# UNIVERSITY OF SOUTHAMPTON

# Studies into the Antiapoptotic Signalling of Protein Kinase Bγ

by

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ABSTRACT

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One of the most important roles for growth factors such as insulin and IGF-1 in certain cells is the promotion of cell survival and protection against apoptosis. Growth factors achieve this antiapoptotic signalling via complex intracellular signalling pathways mediated by protein kinases and phosphatases. A recently cloned protein kinase, protein kinase B (PKB) has been shown to be a key component of growth factor signalling pathways and in particular has been implicated as a crucial mediator in antiapoptoic signalling. In this study I report the cloning of PKBγ, the third and least studied of the PKB isoforms, from rat and mouse brain tissue and production of two different constitutively active mutants of PKBγ, the first a membrane targeted mutant and the second a phosphorylation site active mutant. These mutants were then used to stabily transfect Madin-Darby Canine embryonic Kidney (MDCK) cells in order to investigate the role of PKBγ in antiapoptotic signalling. Using these stable cell lines it was established that GSK-3 is a downstream target of PKBγ and expression of the constitutively active PKBγ mutants was found to protect the stable cells against apoptosis induced by growth factor withdrawal. In addition, a dominant negative rat PKBγ was cloned and found to induce apoptosis when transfected into wild-type MDCK cells, even in the presence of serum. Taken together these results suggest that PKBγ has a crucial role to play in growth factor stimulated cell survival signalling. This research may have important implications for research into cancer, diabetes and neurodegenerative disease since PKBγ has been found to be upregulated in breast cancer and prostate cancer cell lines and downregulation of PKB may be a central cause of insulin resistance and Alzheimers disease.

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# ABBREVIATIONS

| | |
|---|---|
| ADP | Adenosine Bisphosphate |
| AIDS | Acquired Immunodeficiency Syndrome |
| Ala | Alanine |
| ALS | Amyotropic Lateral Sclerosis |
| Arg | Arginine |
| Asp | Aspartate |
| ATP | Adenosine Trisphosphate |
| bp | base pair |
| C | Celcius |
| CAD | Caspase Activated DNase |
| cDNA | Complementary Deoxynucleic Acid |
| dATP | deoxy Adenine Triphosphate |
| DEPC | Diethyl Pyrocarbonate |
| DMEM | Dulbecco's Modified Eagles Medium |
| DNA | Deoxyribonucleic Acid |
| dNTP | deoxy Nucleic Triphosphate |
| DTT | Dithiothreitol |
| EDTA | Ethylene-diamine-tetra-acetate |
| EGF | Epidermal Growth Factor |
| FBS | Fetal Bovine Serum |
| FITC | Fluorescein Isothiocyanate |
| G418 | Geneticin |
| Gly | Glycine |
| GS | Glycogen Synthase |

| | |
|---|---|
| GSK-3 | Glycogen Synthase Kinase-3 |
| HA | Haemagglutinin-Antigen |
| Hyd | Hydrophobic residue |
| ICAD | Inhibitor of CAD |
| IGF-1 | Insulin-like Growth Factor 1 |
| IGFBP-1 | IGF-binding protein-1 |
| IRS-1 | Insulin Receptor Substrate 1 |
| kb | Kilo base |
| kDa | Kilo Dalton |
| LB | Millers Luria broth |
| Lys | Lysine |
| M | Molar |
| MAP kinase | Mitogen Activated Protein kinase |
| MDCK | Madin-Darby Canine embryonic Kidney cells |
| mins | minutes |
| M-MLV RT | Moloney Murine Leukemia Virus Reverse Transcriptase |
| MTS | [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt |
| Myr-Palm | Myristoylation/Palmitoylation |
| NGF | Nerve Growth Factor |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PDGF | Platelet Derived Growth Factor |
| PDK | Phosphatidylinositol-Dependent Protein kinase |
| PEPCK | Phosphoenolpyruvate carboxykinase |
| PES | Phenazine ethosulphate |

| | |
|---|---|
| PH | Pleckstrin Homology |
| Pi | Phosphate group |
| PI 3-K | Phosphatidylinositol 3'-Kinase |
| PI(3,4,5)P3 | Phosphotidylinositol (3,4,5) Trisphosphate |
| PI(4,5)P2 | Phosphotidylinositol (4,5) Bisphosphate |
| PKA | cAMP dependent Protein Kinase |
| PKB/Akt | Protein Kinase B |
| PKC | Protein Kinase C |
| PMSF | Phenylmethylsulphonylfluoride |
| PP2A | Protein Phosphatase 2A |
| PTEN | Phosphatase and Tensin homologue deleted from chromosome 10 |
| PS | Phosphatidylserine |
| RNA | Ribonucleic Acid |
| RNase A(H) | Ribonuclease A(H) |
| rpm | revolutions per minute |
| RT PCR | Reverse Transcriptase Polymerase Chain Reaction |
| SDS-PAGE | Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis |
| Ser | Serine |
| SGK | Serum- and Glucocorticoid- regulated protein kinase |
| SH2(3) | Src Homology 2 (3) |
| SOS | Son Of Sevenless |
| TAE | Tris-acetate-EDTA |
| TEMED | N,N,N',N',-tetrametylenediamine |
| Thr | Threonine |
| TNF | Tumor Necrosis Factor |

# CHAPTER 1

# GENERAL INTRODUCTION

# CHAPTER 1: General Introduction.

## 1.1 Reversible protein phosphorylation.

Reversible phosphorylation of proteins is the major control mechanism adopted by eukaryotic cells. About one third of mammalian proteins contain covalently bound phosphate and $\approx 5\%$ of genes encode protein kinases and phosphatases, the enzymes responsible for the transfer of phosphate causing this phosphorylation/ dephosphorylation (Hunter, 1995). The phosphorylation of proteins on serine, threonine and, to a lesser extent, tyrosine residues alters the proteins' shape and activity (Figure 1.1). This results in the regulation of a wide variety of cellular processes including the control of metabolism, cell growth, gene expression, the immune response and even the acquisition of memory!

