal calibre and hence transmission speed (Guidato *et al.* 1996). Neurofilaments and especially NF-H have been found to be hyperphosphorylated in motor neurone disease and Lewy body disease (which is closely related to Parkinson's disease) (Guidato *et al.* 1996) further suggesting links between GSK-3 activity and the progression of neurodegenerative diseases.

GSK-3 also phosphorylates neuronal cell adhesion molecule, nerve growth factor and synapsin-1 in each case apparently bringing about some degree of regulation which is as yet not clearly determined (reviewed in Plyte *et al.* 1992). Therefore, from the available evidence to date it is apparent that GSK-3 plays many important roles in the nervous system with an apparent link between excessive or abnormal GSK-3 activation/expression and many common neurological diseases.

6.1.2.5 Other Possible GSK-3 substrates:

GSK-3 has been proposed to phosphorylate several nuclear substrates including transcription factors. For example, GSK-3 has been found to phosphorylate c-Jun at 3 serine residues, leading to a reduction in the DNA binding activity of c-Jun homodimers towards TRE-containing DNA, and hence inhibiting transcriptional activation. GSK-3 also phosphorylates Jun D at 3 similar sites and Jun B at 2 (de Groot *et al.* 1992, Boyle *et al.* 1991). GSK-3 has also been found to phosphorylate and inhibit the AP-1 complex and to phosphorylate l-*myc* and c-*myc* and this is likely to modulate their activity in some as yet unknown way (reviewed in Plyte *et al.* 1992). GSK-3 has recently been shown to phosphorylate the transcription factor NF-AT with this serine phosphorylation promoting nuclear export of this factor preventing the activation of early response genes by NF-AT (Beals *et al.* 1997). Another transcription factor shown to be acted on by GSK is CREB, which GSK-3 phosphorylates at serine 12 after prior phosphorylation on serine 16 of CREB by PKA (Benjamin *et al.* 1994, Ross *et al.* 1999). Interestingly GSK-3 activity and/or expression has been found to be low in the middle and latter stages of cell differentiation when these nuclear substrates are required to be at their most active (Benjamin *et al.* 1994).

GSK-3 also phosphorylates the translation factor eIF2B leading to inhibition of the translation factor and hence an inhibition of protein synthesis (Welsh *et al.* 1993). Other possible GSK-3

substrates include, citrate lyase, inhibitor-2, microtubule MAP 1B, the G subunit of protein phosphatase 1, dynamin like proteins and the neuronal protein β-catenin (Goold *et al.* 1999, Hughes *et al.* 1992, reviewed in Plyte *et al.* 1992). Many of the potential GSK-3 substrates have only been identified in studies *in vitro* or using GSK-3 overexpression and therefore further work is needed to clarify the potential substrates GSK-3 and hence cellular roles.

6.1.3 Control of GSK-3 Activity

6.1.3.1 Growth Factor Induced / Phosphorylation Dependent Control of GSK-3 Activity

GSK-3 has been found to be constitutively active in all cell types studied and to exhibit a high activity in quiescent cells. In unstimulated cells both GSK-3 isoforms have been found to be phosphorylated on a specific tyrosine residue 216 in β and 272 in α (Hughes *et al.* 1993). This tyrosine phosphorylation site has been proposed to have some role in GSK-3 regulation since it is conserved throughout the GSK-3 family of homologues (Hughes *et al.* 1993, Murai *et al.* 1996, Yu *et al.* 1997, Cross *et al.* 1994). In fact, tyrosine phosphorylation at this residue has been shown to increase the activity of GSK-3β in a variety of cell lines (Yu *et al.* 1997, Murai *et al.* 1996). However, recent evidence has questioned the importance of this residue in controlling GSK-3 activity since no change in GSK-3 phosphotyrosine content was observed when GSK-3 activity was inhibited in IGF-1 or insulin stimulated 293 cells (Shaw *et al.* 1997). Therefore the relative importance of tyrosine de/phosphorylation in control of GSK-3 activity is controversial and may be confined to assisting the regulation of GSK-3 in certain cell types or in response to certain growth factors (Yu *et al.* 1997).

Conversely, in response to many growth factors both GSK-3 isoforms have been found to be phosphorylated on several serine residues, notably serine 9 in GSK-3β and serine 21 in GSK-3α (Wang *et al.* 1994). The growth factor induced phosphorylation of GSK-3 at these sites was found to lead to a significant decrease in GSK-3 activity suggesting this sites are critical in regulating GSK-3 activity (Wang *et al.* 1994, Stambolic *et al.* 1994). This serine phosphorylation site has also been found to be conserved in all GSK-3 homologues identified and shown to play a key role in the growth factor induced negative regulation or inhibition of these GSK-3 homologues.

Insulin stimulation has been found to cause the inhibition of activity of both GSK-3 isoforms in a variety of cell types (Borthwick *et al.* 1995, Welsh *et al.* 1993 & 1994). GSK-3 activity has also been found to be decreased by phorbol esters (TPA), epidermal growth factor (EGF) and serum with each factor causing the inhibition of GSK-3 activity to varying degrees (Welsh *et al.* 1994, Saito *et al.* 1994). For example, in CHO-7 cells, insulin or serum stimulation decreased the GSK-3 activity by 70%, however TPA caused only a 40-45% inhibition of GSK-3 activity in these cells (Murai *et al.* 1996). In contrast, in NIH 3T3 cells, EGF decreased the activity of GSK-3β by 50% (Eldar-Finkelman *et al.* 1995), whilst in CHO-IR cell lines a TPA treatment caused 30% reduction in GSK-3 activity with a 50% reduction observed with insulin treatment. In all cases, the actions of these factors appears to be mediated by proteins which bring about phosphorylation of serine 9 in GSK-3β and serine 21 in GSK-3α emphasising the importance of these sites in the growth factor induced inhibition of GSK-3 activity (Wang *et al.* 1994, Saito *et al.* 1994, Cross *et al.* 1995, Stambolic *et al.* 1994).

Evidence from studies in the last few years have shown that when insulin acts to decrease GSK-3β activity an increase in serine 9 phosphorylation is also observed indicating a inhibitory/regulatory role for this serine (Stambolic *et al.* 1994). Mutation of this serine to an alanine was shown to generate GSK-3 which was refractory to the inhibitory effect of IGF-1 or insulin and therefore maintained a high GSK-3 activity, even in the presence of growth factor stimulation (Murai *et al.* 1996, Shaw *et al.* 1997). The inhibitory effects of growth factor treatment on GSK-3 activity could be completely reversed by treatment with protein phosphatse-1, again indicating that serine/threonine phosphorylation is the key event in the inactivation of GSK-3 (Saito *et al.* 1994). Therefore, it seems apparent that serine phosphorylation leads to GSK-3 inactivation/ inhibition and thus this is likely to be the major control point for GSK-3. Hence, the involvement of specific kinases and phosphatases is implicated in the control of GSK-3 activity in response to growth factors and other stimuli with the strong possibility of cell type/factor specific mechanisms involved in this regulation. It is therefore important to establish the upstream pathways involved in the phosphorylation and inactivation of GSK-3, and identify the upstream kinases which act on the serine 9 and serine 21 sites of GSK-3α and GSK-3β respectively.

6.1.3.2 Potential GSK-3α/β Serine 21/9 kinases

The regulatory serine site (S9/21) of GSK-3 can be phosphorylated *in vitro* by $p90^{rsk}$ (RSK) (Sutherland *et al.* 1993), S6 Kinase (S6K) (Sutherland *et al.* 1994), PKB (Cross *et al.* 1995), SGK (Kobayashi *et al.* 1999a and b, Park *et al.* 1999) and PKC isoforms (Goode *et al.* 1992). Overexpression of these kinases have also indicated that these proteins could act on GSK-3 *in vivo* and therefore inhibitors of these distinct pathways have been used to try to establish the roles of these proteins and their connected pathways in GSK-3 regulation.

Rapamycin has been used to block the S6K pathway in a variety of cell types (Welsh *et al.* 1993b), with this inhibition shown to have no effect on the inhibition of GSK-3 via growth factor mediated pathways in most cell types (Cross *et al.* 1995, Hurel *et al.* 1996). However, in some cells, for example, 3T3-L1 adipocytes, some rapamycin sensitive activity was present in the pathways acting to inhibit GSK-3 after growth factor stimulation (Shepherd *et al.* 1995). Therefore, it is unlikely that S6K has a major *in vivo* role in the regulation of GSK-3 via growth factor mediated signal transduction in most cell types. Rapamycin, however, has no inhibitory effect on the MAP kinase/RSK pathway, the PKB/SGK pathway or the PKC pathways and so the decrease in GSK-3 activity and increase in serine phosphorylation seen in insulin stimulated cells treated with rapamycin could still be attributed to these pathways (Cross *et al.* 1997).

The possible role of the MAP kinase pathway and hence RSK in GSK-3 regulation has been investigated using the MAP kinase kinase (MEK) inhibitor PD98059 which had previously been shown to inhibit the MAP kinase isoforms Erk-1 and Erk-2 (Dudley *et al.* 1995). Using this inhibitor in myoblasts and L6 myotubes, growth factor induced activation of RSK is almost completely blocked but the growth factor stimulated inhibition of GSK-3 and activation of glycogen synthase appears to be unaffected (Hurel *et al.* 1996, Larzar *et al.* 1995, Cross *et al.* 1995). The time course of Erk-2 and RSK maximal activation by insulin in human myoblasts at around 25 minutes does not fit the time course for insulin induced inhibition of GSK-3 of around 2 minutes or the activation of glycogen synthase of around 10 minutes in the same cells (Hurel *et al.* 1996). However, there is evidence indicating that the MEK inhibitor PD98059 may not be as strong an inhibitor as originally thought and so some MAP kinase isoforms and hence their downstream components may still be activated in its presence. Recently in Swiss 3T3 cells, Erk1/2

activity has been implicated in the PMA induced inhibition of GSK-3α and the transient inhibition of GSK-3α activity by EGF stimulation (Shaw *et al.* 1999). With PMA treatment the MAPK pathway appears to be solely responsible for GSK-3α inhibition whereas with EGF stimulation MAPK appears to make an initial transient contribution to GSK3α inhibition and secondly acts to shorten the duration of GSK-3α inhibition mediated by other kinases (Shaw *et al.* 1999).

A role for PKC in regulating GSK-3 activity is also possible since TPA is known to activate PKC and also gives some degree of GSK-3 inhibition, whilst *in vitro* PKC isoforms have been shown to phosphorylate GSK-3 (Murai *et al.* 1996, Tsujio *et al.* 2000). Use of the PKC inhibitor PDBu has been shown to remove TPA induced inhibition of GSK-3 indicating a likely role for PKC in this regulation at least in some cell types (Murai *et al.* 1996). PKCδ has also recently been shown to directly phosphorylate GSK-3 and inactivation of this PKC isoform was found to lead to continuous activation of GSK-3 (Tsujio *et al.* 2000). Therefore it is possible that PKC isoforms may have a role to play in GSK-3 phosphorylation and inactivation in response to certain stimuli or in different cell types.

Perhaps the most promising direct regulator of GSK-3 activity in most cells based on current experimental evidence is PKB (Cross *et al.* 1995). Initial studies on the growth factor induced phosphorylation and inactivation of GSK-3 revealed that the PI3K pathway is the major mediator of these inhibitory effects with the PI3K inhibitors wortmannin and LY29004 found to prevent growth factor induced inactivation of GSK-3 (Hurel *et al.* 1996, Welsh *et al.* 1994). Subsequent studies using wortmannin have also shown that when PI3K is inhibited, GSK-3 activity can not be inhibited by insulin or other growth factor treatment (Cohen *et al.* 1997). PKB also phosphorylates GSK-3 at the regulatory serine not only *in vitro* but also in when overexpressed and in response to growth factors *in vivo* (Cross *et al.* 1995). Studies have revealed that in human myoblasts the insulin induced activation time of PKB at around 1 minute fits with the time course of inactivation of GSK-3 by insulin (Hurel *et al.* 1996) In rat epididymal fat cells, PKB and GSK-3 responses to insulin and isoproterenol stimulation and wortmannin or LY29004 treatment also correlated, further suggesting a link between these two kinases (Moule *et al.* 1997).

Overexpression of a constitutively active PKB in L6 myotubes, A431 or 293 cells has been shown to inactivate GSK-3 activity whilst a dominant negative PKB mutant was found to prevent growth

factor induced inactivation of GSK-3 (Ueki *et al.* 1998, Cross *et al.* 1995, Shaw *et al.* 1997). Interestingly, overexpressed PKB and GSK-3 have been shown to co-immunoprecipitate from A431 cells, again suggesting a direct link between these two proteins (Shaw *et al.* 1997). In Swiss 3T3 cells, PKB has also been proposed to be solely responsible for IGF stimulated GSK-3 inactivation and to be mainly responsible for EGF induced inactivation along with the previously mentioned contribution of MAPK (Shaw *et al.* 1999).

Despite the mounting evidence for a major role for PKB in mediating growth factor induced GSK-3 inactivation some caution in interpreting these results is required. Firstly, all the studies suggesting an involvement of PKB are based on overexpression experiments, so are not analysing endogenous PKB and GSK-3 levels and hence not the true physiological conditions. Use of PI3K inhibitors and PDK1 mutants have indicated that the PI3K/PDK1 pathway is key to mediating growth factor induced GSK-3 inhibition (Shaw *et al.* 1997). This experiments also suggested that PKB is the downstream factor of this pathway, signalling directly to GSK-3 (Shaw *et al.* 1997). However, the recent discovery of the PKB related kinase SGK has clouded the situation (Park *et al.* 1999, Kobayashi *et al.* 1999a). SGK isoforms have been found to be 45 to 55% identical to PKB but lack the PH domain (Kobayashi *et al.* 1999b). SGK is also activated in response to growth factors via the PI3K/PDK-1 pathway with a similar activation profile (time/level) to PKB (Park *et al.* 1999). Interestingly, SGK has also been shown to phosphorylate the same substrate motif targeted by PKB (RRXRXXS/T) and raise the possibility that some of the proposed physiological roles of PKB may in fact be mediated by SGK (Kobayashi *et al.* 1999a). SGK has also recently been shown to phosphorylate GSK-3 *in vitro* and in co-transfection experiments at similar rates to those observed with PKB (Kobayashi *et al.* 1999a & b, Park *et al.* 1999).

Therefore, a great deal of work needs to be done to establish how GSK-3 is regulated and in the exact roles of these potential GSK-3 kinases. The use of the antisense probes against some of the possible GSK-3 regulators (i.e. PKB and Erk) should help to identify the major physiological kinases directed against GSK-3 and involved in the growth factor induced negative regulation of GSK-3. Therefore, it is important to initially establish a suitable method of analysing GSK-3 activity in the 3T3-L1 cells in response to various growth factors and the antisense treatments.

6.2 Results

6.2.1 Design and Development of a Suitable GSK-3 assay

Initially, the activity of GSK-3 in crude cellular extracts was assessed using a commercial phosphopeptide substrate based on the region of the CREB protein that GSK-3 phosphorylates. Unfortunately, this substrate proved to be an unsuitable for GSK-3 under these conditions, as it may have been phosphorylated by other active kinases in the cell extract (data not shown). Purification of GSK-3 from the cells extracts by MonoQ/MonoS FPLC was also attempted because purified GSK-3 is known to phosphorylate the CREB based substrate effectively. However, the FPLC procedure was very long winded and complicated and only produced a very low and impure recovery of GSK-3 in the cell lines used (data not shown). This method was therefore deemed unsuitable for use in these studies, due to large amounts of extract and hence antisense/factor treatment required to yield levels of GSK-3 sufficient for assaying. Therefore, a new method of assaying GSK-3 needed to be developed.

In 1997 Welsh and Proud published details of a new assay for GSK-3 activity which could be performed in crude extracts making it quicker and easier to assay GSK-3 activity (Welsh *et al.* 1997). This assay method used a newly developed phosphopeptide based on the specific GSK-3 targeted phosphorylation site of eukaryotic initiation factor 2B (eIF2B) (see below). With this peptide, which contains a pre-phosphorylated serine residue four residues C-terminal of the GSK-3 site (e.g. group 1 motif S-X-X-X-S(P)), the assay of GSK-3 activity in crude extracts was shown to be possible. A matched control peptide (see below) that contains an alanine residue instead of the phosphoserine and hence does not have the GSK-3 target motif was also used to show the specificity of GSK-3 only for the phosphopeptide and to account for non-specific phosphorylation (Welsh *et al.* 1997). These experiments also showed that many of the commercially available GSK-3 substrate peptides (e.g. a glycogen synthase peptide) are not suitable for assaying GSK-3 activity in crude cell extracts due their lack of absolute specificity for GSK-3 (Welsh *et al.* 1997)

EIF-2B based GSK-3 peptide substrate/control:

Substrate RRAAEELDSRAGS^{P}PQL (2B-[S^{P}].);

Control RRAAEELDSRAGAPQL (2B-[A].):

Therefore, it was decided to synthesis these two peptides by a solid support synthesis protocol, using Fmoc chemistry. In order to generate the GSK-3 specific phosphopeptide the second serine residue had to be selectively phosphorylated prior to cleavage using a selective deprotection of this residue followed by its phosphorylation using phosphoarmordite chemistry (Pullen 1990) (see Section 2.3.4.2). After cleavage and HPLC purification these two peptides were analysed for their content and purity using HPLC and mass spectroscopy analysis (assisted by Dr. Robert Broadbridge) (see Figures 6.1 & 6.2). Mass spectroscopic analysis showed that the peptides generated corresponding exactly to required sequence and did not contain any contaminating fragments indicating a high degree of purity (Figures 6.1 & 6.2). The purity of the two synthesised peptides was confirmed using HPLC analysis which showed both these peptides had a greater than 95% purity suggesting that this synthesis route generated high quality peptides for use in the assay of GSK-3 activity (Figures 6.1 & 6.2).

Having successfully synthesised the GSK-3 specific phosphopeptide and its matched control peptide the task was now to investigate the use of these peptides in the ^{32}P-ATP based assay of GSK-3 and subsequently to lay down the optimum conditions for using this assay in measuring GSK-3 activity. Initial experiments analysed the most suitable lysis buffer and general components of the GSK-3 assay for use with the 3T3-L1 fibroblasts (data not shown). These results established the optimum lysis buffer and assay components to be those detailed in section 2.4.2 and emphasised the importance of having kinase and phosphatase inhibitors present throughout the extraction and assay protocols. A peptide concentration of 200μM was used throughout the experiments, as using this concentration meant that the level of substrate peptide present was never limiting in the GSK-3 assays (data not shown)

Figure 6.1 - High performance liquid chromatography and Mass Spectroscopy Purification of Control (alanine) peptide. A. HPLC elution profile of purified control peptide (elution time 13.5mins) from a C2-analytical column. **B.** Electron spray mass spectroscopy trace for purified control peptide showing parent ion (+1/Mr 1741) and doublely charged daughter ion (+2/Mr 872) in a 3meV flight.

Figure 6.1a HPLC trace of purified control (alanine) peptide (2B-[A]).

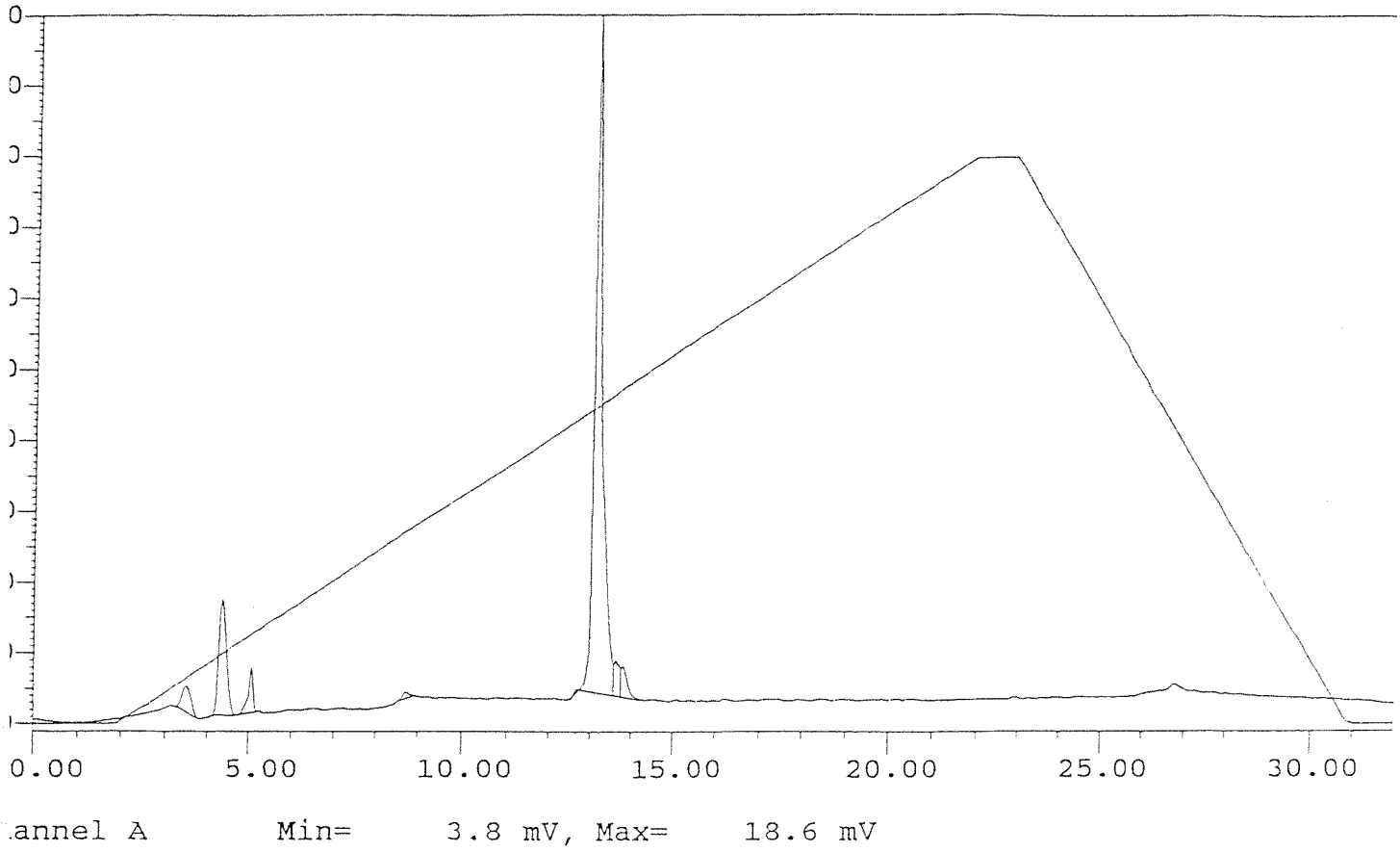


Figure 6.1b. Mass spectroscopy readout for control (alanine) peptide (2B-[A]).