## 1.2 Reversible protein phosphorylation and signal transduction

Signal transduction is the process whereby an extracellular chemical signal evokes an intracellular metabolic response (Figure 1.2). Such signals include hormones, neurotransmitters, growth factors and cytokines. Even though these substances circulate the body at extremely low concentrations (nmolar or less) they can cause very dramatic changes in the cells' metabolic behaviour within a matter of seconds or minutes. The extracellular signal binds to its receptor located at the cells plasma membrane resulting in activation of the receptor. Once active, the receptor then initiates the production of a 'second-messenger' inside the cell. These second-messengers are chemical mediators that regulate the activity of the protein kinases and phosphatases. In this way these kinases and phosphatases alter the level of phosphorylation of their target intracellular proteins resulting in particular biological
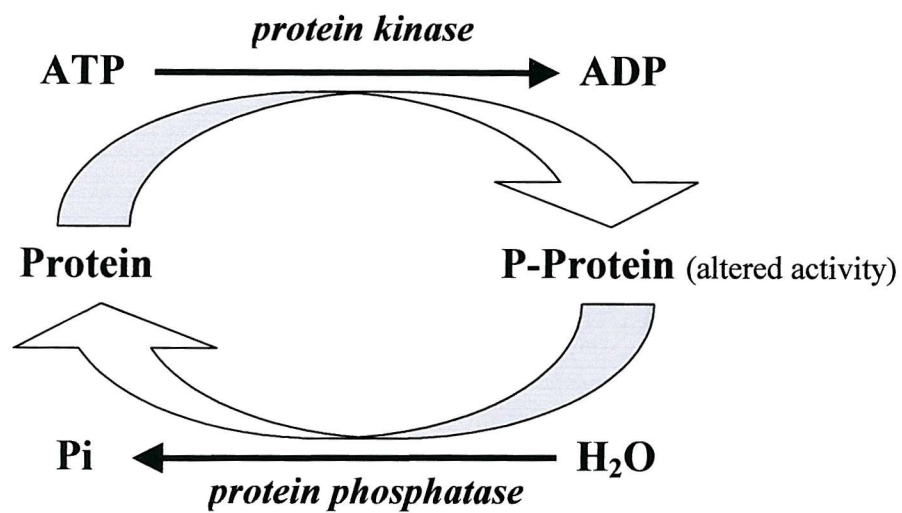
**Figure 1.1    The protein phosphorylation cycle.**

Phosphorylation of substrate proteins by protein kinase enzymes causes a conformational change resulting in either an increase or a decrease in the proteins activity. Dephosphorylation by protein phosphatase enzymes returns the protein to its original state.

responses. This all explains how one signal can produce such a diversity of responses (Cohn, 1982, 1985) and in addition the diversity and amplification of the response is enhanced when several protein kinases become linked together in a sequence to form a protein kinase 'cascade'. This situation is made more complicated since the discovery that these kinase cascades may interact with one another in a process called 'cross-talk' whereby the kinases and phosphatases of one cascade can regulate proteins from a different cascade. For example, in NIH3T3 fibroblasts, activation of the MAP kinase pathway is inhibited by activation of PKA at the level of Raf-1 activation, possibly as a result of direct inhibitory phosphorylation of Raf-1 by PKA (Hafner *et al.*, 1994). As well as signal amplification, branching and cross-talk, the kinase cascades also exhibit feedback mechanisms both positive and negative in order to regulate the response (for review see: Hunter, 1995 and references therein).

## 1.3 Growth factor receptor tyrosine kinases

Growth factors such as Insulin, IGF-1, EGF and PDGF bind to receptors which consist of an extracellular binding domain, a transmembrane domain and an intracellular catalytic domain (Fanti et al., 1993; Malarkey *et al.*, 1995). A great advance in our understanding of the field came with the discovery that these receptors were tyrosine kinases. Once agonist binding occurs tyrosine residues on the intracellular C-terminal of the receptor become transautophosphorylated. Transautophosphorylation is accompanied by dimerisation of the receptor and activation of the tyrosine kinase activity (Fanti et al., 1993; Kazlauskas and Cooper, 1989). The phosphorylation on tyrosine residues paves the way for interaction with downstream target proteins.

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**Figure 1.2    The insulin receptor signalling pathways.**

As an example of how complicated intracellular signalling pathways can be, this is a schematic diagram of the insulin receptor signalling mechanism. Insulin (or IGF-1) binds to its receptor at the plasma membrane causing transautophosphorylation of the receptor and activation of its tyrosine kinase activity. Downstream adaptor proteins such as IRS-1 and shc bind to the phosphorylated receptor via their SH2 domains and become phosphorylated on tyrosine resides allowing for further phophotyrosine-SH2 interactions (and SH3 interactions) with downstream effector signalling molecules. In this way the insulin receptor controls various enzyme pathways, including the p70S6 kinase, MAP kinase and glucose transporter translocation. The end result is an increase in metabolic activity such as protein synthesis, glucose uptake, glycogen synthesis, gene expression and many more.
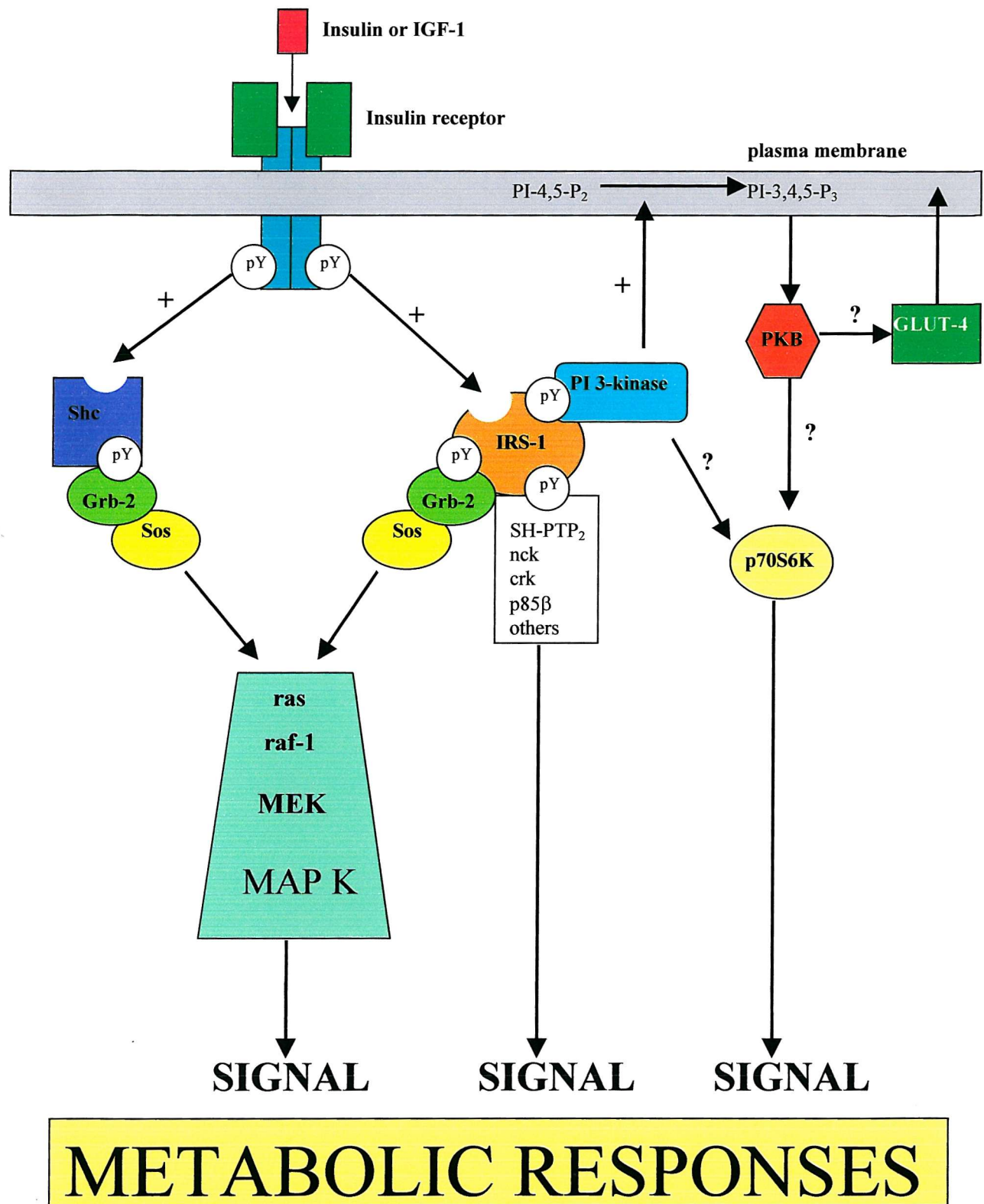
Figure 1.2    The insulin receptor signalling pathways.

## 1.4 SH-2 and SH-3 domains

Many proteins contain regions of about 100 amino acids with considerable homology to the non-catalytic region of the *src* family of protein tyrosine kinases. These regions called Src homology-2 (SH-2) domains and allow the proteins to bind to specific phospho-tyrosine residues on other proteins. In this way following transautophosphorylation, the receptor tyrosine kinases may bind to a wide range of downstream targets such as Insulin receptor substrate proteins (IRS-1, IRS-2 and IRS-3), phosphatidylinositol (PI) 3'-kinase, Grb2/Sos and Shc (Figure 1.2). In many cases, receptor binding results in the tyrosine phosphorylation and a change in the activity of the target protein. However, the target protein is phosphorylated on residues distinct from those in the SH-2 domain. For example, binding of PLCγ to the PDGF receptor on Tyr1021 stimulates phosphorylation upon tyrosine residues at positions 771, 783, and 1254, increasing the catalytic activity of PLCγ (Kim et al., 1991).

An important family of SH-2 proteins are the Insulin Receptor Substrate (IRS) proteins (White, 1997). These act as adapter proteins linking the insulin receptor to other downstream effector proteins such as PI 3'-kinase (Figure 1.2). Proteins such as Grb2 and p85 do not posses a catalytic domain. These proteins serve as adaptor proteins to link the receptor tyrosine kinase to other intermediates. Such proteins contain additional Src homology-3 (SH-3) domains. SH-3 domains are composed of about 60 amino acid residues containing a proline-rich region of 10 amino acids. Both secondary and tertiary structures vary between SH-3 domains of different proteins, suggesting specificity of interaction with downstream target proteins. SH-3 domains may also be involved in other processes such as the targeting of proteins to specific sub-cellular locations. For example, the cytoskeletal protein spectrin

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contains a SH-3 domain that is responsible for the membrane targeting of spectrin (Wasenius et al., 1989).

## 1.5 Phosphatidylinositol (PI) 3'-kinase

PI 3'-kinase has emerged as a key signalling molecule within growth factor signalling pathways (Shepherd et al., 1998). This enzyme is a lipid protein kinase catalysing the formation of the 'second messenger' phosphatidylinositol 3',4',5'-trisphosphate ($PI(3,4,5)P_3$) from phosphatidylinositol 4',5'-bisphosphate ($PI(4,5)P_2$). There are 3 lines of evidence to suggest that the lipid product $PIP_3$ plays a direct role in cell signalling: (1) The rise in $PI(3,4,5)P_3$ correlates with activation of downstream responses (Cross et al., 1997; Vanderkaay et al., 1997); (2) This rise in $PI(3,4,5)P_3$ is transient (Stevens et al., 1991); and (3) $PI(3,4,5)P_3$ can directly modulate downstream proteins via so-called Pleckstrin Homology (PH) Domains (see below).

Following the rise in $PI(3,4,5)P_3$ levels there is an elevation in $PI(3,4)P_2$ levels which occurs due to the degradation of $PI(3,4,5)P_3$ via a phosphatidylinositol 3,4,5-trisphosphate 5'-phosphatase. This elevation in $PI(3,4)P_2$ may also be important in activating other downstream pathways (Woscholski et al., 1997; Guilherme et al., 1996). There are 4 classes of PI 3'-kinase: 1a, 1b, 2 and 3 (Shepherd et al., 1998). The class 1a PI 3'-kinases are involved directly with growth factor receptor signalling complexes and are heterodimers which consist of a p85 adapter/regulatory subunit tightly associated with a p110 catalytic subunit. The p85 adapter subunit contains 2 SH-2 domains and 1 SH-3 domain. The catalytic subunits of the class 1a PI 3'-kinases share significant sequence homology with the class 1b PI 3'-kinase catalytic subunits, however, the class 1b adapter p101 subunit differs

significantly from the class 1a p85 subunit both in structure and function. The other two classes of PI 3'-kinase do not use $PI(4,5)P_2$ as their substrate and do not produce $PI(3,4,5)P_3$ as a lipid product, therefore these will not be discussed further.

### 1.6.1 Activation of PI 3'-kinase by direct association with receptor tyrosine kinases

As mentioned, the class 1a p85 subunit contains 2 SH-2 domains and can therefore associate with the phosphotyrosine residues on activated receptor tyrosine kinases, in particular to YxxM motifs (Shepherd et al., 1998). This binding of p85 to the receptor has two functions: (1) It facilitates the activation of the p110 catalytic subunit of PI 3'-kinase by causing a conformational change upon binding; and (2) It recruits the PI 3'-kinase to the plasma membrane where its substrate is located.

In the case of the insulin and IGF-1 receptors, the p85 subunit binds via its SH-2 domains to phosphotyrosine residues on the IRS proteins rather than to the receptors directly. Once again this will result in activation of the p110 catalytic subunit (White, 1997).