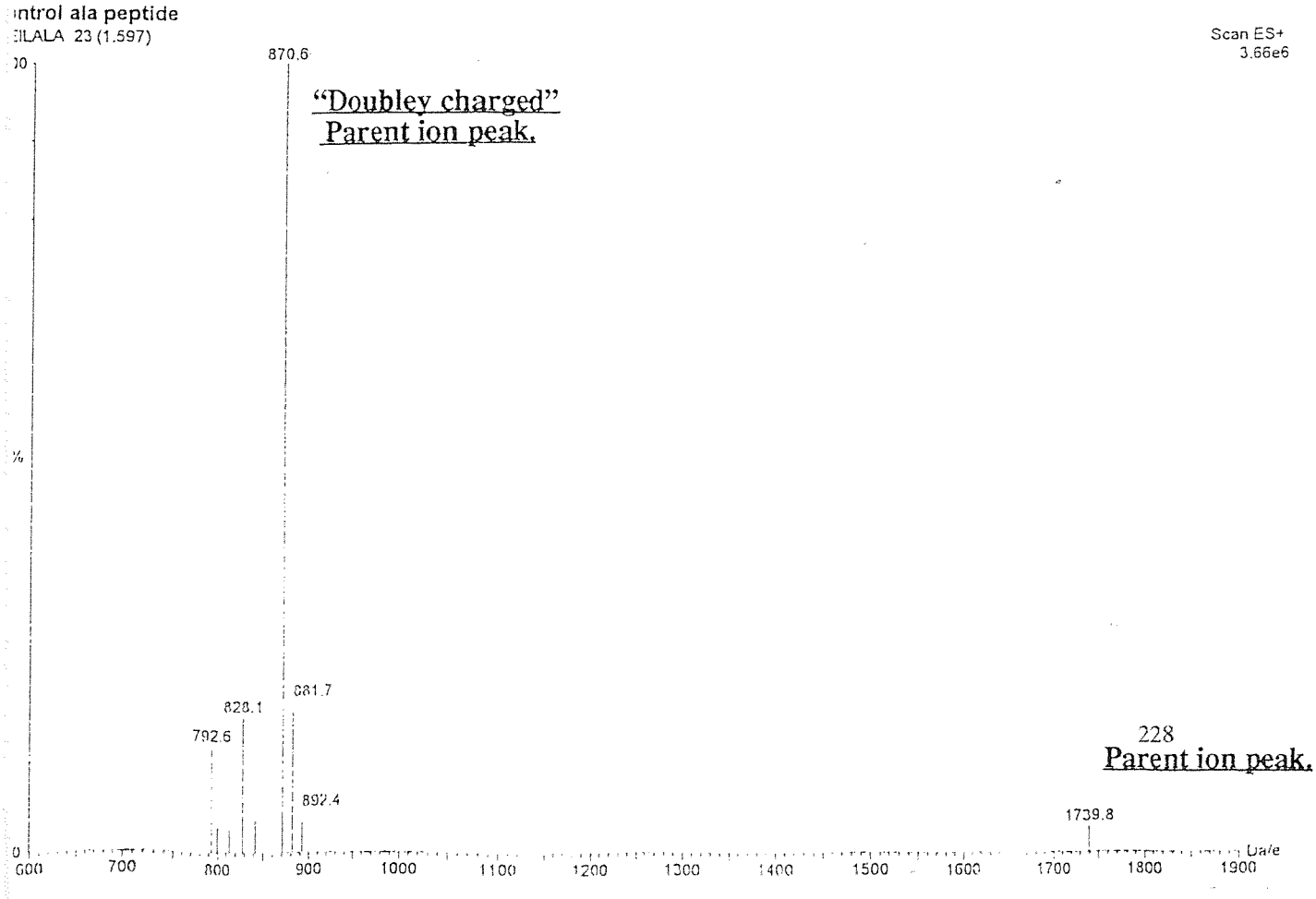


Figure 6.2 - High performance liquid chromatography and Mass Spectroscopy Purification of Phosphopeptide (Pser). A. HPLC elution profile of purified phosphopeptide (elution time 12.5mins) from a C2-analytical column. **B.** Electron spray mass spectroscopy trace for purified control peptide showing parent ion (+1/Mr 1821) and doublely charged daughter ion (+2/Mr 917) in a 3meV flight.

Figure 6.2a HPLC trace of purified phospho- (Pser) peptide (2B[S^{p}])

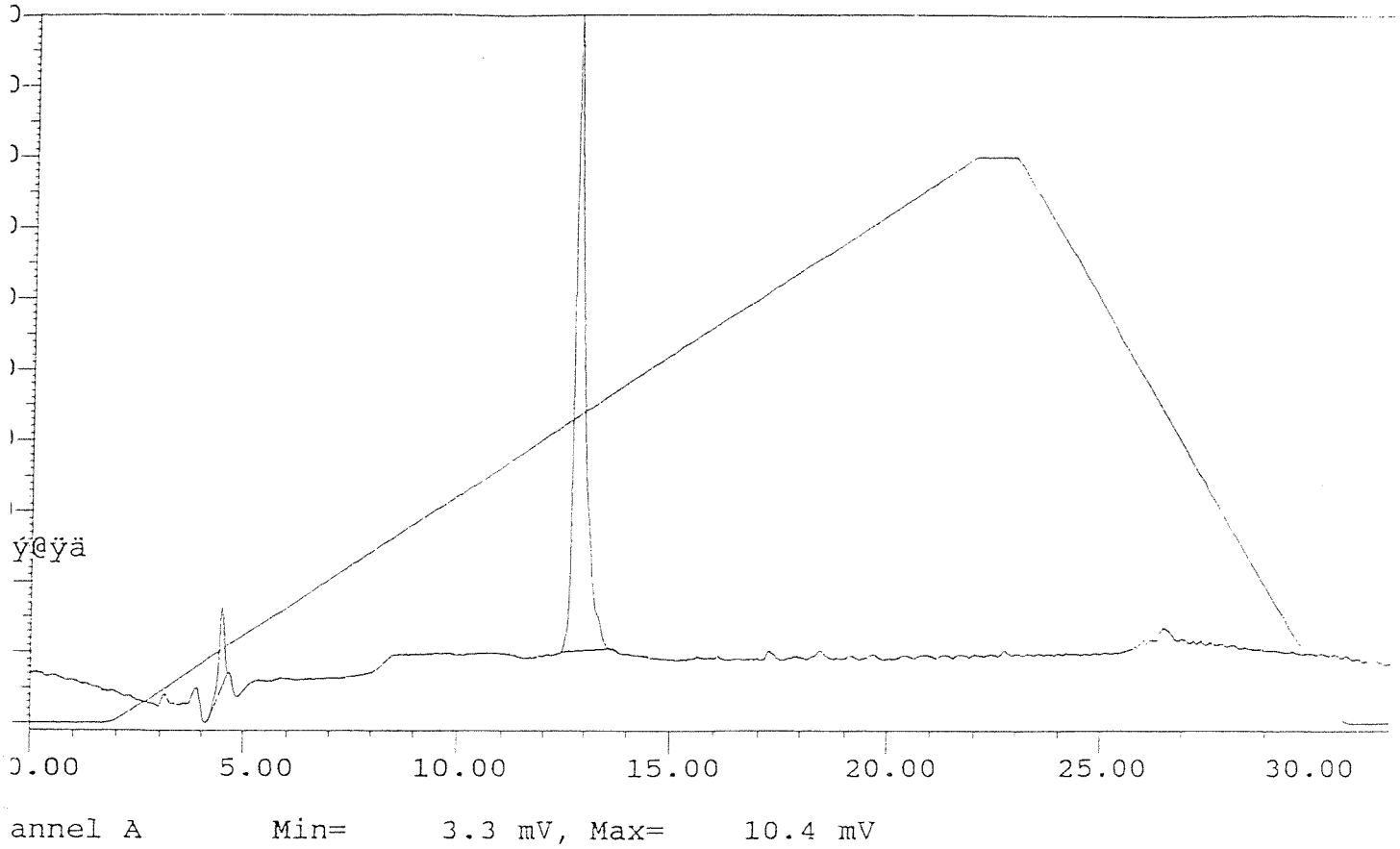
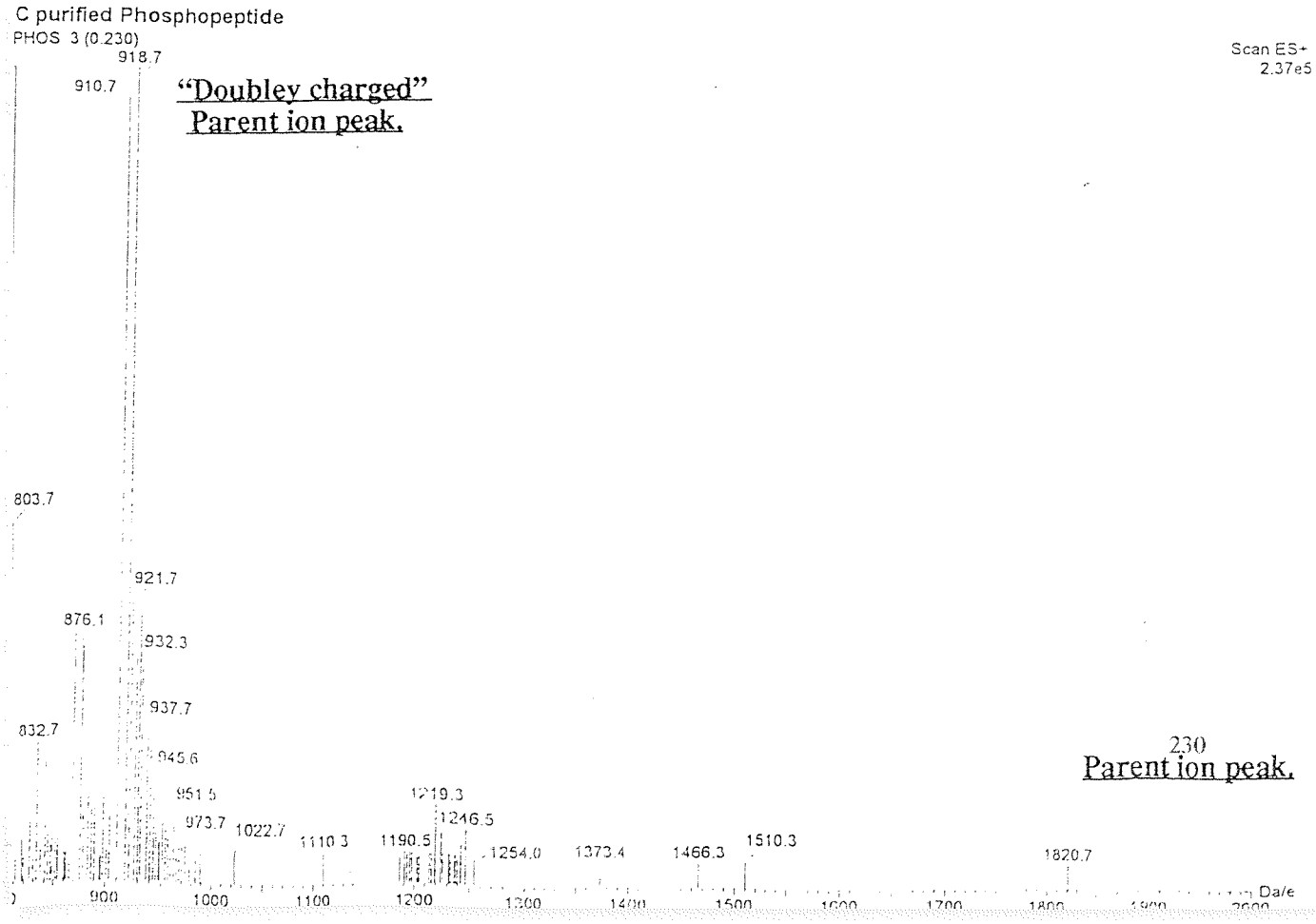