### 1.6.2 Activation of PI 3'-kinase by Ras

PI 3'-kinase can also be activated by the Ras oncogene product (Rodriguez-Viciana et al., 1994). It has been demonstrated that activated GTP-loaded Ras interacts directly with the p110 catalytic subunit of PI 3'-kinase and that this interaction activates PI 3'-kinase. Also, since Ras is a membrane-anchored protein, it is conceivable that the Ras-p110 interaction could play a role in localising PI 3'-kinase to the plasma membrane and in doing so brings it into contact with its lipid substrate. Insulin activates Ras through binding of Grb2/Sos

complexes to phosphorylated SHC and/or IRS proteins (Cheatham et al., 1995) so it has been proposed that the activation of PI 3'-kinase by Ras may provide an alternative effector system to the well established Raf/MAP kinase kinase (MEK)/MAP kinase cascade. Therefore this pathway could make a contribution to the overall activation of PI 3'-kinase by insulin.

### 1.6.3 PH domains

$PI(3,4,5)P_3$ and $PI(3,4)P_2$ bind to target proteins via motifs of about 120 amino acids called Pleckstrin Homology (PH) domains. However not all PH domains bind $PI(3,4,5)P_3$ and there is a great deal of variability in the relative preference of PH domains for particular phosphoinositides (Lemmon, 1999; and references therein). By binding to the protein $PI(3,4,5)P_3$ (or $PI(3,4)P_2$) may cause a conformational change altering the proteins' activity. A second possibility is that the conformational change will expose regulatory sites of phosphorylation within the protein. Finally, the binding of $PI(3,4,5)P_3$ (or $PI(3,4)P_2$) may aid in the co-localisation of the protein to other proteins in the signalling pathway at membranes.

### 1.7 Downstream of PI 3'-kinase: Protein kinase B

The activation of PI 3'-kinase results in a wide range of biological responses via many signalling pathways (Shepherd et al., 1998). However, it is known that many of the downstream signalling events are controlled by serine/threonine kinase cascades so, how does a lipid kinase translate its signal into a protein kinase cascade? The answer to this question lies with the binding of the lipid products $PI(3,4,5)P_3$ and $PI(3,4)P_2$ to the PH domains of downstream kinases resulting in their activation. One of the most important discoveries in recent years has been the identification of Protein kinase B (PKB), a 57 kDa serine/threonine

kinase containing a PH domain which acts immediately downstream of PI 3'-kinase. PKB is emerging as one of the central players in growth factor signalling and is the focus point for the remainder of this study.

**1.7.1 The discovery of PKB**

In 1991, 3 groups working independently discovered PKB. Two groups identified the kinase as a result of its homology to both protein kinase A (68% homology to the PKA kinase domain) and protein kinase C (73% homology to the PKCε kinase domain) hence the nomenclature protein kinase B (PKB) (Coffer and Woodgett, 1991) and RAC-PK (related to the A and C kinases) (Jones et al., 1991). The third group identified the kinase as the homologue of the oncogene *v-akt* of the acute transforming retrovirus AKT8 found in a rodent T-cell lymphoma (Bellacosa et al., 1991). The retroviral oncogene encodes a fusion of the cellular PKB/Akt protein to the viral structural protein Gag. Mammalian genomes contain 3 genes encoding PKB's (termed α/AKT1, β/AKT2, and γ/AKT3). PKBβ and γ are approximately 82% identical with the α isoform, although rat PKBγ was found to lack 23 amino acids at the C-terminus compared with the others (Konishi et al., 1995). Homologues have also been identified in the nematode worm Caenorhabditis elegans (Waterston et al., 1992) and the fruitfly Drosophila melanogaster (dAkt) (Franke et al., 1994). PKBα and PKBβ are expressed in almost all tissues with highest levels of expression in the brain, thymus, heart and lung (Coffer and Woodgett, 1991; Jones et al., 1991; Bellacosa et al., 1993). PKBγ exhibits high levels of expression in brain, testes, heart and placental tissue with smaller but detectable amounts found in skeletal muscle, kidney and pancreas (Konishi et al., 1995;Nakatani et al., 1999).

## 1.7.2 The structure of the PKB isoforms

Analysis of the amino acid sequence of PKBα shows that it is composed of three distinct regions (Figure 1.3). At the N-terminus there exists a PH domain (Haslam et al., 1993), followed by a central catalytic domain (kinase domain), and a C-terminus domain. The catalytic domain of PKBα is homologous to that of PKA and PKC and the C-terminus shows similarity to a regulatory region found in some members of the PKC family (Bellacosa et al., 1993). As mentioned briefly earlier, rat PKBγ has a truncated C-terminus and is therefore missing the regulatory serine phosphorylation site found on all the other isoforms. The recently cloned human and mouse PKBγ isoforms do however contain the full length C-terminus including this serine residue (Brodbeck et al., 1999; Nakatani et al., 1999) suggesting that the rat PKBγ is actually a product of alternative mRNA splicing.

The sequence analysis of v-Akt cDNA reveals that it is a fusion between a truncated tripartite viral group-specific antigen gag (p15, p12 and truncated p30) and PKBα. These two domains are joined by a 21 amino-acid long peptide encoded by sequences from the 5'-untranslated sequence of PKBα with an added 3 nucleotides at the recombination breakpoint. The addition of the viral gag protein is important to the oncogenic nature of v-Akt. Since the N-terminus of gag is myristoylated ( Bellacosa et al., 1991) this causes it to be targeted to the membranes (for review of N-terminal myristolytion see Gordon et al., 1991). This results in the consitutive activation of the v-Akt.
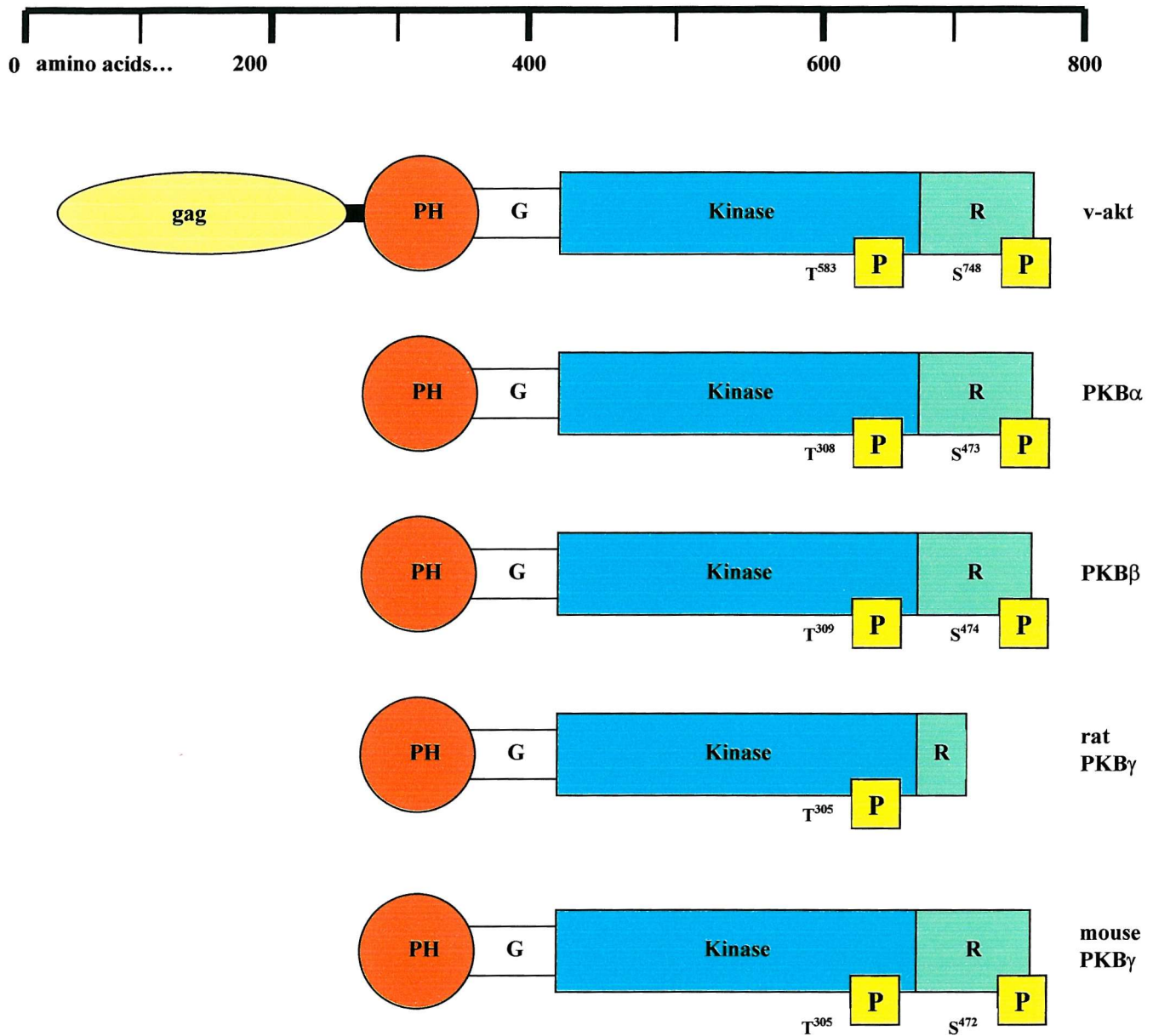
**Figure 1.3    Structure of the PKB isoforms.**

The domain structure and size of v-akt, and PKB isoforms ($\alpha, \beta$, truncated and full length $\gamma$). v-akt is a fusion of PKB with a viral gag protein. PKB contains a PH domain (PH) and a glycine rich region (G). The phosphorylation sites of the regulatory domain (R) are also indicated.

## 1.7.3 Activation of PKB by phospholipids and phosphorylation.

Following stimulation by growth factors all three isoforms of PKB become active within a minute. This activation is inhibited by inhibitors of PI 3'-kinase or by over-expression of dominant negative forms of PI 3'-kinases (Burgering and Coffer, 1995; Franke et al., 1995). To back up these findings PKB has been found to interact with $PI(3,4,5)P_3$ or $PI(3,4,)P_2$ at low micromolar concentrations in vitro. $PI(3,4,)P_2$ interaction is about 10-fold weaker and many other phospholipids do not bind at all (Alessi et al., 1997a). Therefore, $PI(3,4,5)P_3$ and $PI(3,4,)P_2$ have an important role to play in the activation of PKB (Figure 1.4).

The activation of PKB is accompanied by phosphorylation on specific residues. PKBα becomes phosphorylated at Thr308 and Ser473 (Alessi et al., 1996). PKBβ becomes phosphorylated on Thr309 and Ser474 (Meier et al., 1997; Walker et al., 1998). The full length human PKBγ is phosphorylated on Thr305 and Ser472 (Brodbeck et al., 1999; Nakatani et al., 1999) while the truncated rat PKBγ is phosphorylated on Thr305 only (Walker et al., 1998). Phosphorylation of Thr308 and Ser473 is abolished if the cells are incubated with PI 3'-kinase inhibitors prior to growth factor stimulation (Alessi et al., 1996). In addition, mutation of Thr308 or Ser473 to Ala inhibits activation of PKBα by insulin or IGF-1 by 95% and 85% respectively. Conversely, mutation of either Thr308 or Ser473 to Asp (which mimics the effect of phosphorylation by introducing a negative charge) increases PKBα activity 5-fold in unstimulated cells. Mutation of both residues to Asp produces a 20-fold increase in activation in unstimulated cells (Alessi et al., 1996). Interestingly, maximal activation of PKB can be achieved by attaching a membrane-targeting signal to the N-terminus (Hajduch et al., 1998; Meier et al., 1997; Andjelkovic et al., 1997). This occurs because both Thr308 and Ser473 become highly phosphorylated by PDK1 and PDK2.

## 1.7.4 The Protein kinase B kinases (PDK1 and PDK2).

Until very recently, the kinase(s) that phosphorylate PKB remained elusive. However a novel 67kDa protein kinase termed 3-phosphoinositol-dependent protein kinase 1 (PDK1) has been purified which activates the PKB isoforms by phosphorylation at Thr308 (PKBα) (Alessi et al., 1997a), Thr309 (PKBβ) (Walker et al., 1998), and Thr305 (PKBγ) (Walker et al., 1998). The name 3-phosphoinositol-dependent protein kinase 1 stems from the observation that this enzyme is only active in the presence of lipid vesicles containing $PI(3,4,5)P_3$ or $PI(3,4)P_2$ (Alessi et al., 1997a) .