Figure 6.2b Mass spectroscopy readout for phospho- (Pser) peptide (2B[S^{p}])



The next task was to establish the optimum time course and lysate concentrations for use in the GSK-3 assay. Firstly, both peptides were incubated at 30°C with 10mg of cell lysate and the other assay components for a range of times up 30 minutes (Figure 6.3). These experiments showed that both peptides showed increased ^{32}P incorporation in a linear fashion up to around 20 minutes followed by a slower increase in phosphorylation rate up to 30 minutes (Figure 6.3). The rate/extent of phosphorylation of the GSK-3 specific phosphopeptide was 2-3 times faster than that seen with the non-specific control peptide indicating that this peptide was phosphorylated by GSK-3 (Figure 6.3). The fact that the control peptide was slowly phosphorylated in these experiments indicated other kinases can act non-specifically at this site at a much slower rate and suggest that the level of ^{32}P incorporation/phosphorylation of this peptide should be used as a background rate and subtracted to give the GSK-3 specific rate. From these experiments, a 10-minute incubation time was established as the optimum assay time for analysis of GSK-3.

The optimum lysate concentration for use in the GSK-3 assay was established by incubating various concentrations of cell lysate up to 20mg with the peptides and other assay components for 10 minutes (see Figure 6.4). This showed a concentration dependent linear increase in ^{32}P incorporation indicating that the level of phosphorylation of both peptides is directly proportional to the protein concentration present (Figure 6.4). These experiments also again showed that the GSK-3 specific phosphopeptide is phosphorylated at much faster and higher levels than the control peptide, which is still phosphorylated in a concentration dependent fashion (Figure 6.4). This suggests that the minimal ^{32}P incorporation seen with the control peptide should be subtracted from the levels of ^{32}P incorporation into the GSK-3 phosphopeptide, in order to establish the rate of GSK-3 specific phosphorylation and the GSK-3 activity. Because the level of ^{32}P incorporation was directly dependent on the protein concentration in the lysate, it was decided that a fixed volume of 10µl (about 6-11µg of protein) of cell lysate could be used with the obtained values corrected for protein concentration at later date to establish the GSK-3 activity per µg of protein.

Therefore, the optimum conditions for assay of GSK-3 in 3T3-L1 fibroblasts were established as a 10-minute assay time using10µl of cell extract (~8mg). In order to calculate the specific GSK-3 rate, several controls were included in the assay and accounted for in rate activity calculations.

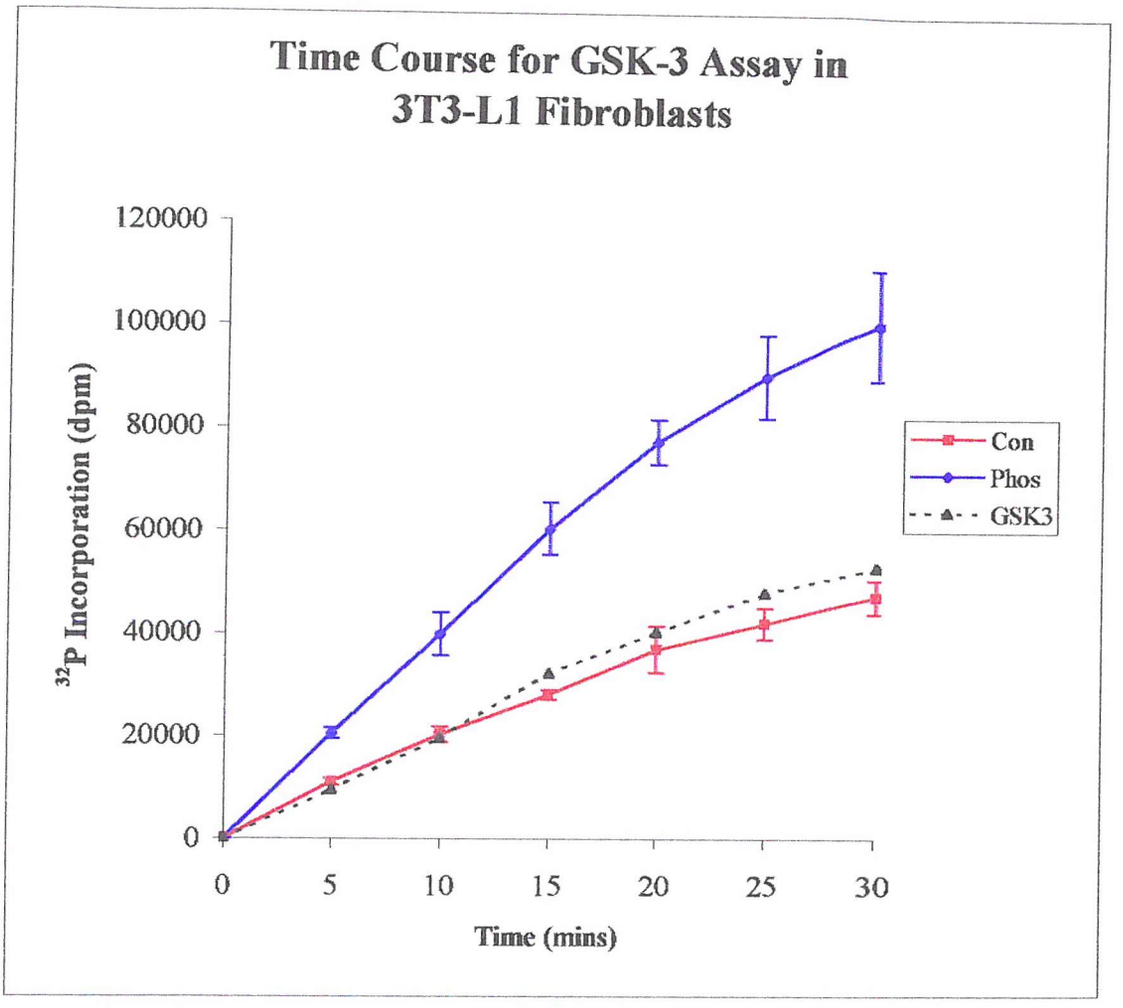


Figure 6.3 - Determination of the incubation time specific linearity of the GSK-3 phosphorylation assay. 3T3-L1 fibroblasts (~95% confluent) were extracted into NP-40 lysis buffer (see sections 2.4.2, 2.9.1) and 10µl of lysate incubated at 30°C with 200µM of either the GSK-3 phosphopeptide or the control peptide. At the times indicated 25µl aliquots were removed from the reaction mixture and spotted onto p81 phosphocellulose paper. Following extensive washing, the strips were dried and the ^{32}P incorporation into each peptide was quantitated by liquid scintillation counting. The graph is representative of 3 experiments, with the specific GSK-3 activity (---) calculated as the difference between the ^{32}P incorporation into the phospho and control peptides at each time point. Control Peptide (-----), Phosphopeptide (-----).

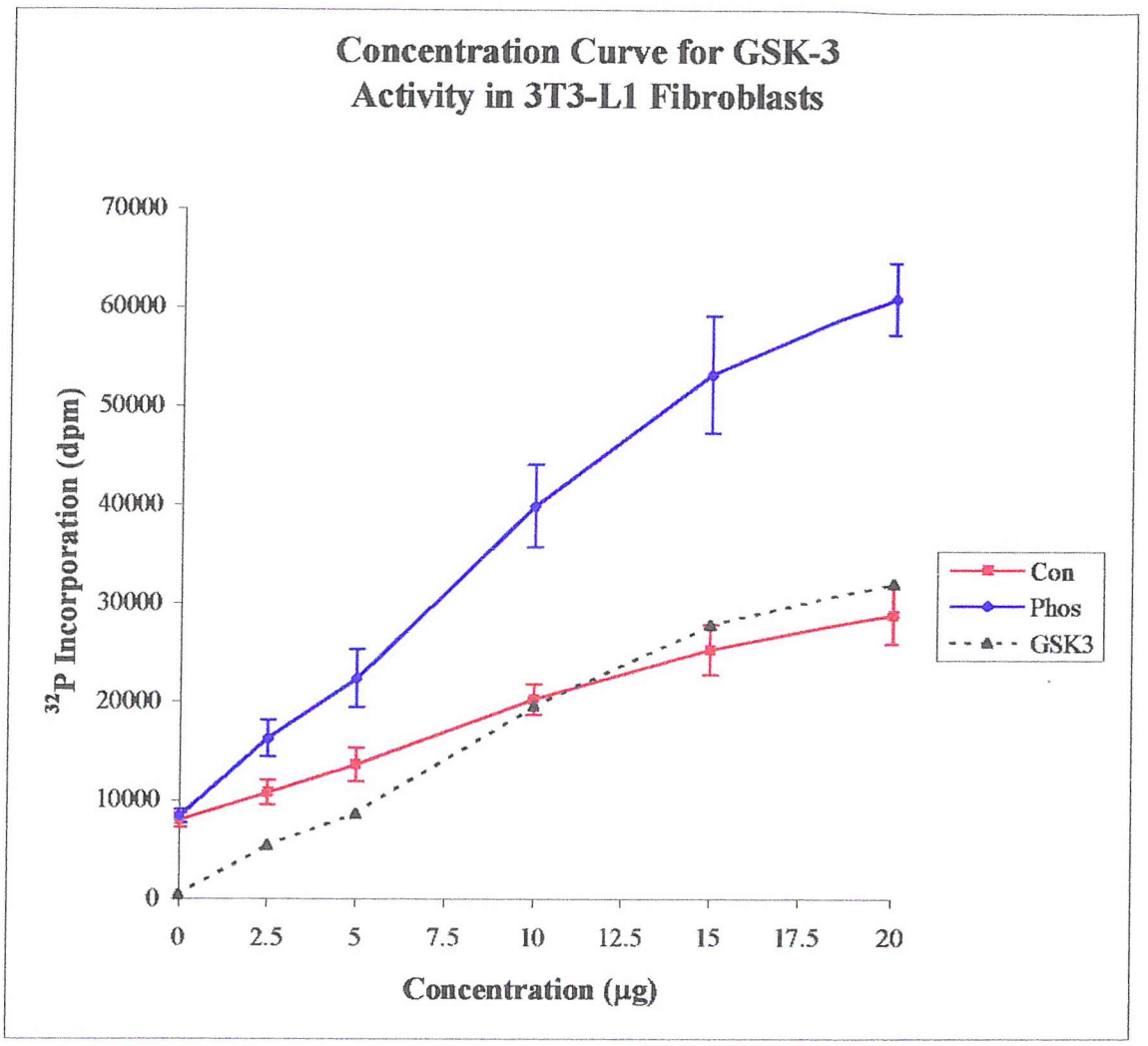


Figure 6.4- Determination of the cell lysate concentration specific linearity of the GSK-3 phosphorylation assay. 3T3-L1 fibroblasts (~95% confluent) were extracted into NP-40 lysis buffer (see sections 2.4.2, 2.9.1) and the concentrations of lysate shown incubated at 30°C with 200µM of either the GSK-3 phosphopeptide or the control peptide. After 10 minutes 25µl aliquots of each condition was removed from the reaction mixture and spotted onto spotted onto p81 phospho-cellulose paper. Following extensive washing, the strips were dried and the ^{32}P incorporation into each peptide was quantitated by liquid scintillation counting. The graph is representative of 3 experiments, with the specific GSK-3 activity (---) calculated as the difference between the ^{32}P incorporation into the phospho and control peptides at each lysate concentration. Control Peptide (-----), Phosphopeptide (-----).

Firstly a lysis buffer only control was incubated against both peptides to account for background ^{32}P levels. The respective ^{32}P incorporation into each peptide with this control was subtracted from the values obtained with each peptide in cell lysate containing assays. The GSK-3 specific phosphopeptide and control peptide values for each assay were then corrected for protein content to give standardised results. In order to establish the GSK-3 specific rate, the control peptide ^{32}P incorporation value was finally subtracted from the GSK-3 phosphopeptide value. The final calculated GSK-3 values could then be compared between untreated and treated cells to establish the percentage change in GSK-3 activity with various treatments.

6.2.2 Use of the GSK-3 Assay to Establish GSK-3 Responses in 3T3-L1 Cells

Having established the optimum conditions for GSK-3 assay, defined the appropriate controls and calculations for obtaining the GSK-3 specific activity, this assay was used to analyse the GSK-3 activity in 3T3-L1 cells. Experiments were initially performed in 3T3-L1 fibroblasts and GSK-3 activity in these cells was analysed following treatment with a variety of factors (see Figure 6.5). These experiments showed that in 3T3-L1 fibroblasts, GSK-3 activity was inhibited by treatment with serum (59%), vanadate (58%), PDGF (54%), EGF (52%), and to a lesser extent insulin (41%) (figure 6.5). This indicates that all these factors act to stimulate the activity of upstream pathways that ultimately bring about the inhibition of GSK-3. These results also show that in 3T3-L1 cells GSK-3 clearly lies downstream of proteins activated by a variety of signalling factors and that GSK-3 activity is significantly diminished in response to these factors. The position of GSK-3 downstream of PI3K was also confirmed in experiments using the PI3K inhibitor, wortmannin. This inhibitor was found to partially restore GSK-3 activity in serum treated fibroblasts indicating the involvement of the growth factor stimulated PI3K pathway as a negative regulatory of GSK-3 activity (Figure 6.5). Therefore, this established that the GSK-3 assay is suitable for use in the 3T3-L1 fibroblasts and that GSK-3 activity could be modulated in these cells in response to a variety of growth factors and by inhibitors of the pathways these factors stimulate.

The suitability of the GSK-3 assay system was then analysed in the related 3T3-L1 cell line 3T3-L1 adipocytes again in response to a variety of factors. In these, as in the fibroblasts, a variety of growth factors were tested and found to inhibit GSK-3 activity (see figure 6.6).

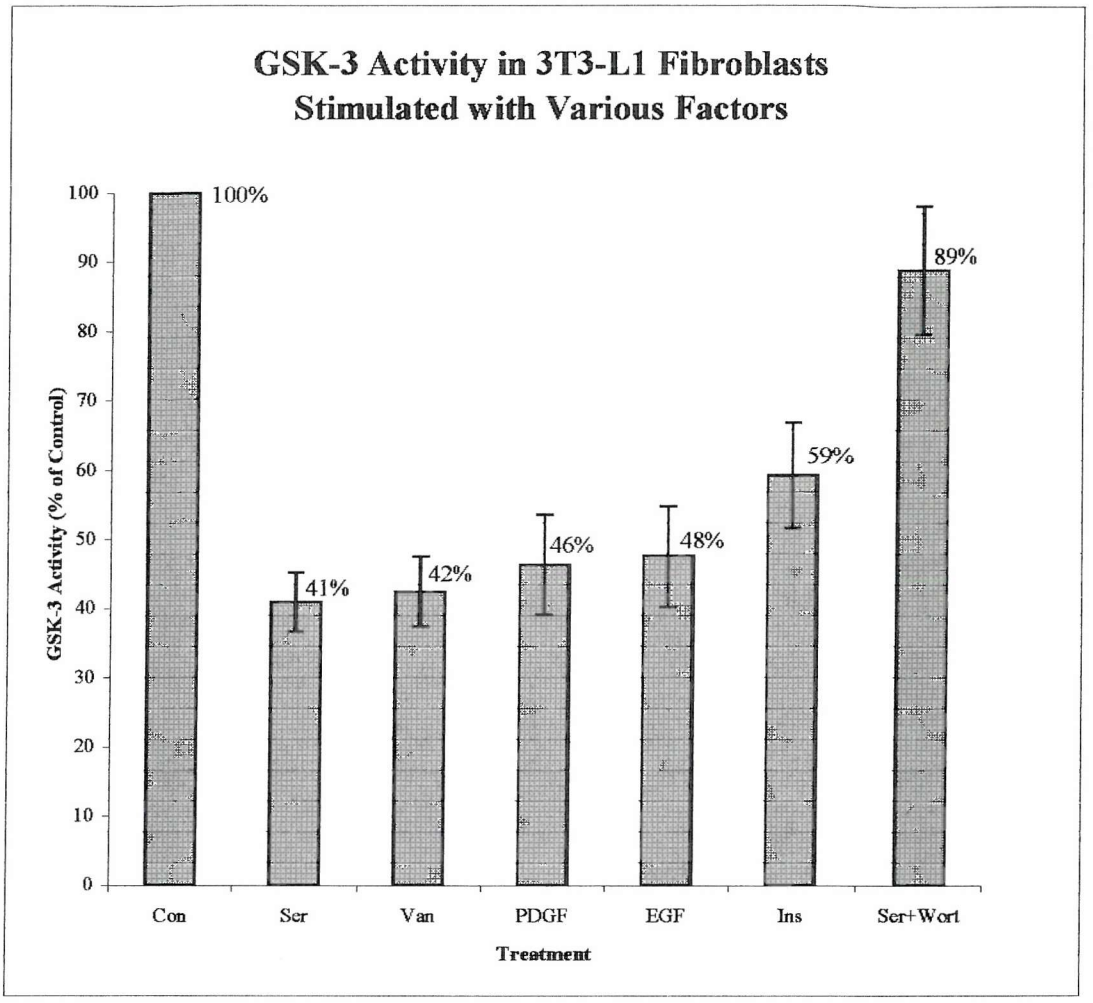


Figure 6.5- The level of agonist-induced inhibition of GSK-3 activity varies with the agonist used and can be attenuated by prior treatment of wortmannin. 3T3-L1 fibroblasts (~95% confluent) were serum starved for 24 hours prior to stimulation (37°C) prior to stimulation with the appropriate agonist or control (basal level). Where necessary the cells were treated with 100nM wortmannin for 40 minutes prior to agonist stimulation. Cells were extracted into NP-40 lysis buffer and GSK-3 activity assessed using the developed GSK-3 activity. ^{32}P incorporation into either the phospho or control peptides was measured by liquid scintillation counting and the specific GSK-3 activity calculated. Results are expressed as a percentage GSK-3 activity of the basal level. **PDGF** (20ng.ml^{-1}/6mins), n=3; **Insulin** (100ng.ml^{-1}/10mins), n=3 **Vanadate** (10mM/10mins), n=5; **EGF** (100ng.ml^{-1}/6mins), n=3; **FBS** (20%^{v}/v/10mins), n=8; **Wortmannin** (100nM/40mins) + **FBS** (20%/10mins), n=2.

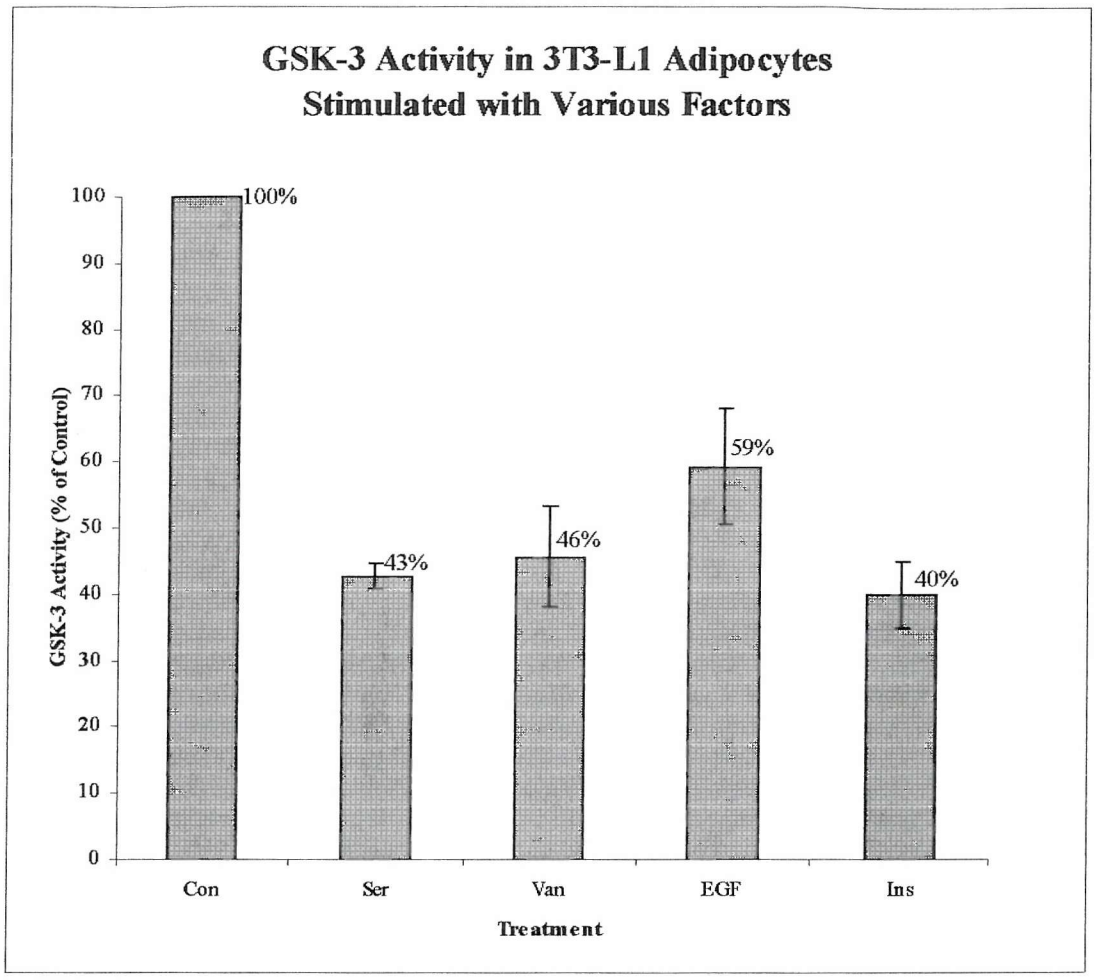


Figure 6.6- The level of agonist-induced inhibition of GSK-3 activity in 3T3-L1 adipocytes varies with the agonist used. 3T3-L1 adipoctyes were serum starved for 24 hours prior to stimulation (37°C) prior to stimulation with the appropriate agonist or control (basal level). Cells were extracted into NP-40 lysis buffer and GSK-3 activity assessed using the developed GSK-3 activity. ^{32}P incorporation into either the phospho or control peptides was measured by liquid scintillation counting and the specific GSK-3 activity calculated. Results are expressed as a percentage GSK-3 activity of the basal level. **Insulin** (100ng.ml^{-1}/10mins), n=3 **Vanadate** (10mM/10mins), n=3; **EGF** (100ng.ml^{-1}/6mins), n=3; **FBS** (20%^{v}/v/10mins), n=3;