The structure of PDK1 mirrors that of PKB since it consists of a N-terminal catalytic domain (similar to that found in protein kinases such as PKA, PKB and PKC) and a C-terminal PH domain ( Alessi et al., 1997b). Mammalian PDK1 has been found to be highly homologous to a Drosophila counterpart termed DSTPK61 (Alessi et al., 1997b). DSTPK61 has the ability to phosphorylate PKBα in vitro and has been implicated in the regulation of sex differentiation, oogenesis, and spermatogenesis in Drosophila since the gene coding for DSTPK61 is differentially spliced in male and female flies. This generates many different transcripts that all have the same open reading frame (and therefore produce the same protein), but differ significantly in their 5' and 3' untranslated regions. Therefore this leads to differing levels of DSTPK61 expression in male and female flies ( Alessi et al., 1997b).

It is as yet unclear whether or not PDK1 is responsible for the phosphorylation of Ser473 (PKBα) since the phosphorylation status of this residue is unaffected in cells over-expressing PDK1 (Alessi et al., 1997a). In fact the protein kinase that phosphorylates Ser473 (PKBα),

Ser474 (PKBβ) and Ser472 (human PKBγ) remains unidentified and is tentatively named phosphoinositide-dependent protein kinase 2 (PDK2). Recently it has been suggested that PDK-1 could actually interact with other proteins in order to modify its function. PDK-1 may complex with a fragment of another kinase, PRK-2 (Balendran et al., 1999). This PDK-1/PRK-2 complex is then capable of phosphorylating PKBα at Ser473. In addition, this complex is upregulated by PI-3 kinase –generated 3'-phosphorylated phospholipids. Other evidence suggests that Integrin-linked kinase (ILK) has the ability to phosphorylate Ser473 of PKBα *in vitro* (Delcommenne et al., 1998) and since this enzyme is activated by PI 3'-kinase it may well be PDK2.

### 1.7.5 The negative regulation of PKB.

### PP2A

The phosphorylation of PKBα on Thr308 and Ser473 is only short-lived suggesting that PKB is negatively regulated by dephosphorylation (Andjelkovic et al., 1996; Meier et al., 1997). Meier and co-workers used hyperosmotic shock to rapidly inactivate PKB and showed that this inactivation was preceded by dephosphorylation. Most importantly, they then prevented the dephosphorylation and inactivation of PKB by adding calyculin A, a relatively specific inhibitor of PP2A (Meier et al., 1998). These results proved that PP2A was a key enzyme involved in the negative regulation of PKB.

### PTEN

PTEN (phosphatase and tensin homologue deleted from chromosome 10) is a tumor supressor protein mutated or deleted in a wide range of human cancers (Cantley and Neel,

1999). PTEN shares homology with dual-specificity phosphatases that can dephosphorylate serine, threonine and tyrosine residues. However, attempts to confirm PTEN as a protein phosphatase revealed only weak phosphatase activity (Li and Sun, 1997; Myers et al., 1997). It was soon realised that PTEN was in fact a lipid phosphatase and NOT a protein phosphatase (Maehama and Dixon, 1998; Myers et al., 1998). Recombinant PTEN dephosphorylates 3-phosphoinositides specifically at position 3 of the inositol ring and has the highest activity for PI(3,4,5)P$_3$ converting it to PI(4,5)P$_2$ (Maehama and Dixon, 1998). In addition, over-expression of PTEN significantly reduced PI(3,4,5)P$_3$ production induced by insulin, and PTEN-null cells have higher levels of PI(3,4,5)P$_3$ (Haas-Kogan et al., 1998; Maehama and Dixon, 1998; Stambolic et al., 1998). Therefore, since PTEN was obviously antagonising the effects of PI 3-kinase it was hypothesised that its role in tumour suppression involves the negative regulation of PKB. This was confirmed using tumour cell lines with inactive PTEN, and in PTEN-null fibroblasts since these cells showed higher-than-normal levels of basal PKB activity (Myers et al., 1997; Maehama and Dixon, 1998; Myers et al., 1998; Haas-Kogan et al., 1998; Stambolic et al., 1998; Li and Sun, 1998; Wu et al., 1998).


**SHIP**

A second lipid phosphatase which was found to negatively regulate PKB activity is SHIP-an inositol 5'phosphatase that hydrolyses PI(3,4,5)P$_3$ to PI(3,4)P2. Over-expression of SHIP inhibits PKB activity while SHIP-null cells exhibit prolonged activation of PKB upon stimulation (Aman et al., 1998; Liu et al., 1999).


In addition to these protein and lipid phosphatases, PKB may also be negatively regulated simply by its sub-cellular location. Following activation by growth factor stimulus, PKB rapidly translocates to the plasma membrane by virtue of its PH domain binding PI(3,4,5)P$_3$

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(Andjelkovic et al., 1997; Meier et al., 1997). However, following phosphorylation by PDK1 and/or PDK2, PKB detaches from the plasma membrane and translocates to the nucleus. This re-location of PKB may be to bring it into contact with nuclear substrates such as transcription factors, but it may also be to negatively regulate its activity by taking it away from cytosolic substrates (Andjelkovic et al., 1997).

**1.7.6 The mechanism of activation of PKB.**

Figure 1.4 outlines the current basic mechanism of activation of PKB leading to its many downstream effects. The precise mechanism of PKB activation remains a controversial topic. However, it is clear that this process relies on the binding of $PI(3,4,5)P_3$ and/or $PI(3,4)P_2$ to the PH domains on both PKB and PDK1 following stimulation with growth factors. The binding of $PI(3,4,5)P_3$ and/or $PI(3,4)P_2$ to the PH domains of PKB and PDK1 may have several functions:

(1) It causes co-localisation of PKB and PDK1 at the plasma membrane (Stokoe et al., 1997). Following stimulation by growth factors PKB binds $PI(3,4,5)P_3$ or $PI(3,4)P_2$ and translocates from the cytosol to the plasma membrane where it is co-localised with PDK1 and is phosphorylated by PDK1. Translocation is prevented by inhibitors of PI 3'-kinase or if the PH domain of PKB is deleted (Andjelkovic et al., 1997). Also, membrane targeting of PKB by the addition of a N-terminal myristoylation signal results in the phosphorylation of PKBα at Thr308 and Ser474 making it constitutively active even in unstimulated cells (Andjelkovic et al., 1997; Hajduch et al., 1998).

(2) The binding of $PI(3,4,5)P_3$ or $PI(3,4)P_2$ to the PH domain of PDK1 may be important in stimulating its kinase activity towards PKB. Deletion of the PH domain of PDK1 results in a

30-fold reduction in its ability to activate PKB in vitro (Stokoe et al., 1997; Alessi et al., 1997B).

(3) In addition, the binding of PI(3,4,5)P$_3$ or PI(3,4)P$_2$ to the PH domain of PKB may alter its conformation so that the phosphorylation sites may become more accessible to PDK1 and PDK2 (Stokoe et al., 1997).