However, in this insulin responsive cell line, insulin had a much more significant inhibitory effect (60%) whilst EGF (41%) was found to have a reduced effect on GSK-3 inactivation than that seen in the fibroblasts (see figure 6.6). The other tested factors, serum (57%) and vanadate (54%) were also all shown to inhibit GSK-3 activity in the adipocytes (see figure 6.6). Therefore, it was clearly established that the GSK-3 assay was suitable for analysing GSK-3 activity in the adipocytes and that in these cells, as in the fibroblasts GSK-3 activity can be modulated by differing amounts by a wide variety of factors

6.2.3 Potential Role of Erk1/2 in Growth Factor Induced GSK-3 Inhibition

In order to examine the potential role of Erk1/2 in the phosphorylation and inactivation of GSK-3, the Erk1/2 isoforms protein levels were depleted using the Erk1/2 antisense probe EAS-1 (Sale *et al.* 1995). This probe has been previously shown to specifically deplete both Erk isoforms by more than 95% in the 3T3-L1 fibroblast and adipocyte cells (Sale *et al.* 1995). The optimum conditions for use of EAS-1 in the 3T3-L1 cells were established and found to be a 4μM/ 96 hour transfection time in fibroblasts and 8μM/ 96 hour in the adipocytes, with EAS-1 showing a concentration dependent depletion of Erk1/2 levels in both cell lines (not shown).

3T3-L1 fibroblasts were transfected with EAS-1 at the optimum conditions prior to growth factor stimulation with serum, vanadate or EGF. GSK-3 activity was then assayed in crude cell extracts to analyse the effect of prior of depletion of Erk1/2 isoforms has on the response of GSK-3 to growth factor stimulation (see figure 6.7). Stimulation of EAS-1 treated fibroblasts with PDGF, insulin or vanadate inhibited GSK-3 by 58%, 43% and 57% respectively whereas non-transfected fibroblasts, stimulated with PDGF, insulin or vanadate inhibited GSK-3 activity by 56%, 41% and 58%. These results showed that prior depletion of Erk1/2 had little effect on the PDGF, insulin or vanadate induced inhibition of GSK-3 activity (see figure 6.7). However in 3T3-L1 fibroblasts stimulated with EGF or serum, prior depletion of Erk1/2 was found to partially attenuate the EGF or serum induced inactivation of GSK-3 giving a only 30% or 35% inhibition of GSK-3, compared to a 52% or 58% inhibition in non-transfected cells treated with this growth factor (see figure 6.7). These results indicate that in 3T3-L1 fibroblasts, Erk1/2 may have a partial role to play in the growth factor induced inhibition of GSK-3.

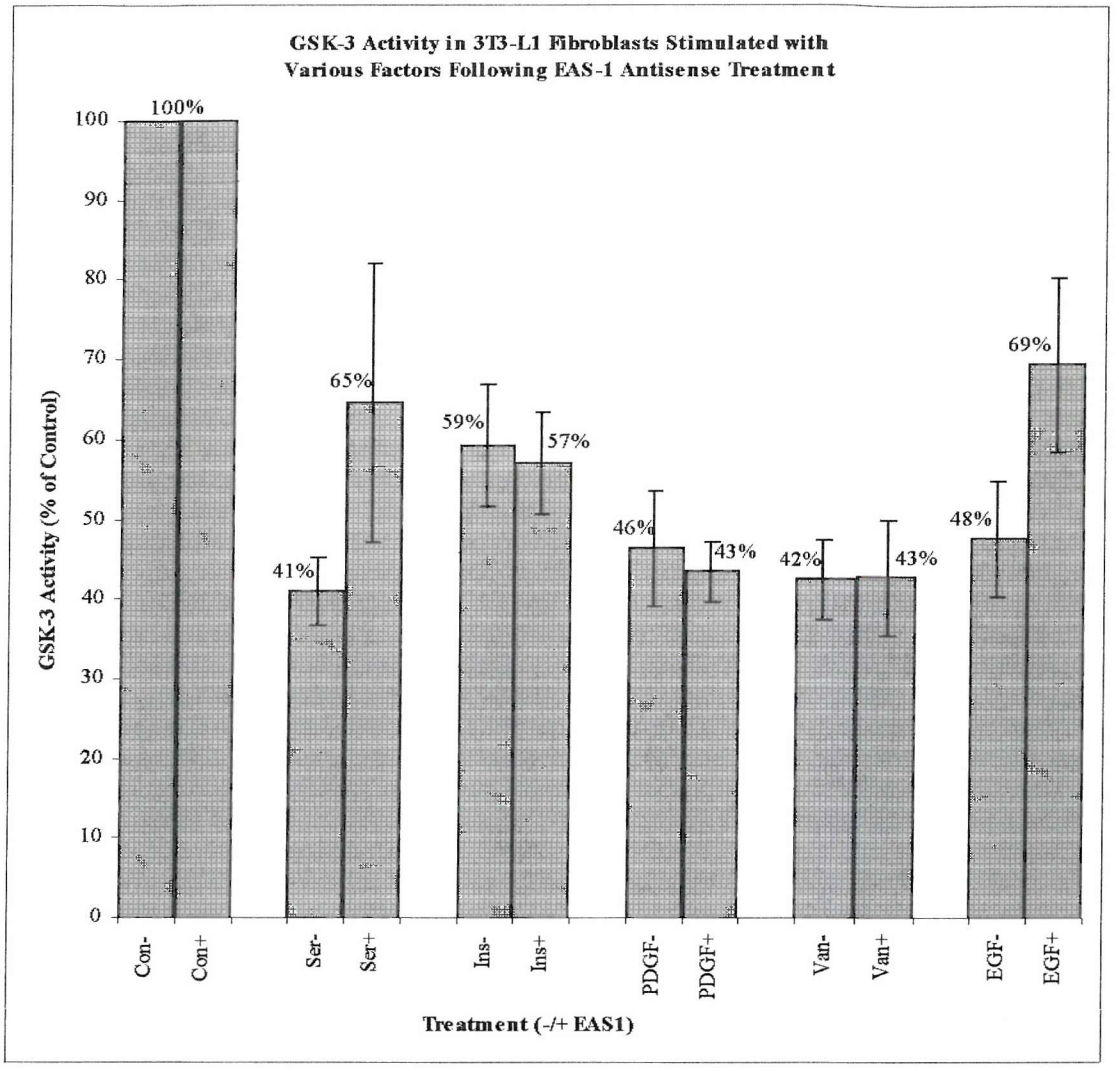


Figure 6.7 - Treatment of 3T3-L1 fibroblasts with the Erk1/2 antisense, EAS-1, can partially attenuate the inhibition of GSK-3 activity induced by certain agonists. 3T3-L1 fibroblasts (~95% confluent) were transfected for 96 hours with EAS1 (4μM) as described in section 2.3.1 prior to agonist stimulation. Cells were extracted into NP-40 lysis buffer and GSK-3 activity assessed using the developed GSK-3 activity. ^{32}P incorporation into either the phospho or control peptides was measured by liquid scintillation counting and the specific GSK-3 activity calculated. Results are expressed as a percentage GSK-3 activity of the basal level. **PDGF** (20ng.ml^{-1}/6mins), n=3; **Insulin** (100ng.ml^{-1}/10mins), n=3; **Vanadate** (10mM/10mins), n=3; **EGF** (100ng.ml^{-1}/6mins), n=3; **FBS** (20%$^{v}/_{v}$/10mins), n=3;

However based on previous results these proteins are not likely to be the major EGF stimulated pathway which brings about the inactivation of GSK-3 in fibroblasts, suggesting the involvement of another EGF stimulated protein kinase cascade. Lack of time prevented testing the effect of antisense depletion of PKB, on the inhibition of GSK-3 induced by a variety of growth factors. However, work into the possible roles of PKB in inhibiting GSK-3 is currently underway using the designed antisense probes αAS3 and βAS2.

6.3 Discussion

6.3.1 GSK-3 Assay Development

The ability to assay GSK-3 in crude extracts is very desirable, since it makes analysis of the effects of various factors on GSK-3 quick and easy. Also, large numbers of GSK-3 assays could be performed at any one time and it should be fairly straight forward to devise suitable controls. The design of a suitable phosphopeptide based on the GSK-3 substrate eIF2B has enabled this study of GSK-3 activity in crude cell extracts (Welsh *et al.* 1997). The GSK-3 substrate peptide designed possessed a phosphoserine residue at a position 4 amino C-terminal to the serine acted on by GSK-3 and served as a specific GSK-3 substrate (Welsh *et al.* 1997). The results presented here demonstrate that this peptide could be synthesised using standard solid phase peptide synthesis and phosphoramidite chemistry (Pullen 1990) and produced in a highly pure form suitable for use in assays. Suitable assay conditions were also laid down for the use of this peptide and its matched control alanine peptide, for the analysis of GSK-3 activity in 3T3-L1 fibroblasts and adipocytes. Interestingly, the incorporation of ^{32}P into the GSK-3 phosphopeptide was shown to be linear with respect to protein concentration and incubation time. Therefore, a set volume (10μl) of crude extract could be used in GSK-3 assay and the varying protein concentrations corrected for in post assay calculations. This enabled lysates to be assayed rapidly post extraction without errors associated with standardising the protein concentrations before the GSK-3 assay, the lysate sitting around for too long prior to assay or the need to freeze the extracts and assay later.

The alanine control peptide possessed an alanine residue instead of the phosphoserine residue at position +4, but still contained the serine site that GSK-3 phosphorylates on eIF2B (Welsh *et al.* 1997). In the original work by Welsh *et al*, immunoprecipitated GSK-3 was found only to phosphorylate the phosphopeptide and not the alanine containing control peptide. This demonstrated that GSK-3 specifically phosphorylates the phosphopeptide only and indicates the importance of the phosphoserine residue at position +4 (Welsh *et al.* 1997). The need for this priming +4 serine phosphorylation in creating a GSK-3 substrates was further emphasised in work by Welsh *et al.*, which showed that immunoprecipitated GSK-3 did not phosphorylate a second control peptide possessing a non-phosphorylated serine residue at position +4 (Welsh *et al.* 1997).

In the results presented here, in crude cell extracts both peptides were found to become phosphorylated at the GSK-3 target site serine, with the phosphopeptide phosphorylate at a much faster rate and to a much greater extent. Similar results to this were obtained by Welsh *et al.* in crude cell extracts from NIH3T3, CHO.T and fat cells, where both the phosphopeptide and the alanine control peptide were phosphorylated but with the ^{32}P incorporation at least 2 fold higher in the phosphopeptide (Welsh *et al.* 1997). Because GSK-3 had been previously shown to only target the phosphopeptide (Welsh *et al.* 1997) this suggested that non-specific kinase could also phosphorylate the GSK-3 target serine in both the control and phosphopeptides. Therefore, in order to calculate the actual GSK-3 activity in the extracts this non-specific phosphorylation had to be accounted for and ^{32}P incorporation into the control peptide was subtracted from the ^{32}P incorporation into the phosphopeptide. This was deemed to be a suitable control, since both peptides contain the serine site and so non-specific kinases would phosphorylate both peptides at equal rates. A second control using lysis buffer instead of extract was also employed to account for background ^{32}P incorporation into the peptide or onto the phosphocellulose paper.

6.3.2 Use of the GSK-3 Assay to Investigate GSK-3 activity in 3T3-L1 Fibroblasts and Adipocytes

In the results presented here, a high GSK-3 activity was detected in unstimulated/serum starved fibroblasts and adipocytes. GSK-3 expression and activity has previously been reported to be high in serum starved or quiescent 3T3-L1 fibroblasts (Benjamin *et al.* 1993, Orena *et al.* 2000) and

therefore these results correlate well with the present findings. The situation in the 3T3-L1 adipocytes is less clear. In the present studies, a clear GSK-3 activity is detected in 3T3-L1 adipocytes, which correlates well with the previous findings of Orena *et al.* and Summers *et al.* (Orena *et al.* 2000 and Summers *et al.* 1999). However Ukei *et al.* have previously been unable to detect any significant GSK-3 activity in adipocytes (Ukei *et al.* 1998) and Benjamin *et al*, have found GSK-3 expression to decrease to undetectable levels during the differentiation of 3T3-L1 adipocytes (Benjamin *et al.* 1993). The current understanding of this is, that the experiments of Orena et al. used a more sensitive antibody to detect GSK-3 levels in the adipocytes and so some GSK-3 activity is likely to present in these cells (Orena *et al.* 2000). It is interesting to note that Orena *et al.* also used the Welsh *et al.* GSK-3 phosphopeptide assay system in a similar assay protocol to the one developed in this results chapter (Orena *et al.* 2000, Welsh *et al.* 1997).

A variety of growth factors were used to stimulate the 3T3-L1 fibroblasts and adipocytes, with all of them being found to inhibit GSK-3 activity to differing but significant degrees. Interestingly, serum, vanadate, PDGF and EGF were the strongest inhibitors or GSK-3 activity in the fibroblasts whilst insulin had a less significant effect in these cells which are known have a fairly limited insulin responsiveness. Conversely, in the highly insulin responsive adipocyte cells, insulin elicited a much stronger inhibition of GSK-3 activity and was one of the most potent inactivators of GSK-3 along with serum and vanadate. In the adipocyte cells, conversely EGF elicited a lower inhibitory effect on GSK-3 activation. This results correlate well with the findings which show EGF to be a stronger activating factor of PI3K cascade in 3T3-L1 fibroblasts, but not a strong activator of this pathway in adipocytes, whilst the inverse was found to be true for insulin (Eldar-Finkelman *et al.* 1995, Saito *et al.* 1994, Ueki *et al.* 1998). Interestingly, the use of the PI3K inhibitor, wortmannin was found to relieve most of the inhibitory effects on GSK-3 activity, growth factor stimulation of 3T3-L1 fibroblasts and adipocytes usually produced (Ueki *et al.* 1998, Shaw *et al*, 1999, *Orena et al.* 2000, Kohn *et al.* 1996). This suggests that in both these cell types growth factor induced GSK-3 inhibition is mediated by the PI3K signalling cascade.

The levels of growth factor induced inhibition of GSK-3 observed in these results correlates well with previous studies into the growth factor regulation of GSK-3 activity in a variety of cells (Hurel *et al.* 1996, Shaw *et al.* 1997 & 1999, Saito *et al.* 1994). For example, in the 3T3-L1 fibroblasts insulin inhibited GSK-3 activity by 41% whereas in the adipocytes this level of GSK-3

inhibition increased to 60%. This correlates well with an insulin-induced inhibition of GSK-3 of 52% in rat adipocytes observed by others (Orena *et al.* 2000). Similar levels of insulin stimulated GSK-3 inactivation have also been observed in other insulin responsive cell lines; i.e. CHO.T (48%), NIH.3T3 (58%), epididymal fat cells (51-56%), L6 myotubes (45-55%) and human myoblasts (35%) (Welsh *et al.* 1997, Yu *et al.* 1997, Moule *et al.* 1997, Ueki *et al.* 1998, Hurel *et al.* 1996). The levels of serum induced GSK-3 inhibition observed in both the fibroblasts (59%) and adipocytes (57%) also correlated with the levels of GSK-3 inhibition observed by others with serum stimulation in a variety of cell lines, for example, serum was found to inhibit GSK-3 activity by 59% in CHO.T cells (Welsh et al. 1997). EGF induced inhibition in the fibroblasts (52%) and to a lesser extent the adipocytes (41%) also correlated well with the EGF induced-inhibition of GSK-3 seen in other cells, for example, in A431 cells (48%) (Saito *et al.* 1994)

Use of the specific and highly effective Erk1/2 antisense EAS-1, to investigate the role of MAPK in GSK-3 inactivation indicated a possible minor role for Erk1/2 in GSK-3 inhibition under certain conditions. In the 3T3-L1 fibroblasts depletion of Erk1/2 by EAS-1 probe, was found to have no significant effect on the PDGF, insulin or vanadate induced GSK-3 inhibition indicating that Erk1/2 do not have roles in the GSK-3 inactivation signalling pathways these factors stimulate. However, prior treatment with EAS-1 was found to partially reduce the inhibitory effects of EGF and serum on GSK-3 activity. This suggests, that in response to EGF or serum stimulation the Erk1/2 pathway is activated and contributes to the signals which ultimately inactivate GSK-3. However, Erk1/2 apparently only makes a relatively small contribution to the EGF or serum induced inactivation of GSK-3, suggesting, that EGF and serum activate other downstream factors which make a more significant contribution to GSK-3 activation. These preliminary findings correlate well with the work of Shaw *et al.* who showed that whilst the Erk1/2 pathway is not involved in mediating the IGF-1-induced inactivation of GSK-3 in Swiss 3T3 cells, Erk1/2 does makes a transient contribution to EGF induced GSK-3 inactivation in these cells (Shaw *et al.* 1999). Shaw *et al.* also showed that in EGF induced inactivation of GSK-3, Erk1/2 also has a secondary role in reducing the length of GSK-3 inactivation mediated by other kinases (Shaw *et al.* 1999). Interestingly, Shaw *et al.* also showed that the Erk1/2 pathway is the major route in Swiss 3T3 cells by which PMA stimulation signals to inhibit GSK-3 activity (Shaw *et al.* 1999). Therefore, it is apparent that the Erk1/2 pathway may be involved in mediating the inactivation of GSK-3 induced by certain factors or in specific cell lines.

The Erk1/2 antisense probe is a well-established and effective antisense probe that has previously been extensively characterised (Sale et al. 1995). This probe has subsequently been effectively used to analyse the roles of Erk1/2 in a variety of cellular roles including differentiation, and RSK activation. In all these examples, the probe EAS-1 was shown to specifically reduce the Erk1/2 levels, causing a marked effect on the downstream functions studied. In contrast, control oligonucleotides did not affect the expression of Erk1/2 or the possible downstream functions of these proteins. Therefore, the specific antisense of EAS-1 against Erk1/2 has been well established.

In order to establish the possible roles Erk1/2 has in the inactivation of GSK-3, further study is needed. It would be interesting to analyse the effects of the Erk1/2 antisense on the GSK-3 inhibition induced by other factors including PMA and IGF-1, in the 3T3-L1 fibroblasts. The role of Erk1/2 in the EGF-induced inactivation of GSK-3 also needs studying to establish whether in the 3T3-L1 fibroblasts, Erk1/2 not only transiently assists other kinases in the inactivation of GSK-3, but also reduces the duration of this inactivation, as was the situation in the Swiss 3T3-L1 cell line (Shaw *et al.* 1999). Study of the possible roles of Erk1/2 in growth factor induced inactivation of GSK-3 in other cell lines (i.e. 3T3-L1 adipocytes) would also be beneficial to determine the relative importance of the Erk1/2 cascade in signalling to GSK-3. In order to confirm any possible roles for Erk1/2 in GSK-3 inactivation, as determined by EAS-1 antisense treatment, control oligonucleotide (i.e. sense, scrambled and mismatch) treatments will also need to be used. The use of these controls would be important to confirm that any observed changes in the GSK-3 inactivation profile could be attributed to the EAS-1 specific depletion of Erk1/2 and not due to non-specific oligonucleotide effects.

Having established a successful and highly specific method for assaying GSK-3 activity in the 3T3-L1 fibroblasts and adipocytes, it important to now investigate the pathways that mediate growth factor induced inactivation of GSK-3. The 3T3-L1 fibroblasts and adipocytes have already been shown to be responsive to a wide variety of growth factors which signal to inactivate GSK-3. In the fibroblasts, the role of the Erk1/2 cascade has initially been studied with these experiments, indicating that this cascade is not the major route by which growth factors signal to inactivate GSK-3. Therefore, it is important to establish which other pathways are involved in GSK-3 inactivation in these and other cells. The findings that PI3K inhibitors inhibit the growth factor-induced inactivation of GSK-3, suggests the involvement of the PI3K cascade in mediating the

inhibition of GSK-3. Therefore, a role for PKB in the inactivation of GSK-3 has been proposed. Initial studies show that PKB can directly phosphorylate GSK-3 *in vitro* and *in vivo* when overexpressed (Cross *et al.* 1995). Overexpression studies using constitutively active or dominant negative mutants also suggest that PKB functions immediately upstream of GSK-3 and can directly phosphorylate and inactivate GSK-3 in response to growth factor treatment (Shaw *et al.*1997, Ueki *et al.* 1998, Cross *et al.* 1995). However, all these studies involve overexpression studies and so do not analyse endogenous protein levels of PKB or GSK-3. Therefore these studies may not be relevant to physiological cellular functioning. The possible involvement of SGK, which is very similar to PKB and can also phosphorylate GSK-3, in this pathway also needs to be resolved (Park *et al.* 1999, Kobayashi *et al.* 1999a & b).