Once PKB has been localised to the plasma membrane in this way, the constitutively active PDK1 and/or PDK2 protein kinases phosphorylate the Thr308 and Ser473 residues (PKBα). This phosphorylation results in a conformational change and the full activation of PKB allowing it to dissociated from the plasma membrane and phosphorylates its downstream targets (Stokoe et al., 1997).

## 1.7.7 The PKB phosphorylation site.

The optimal PKB phosphorylation site was determined by Alessi and co-workers studying the phosphorylation of GSK3α and β by PKB (Alessi et al., 1996). They found that the minimun consensus sequence for efficient phosphorylation by PKB was Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd, where Xaa is any amino acid, Yaa and Zaa are small amino acids other than Glycine, and Hyd is a bulky hydrophobic residue. The knowledge of this consensus sequence was a good starting point towards the identification of other potential PKB substrates. However, this data should be interpreted carefully, since other kinases such as PKA, MAPKAP-K1, p70 S6-kinase, p90RSK and the recently characterised serum- and glucocorticoid-regulated protein kinase (SGK) can also phosphorylate a similar consensus site (Kobayashi and Cohen, 1999).

**Figure 1.4    Model for the growth factor stimulated activation of PKB mediated by PI 3-kinase.**

Growth factors (e.g. insulin and IGF-1) bind to specific receptor tyrosine kinases at the plasma membrane. This activates the receptor tyrosine kinase and the receptor undergoes transautophosphorylation promoting the recruitment and activation of PI 3-kinase. Activated PI 3-kinase at the membrane converts $PI-4,5-P_2$ to $PI-3,4,5-P_3$ (some $PI-3,4-P_2$ is also generated by a inositol 5'-polyphosphatase, PTEN). The $PI-3,4,5-P_3$ (and possibly $PI-3,4-P_2$) bind to the PH domain of inactive PKB recruiting it to the plasma membrane and causing a partial conformational change which exposes the regulatory Thr308 and Ser473 phosphorylation sites. These events allow the PKB to co-localise with its upstream kinases PDK1 and PDK2 which phosphorylate and fully activate PKB. Once active, PKB is released from the membrane to phosphorylate its downstream targets involved in a wide range of cellular processes including cell survival/apoptosis ( ↓BAD); glucose metabolism ( ↓ GSK-3); protein synthesis (↑p70S6K) and gene expression (↑NF-κB).
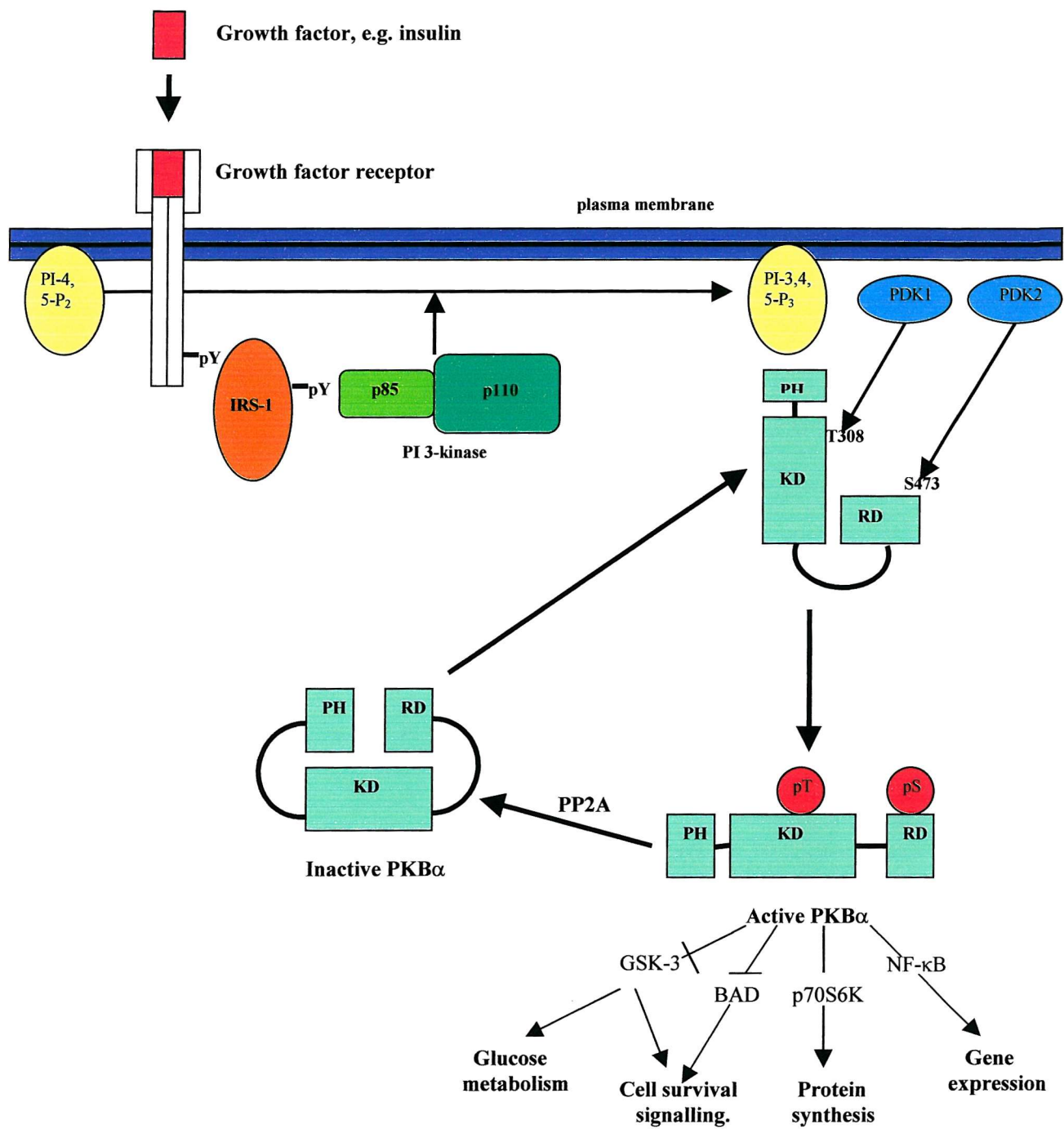
**Figure 1.4    Model for the growth factor stimulated activation of PKB mediated by PI 3-kinase.**

## 1.8 PKB and cell survival.

One of the major functions of PKBα is to promote growth factor-mediated cell survival and to block programmed cell death or apoptosis. In fact, over a wide range of studies PKBα has been shown to consistently and reproducibly demonstrate antiapoptotic activity.