The development of the PKBα and PKBβ antisense probes should therefore help to unravel the GSK-3 signalling pathway. These probes could be used to analyse the individual role of both PKBα and PKBβ in phosphorylation and inactivation of GSK-3 in the fibroblast and adipocytes in response to a variety of growth factors/stimuli. The further development of the PKBγ and multi isoform antisense probes will also assist in deciphering the isoform specific and general roles of PKB in signalling to GSK-3. Therefore, it is critical to use these PKB antisense probes to test roles of PKB in GSK-3 phosphorylation and inactivation and determine the importance of PKB in this pathway. Further study of the GSK-3 pathway and its downstream signalling would also be of great interest to establish the relative importance of GSK-3 in various cellular pathways. The new antisense design rationale developed here should enable the design of specific GSK-3 antisense probes which hopefully could then be used to identify the key roles of GSK-3. Therefore, it is necessary to determine the precise interactions between PKB, MAPK and GSK-3 and to resolve the GSK-3 signalling pathway. The use of specific antisense probes against endogenous levels of these proteins provide a highly specific and effective method for analysis of this pathway in physiological/cellular conditions and may also provide insights into the aberrant functioning of GSK-3 in disease states.

Summary and Future Work

Summary and Future Work.

I have developed effective and specific individual antisense probes against the α and β isoforms of PKB and tested their effectiveness in 3T3-L1 fibroblasts and adipocytes. Further characterisation of these probes has established the optimum conditions for the use of these probes. In the 3T3-L1 fibroblasts, the optimum transfection protocol for the PKBα antisense probe was established to be 4μM αAS3 for 96 hours, conditions which yielded a 92% depletion of PKBα. Interestingly, these conditions were also shown to effectively deplete the PKBα levels by >90% in the adipocytes. The PKBβ isoform specific antisense βAS2 was found to effectively deplete PKBβ by >90% in both cell lines when used at 8μM over a 96 hour transfection period. Suitable control oligonucleotides were designed for each antisense probe and used to show that the two designed probes acted uniquely as antisense agents. Analysis of the effects of these two probes and their respective controls on the levels of expression of other proteins (i.e. Erk1/2 and S6K) confirmed the designed probes to act specifically against the PKB isoform they target. A second effective PKBα antisense probe was also initially developed and found to effectively deplete PKBα levels by >80% in the fibroblasts and adipocytes. Another PKB antisense probe αAS2 was shown to deplete the levels of both the α (60%) and β (30%) isoforms. This probe could also potentially target PKBγ, raising the possibility of developing this probe as a general PKB antisense effective against all 3 PKB isoforms. Possibly isoform specific antisense probes directed against PKBγ have also been designed and are currently undergoing testing. Therefore, these 3 potential antisense agents require further study to characterise their effectiveness as antisense agents and establish whether they could be useful tools in studying the functioning of PKB.

With the development of these PKB antisense probes the first direct effectors of endogenous PKB levels and hence functioning have been developed. To date, no other specific PKB inhibitors are known and therefore these antisense agents represent an important development in the study of functioning of PKB. Therefore, use of these antisense probes to prevent the expression of PKB isoforms will assist in the identification of direct substrates and roles of PKB. In antisense studies, the probes act to deplete the endogenous levels of the target protein by inhibition of translation. Therefore, with the PKB antisense probes the functioning of endogenous PKB can be studied and physiological roles determined. The use of these antisense probes in assignment of the roles of PKB

will not encounter any of the problems associated with overexpression studies. Previous research has indicated that overexpression of PKB may adversely affect the function of the upstream protein PDK-1 (reviewed in Vanhaesebroeck *et al.* 2000). Overexpressed PKB may also act on non-physiological substrates or prevent the action of other proteins which normal target these substrates (reviewed in Vanhaesebroeck *et al.* 2000). Therefore, caution needs to be exercised when assigning functions for PKB based on such studies. The use of antisense to investigate PKB should therefore eliminate these possible problems and enable the identification of the true roles of PKB.

These PKB antisense probes will also help to distinguish between the cellular roles of PKB and those of the related protein SGK which potentially targets a similar group of substrates (Park *et al.* 1999). The ability to design isoform specific antisense probes for PKB is also a unique feature of antisense strategies. The specific nature of the targeting of these probes against only one isoform of PKB also will be important in future studies. As yet, no specific roles for individual isoforms of PKB have been established, although variations in responses to different growth factors have previously been suggested (reviewed in Kandel *et al.* 1999). The lack of information concerning the roles of individual isoforms of PKB is based on the lack of direct inhibitors of PKB. Any possible isoform specific roles for each isoform of PKB will be able to be determined using the probes and the relative contribution each protein makes to a particular function of PKB can be established.

Use of the PKBα antisense probe αAS3 has already confirmed a key role for PKBα in the differentiation of 3T3-L1 fibroblasts into adipocytes. This not only established the importance of PKBα in cellular differentiation but also indicated the effectiveness of αAS3 as an inhibitor of PKBα functionality. Work is continuing in the laboratory to investigate the roles of PKB in cell differentiation. The potential role of PKBβ in the differentiation of the 3T3-L1 fibroblasts into the adipocytes can be investigated using the specific PKBβ antisense βAS2. It would also be interesting to investigate the possible roles of PKBα and PKBβ in the differentiation of other cell types including muscle and neural cell lines. Future work will also include investigating the roles of PKBγ in the differentiation of fibroblasts into adipocytes and in the differentiation other cell types, once the characterisation of the PKBγ antisense is complete.

A shift in the electrophoretic mobility of the SGK isoforms p70S6K and p85S6K has been found to occur in response to growth factor stimulation. This band shift effect has been developed into a

Western blotting technique for analysing the phosphorylation state and potential activation profile of S6K. Treatment of the 3T3-L1 fibroblasts with either αAS3 or βAS2 individually or these two probes in tandem was found not to affect the band-shift of S6K induced upon growth factor stimulation. This showed that in these cells, neither PKBα nor PKBβ individually or together are sufficient to induce a shift in SGK electrophoretic mobility in response to growth factor stimulation. Therefore, these results suggest, that PKBα and PKBβ are not involved in the growth-factor induced activation of S6K in these cells. Future work will therefore include, investigating the potential role of PKBγ in the growth factor induced activation of SGK and analysing the possible involvement of PKB in S6K activation in other cells. Also the potential role of PKB in S6K activation could be analysed using an immunoprecipitation based kinase, to identify if depletion of PKB levels affects the kinase activity of S6K without affecting its phosphorylation state.

A specific peptide based assay for analysing the activity of GSK-3 in crude cell lysates has also been developed based on previous work by Welsh et al. (Welsh et al. 1997). Peptides were synthesised for use in this assay and characterised by HPLC and mass spectroscopy. Optimum conditions for assaying GSK-3 activity were established and the assay shown to be effective in analysing growth factor-induced inhibition of GSK-3 in fibroblast and adipocyte cell lines. Initial studies using the Erk1/2 antisense EAS-1 suggest that the Erk pathway may be partially involved in the inhibition of GSK-3 activity induced by EGF and serum stimulation. However, these studies indicate that the Erk pathway is not involved in mediating vanadate, PDGF and insulin induced inhibition of GSK-3 in the 3T3-L1 fibroblasts. A possible role of the Erk cascade in the inhibition of GSK-3 in response to certain growth factors has been previously proposed (Shaw *et al.* 1999). The involvement of Erk1/2 in the growth factor induced inhibition of GSK-3 requires further investigation using control oligonucleotides. Also the involvement of this pathway in the inactivation of GSK-3 induced by other factors or in other cell lines could be studied.

The main rationale behind the design of this specific GSK-3 assay was to investigate the proposed roles of PKB in the direct phosphorylation and inhibition of GSK-3. Having successfully developed this assay it will now be important to analyse the effects of the PKB antisense probes on the inhibition of GSK-3. These antisense studies are currently ongoing and should enable the establishment of any roles for PKB in growth factor-induced GSK-3 inhibition. The isoform specific nature of the developed probes will also enable the individual roles of each PKB isoforms

in the phosphorylation and inactivation of GSK-3 to be investigated. Phospho-specific GSK-3β (serine 9 site) antibodies are also available which should assist in identifying whether PKB can directly phosphorylate GSK-3

The design and development of specific PKB antisense probes will also enable the elucidation of other roles of the individual PKB isoforms and PKB functioning in general. The roles of PKB in glycogen synthesis and lipid synthesis could be investigated using standard ^{14}C-glucose incorporation studies. PKB has previously been proposed to be involved in this processes based on overexpression studies (Ukei *et al.* 1998). Therefore the antisense targeting of endogenous PKB levels should help to establish the precise roles of PKB in these processes.

PKB has also been proposed to be involved in glucose uptake and GLUT4 translocation (Kohn *et al.* 1996). These roles of PKB in glucose uptake are currently under investigation using radioactive glucose to analyse the effects of the antisense probes on glucose transport. Immunofluorescent antibodies can also be used to analysis the effects of the antisense probes on the translocation of glucose transporter to the plasma membrane and therefore establish if any isoforms of PKB have a role to play in GLUT4 translocation. The possible involvement of PKB in GLUT1 expression could also be investigated by analysing changes in the protein levels of GLUT1 following growth factor stimulation in antisense treated cells and their matched controls.

The possible roles of PKB in protein synthesis could also be studied using the antisense probes. This could include investigating the possible direct action of PKB on mTOR or the possible roles of PKB in the phosphorylation of 4E-BP1 (reviewed in Kandel *et al.* 1999). Towards this objective a band-shift Western blotting protocol to analyse the growth factor induced changes in the phosphorylation state of the translation inhibitor 4E-BP1 has already been developed (data not shown).

The other major field of study for which the PKB antisense probes will be very useful, is in assessing the potential role of PKB as a survival factor (reviewed in Datta *et al.* 1999). The effects of the probes on general apoptosis/cell survival could be assessed, in suitable cell lines, using various apoptotic studies including, DNA fragmentation, apoptotic marker visualisation or flow cytometry. Possible direct targets for PKB implicated in apoptotic control could also been

identified using the antisense probes coupled to appropriate detection techniques. For example, the possible direct phosphorylation and inhibition of the pro-apoptotic protein BAD by PKB could be investigated using an electrophoretic mobility shift assay or phospho-specific BAD (serine 136 site) antibodies. Other possible direct roles for PKB in apoptotic control including, the inhibition of mitochondrial cytochrome C release or caspase activation or the possible induction of Bcl-2 expression could also be studied using the developed antisense agents (reviewed in Datta *et al.* 1999).

The antisense strategy developed in this study also may be of interest in the future design of antisense probes. The targeting of the 3'translated region of the mRNA sequence of each PKB isoform proved to be a very effective target site for potent antisense probes. Directing probes against this site lead to the development of highly effective and specific antisense probes against the α and β isoforms of PKB respectively. Therefore, this site may prove to be an effective target region for the design of other antisense agents. In the future in may be useful to target other proteins involved in the PI3K/PDK-1 cascade to establish the precise roles of this pathway in cell signalling. Antisense probes could possibly be designed against, PDK-1, SGK, mTOR or GSK-3 to elucidate the role of these proteins in complex signalling processes. The ability to target individual isoforms demonstrated by this antisense strategy may also be useful in targeting the isoform specific role of multi-isoform proteins for example PKC.

Therefore, an effective antisense strategy has been developed for the isoform specific targeting of PKB isoforms. This strategy will hopefully lead to the development of other antisense probes against key signalling proteins. The design of PKB antisense probes can be used to determine the precise role of the PKB isoforms on cellular function. Hopefully, these findings will collectively, lead to an increased understanding of the roles of PKB and help to unravel the complex field of cell signalling.

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