## 1.8.1 Apoptosis.

In 1972 Kerr, Wyllie and Currie distinguished between two forms of cell death, necrosis and apoptosis (Kerr et al., 1972). Necrosis is the form of death that results from cellular metabolic collapse, when a cell can no longer maintain ionic homeostasis. Necrosis is not seen in normal development, but is invariably the response to injury or toxic damage and is often accompanied by inflammation. Apoptosis, on the other hand, is a central part of normal development. It is a highly regulated, multistep form of cell death characterised by nuclear and cytoplasmic condensation, DNA fragmentation, dilation of the endoplasmic reticulum and alterations in cell membrane composition (Saraste and Pulkki, 2000; Nagata, 2000). Apoptosis is crucial for maintaining appropriate cell number and tissue organisation in certain cell types such as lymphocytes and neurones (Datta and Greenberg, 1998; Jacobson et al., 1997; Marx, 1993; Barinaga, 1993; Vaux, 1993; Raff, 1992) and is involved in many pathogenic conditions such as acute neurological conditions, neurodegenerative diseases, cardiovascular diseases, imunological diseases, AIDS and cancer (Thompson, 1995).

Apoptosis is a highly regulated process and can be suppressed by a variety of extracellular stimuli. In the 1950's the Nerve Growth Factor (NGF) was purified and found to promote the survival of sympathetic neurones. This was only the tip of the iceberg since a wide variety of

peptide growth factors have been found to promote the survival of a wide variety of cell types both in vitro and in vivo (Levi-Montalcini, 1987).

## 1.8.2 The current model of apoptosis.

The current model of growth factor withdrawal induced apoptosis concerns the Bcl-2 family of proteins and their interaction with the mitochonria (Figure 1.5). Bcl-2 was first identified as a proto-oncogene in folicular B-cell lymphoma. In the lymphoma cells the Bcl-2 gene was found at the breakpoint of the translocation between chromosome 18 and chromosome 14, where the gene is under the control of the immunoglobulin heavy chain intron enhancer (Tsujimoto et al., 1985). This results in translational upregulation of the gene and overexpression of the Bcl-2 protein. Bcl-2 was identified as the mammalian homologue of the apoptosis repressor gene ced-9 in C. elegans and is the prototype for a large family of structurally related proteins that regulate cell death in mammals (Antonsson and Martinou, 2000; Reed, 1998). Some of these Bcl-2 proteins promote cell survival (Bcl-2, Bcl-$X_L$, Bcl-w, Mcl-1 and A1), whereas others in the family promote cell death (Bax, BAD, Bak, Bok, Bcl-$X_S$, Bid, Bik/Nbk, Bim, Krk, Mtd). These various family members are part of a network of apoptotic regulators distributed both in the cytoplasm and at intracellular membranes, such as the outer mitochondrial membrane. *It is the equilibribum within the cell between these family members that decides the fate of the cell.* The road to cell death involves three main stages. Firstly, in the presence of survival factors, the activity of the pro-survival Bcl-2 members prevails over the activity of pro-death Bcl-2 family members. However, apoptotic stimuli, for example the withdrawal of growth factors results in a rapid shift in balance from pro-survival to pro-apoptotic via transcription-dependant and –independent mechanisms. This disequilibrium results in the translocation of pro-apoptotic Bcl-2 members from the

cytoplasm to the mitochondrial outer membrane where they initiate the second stage in cell death, a cell death programme. This involves changes in the mitochondria (mitochondrial stress) that includes the opening of the permeability pore (PTP), decreases in the mitochondrial membrane potential, decreases in nucleotide exchange, and the release of cytrochrome c into the cytoplasm (for review: Antonsson and Martinou, 2000). The release of cytochrome c into the cytoplasm results in the third and final stage of apoptosis, the formation of a multiprotein complex called the apoptosome (Tsujimoto, 1998). The apoptosome consists of the Apaf-1 adapter protein, cytochrome c, dATP, and the aspartyl-directed protease caspase 9, in its inactive form (pro-caspase 9). In the absence of cytochrome c, Apaf-1 is inhibited from interacting with pro-caspase 9. However, following cytochrome c release, cytochrome c binds to Apaf-1 and releases the intramolecular inhibition, resulting in interaction between Apaf-1 and pro-caspase 9. This interaction in turn activates the pro-caspase 9 by cleavage at Asp residues. The released and active caspase 9 then goes on to activate downstream effector caspases (such as caspases 3 and 7). These effector caspases cleave a variety of structural and regulatory proteins resulting in apoptosis as detected by the changes in cell morphology and by DNA fragmentation (Bratton et al., 2000; Thornberry and Lazebnik, 1998; Cohn, 1997; Li et al., 1997; Zou et al., 1997; Nicholson et al., 1995).

Although this is the main pathway towards apoptosis, it must also be noted that Bcl-2 family members may also regulate the apoptosome directly by binding to Apaf-1 (Dragovich et al., 1998). Bcl-$X_L$ can bind to Apaf-1 and block the ability of Apaf-1 to activate procaspase 9. The binding of Bcl-$X_L$ to Apaf-1 may be antagonised by Bax. In addition, in some cells growth factor withdrawl triggers cell death via cell surface death receptors (Le-Niculescu et al., 1999). Activation of these receptors, acting through adaptor proteins such as FADD, results in the activation of caspase 8 which then activates caspase 3 initiating apoptosis independently of the apoptosome (Green 1998).

**Figure 1.5    A general overview of the apoptotic pathway leading to Caspase activation and substrate cleavage.**

Apoptotic stimuli such as growth factor withdrawal result in the balance between pro-survival and pro-apoptoic Bcl-2 family members tipping towards the pro-apoptotic members (e.g. BAD, tBID and BAX), which translocate to the mitochonrial outer membrane. This causes mitochondrial stress inducing the release of cytochrome c into the cytoplasm. Cytochrome c and dATP induce a conformational change in Aparf-1 that allows it to oligomerise into a complex capable of recruiting and activating the initiator caspase, Caspase 9. Once active, Caspase 9 goes on to cleave and activate the downstream effector caspases (e.g. Caspase 3 and Caspase 8). The ultimate result is the cleavage of a wide range of vital cellular proteins causing the onset of apoptosis observed as membrane blebbing, chromatin condensation, DNA fragmentation and formation of apoptotic bodies. Cells that have undergone apoptosis are either removed by phagocytosis or remain as apoptotic bodies.
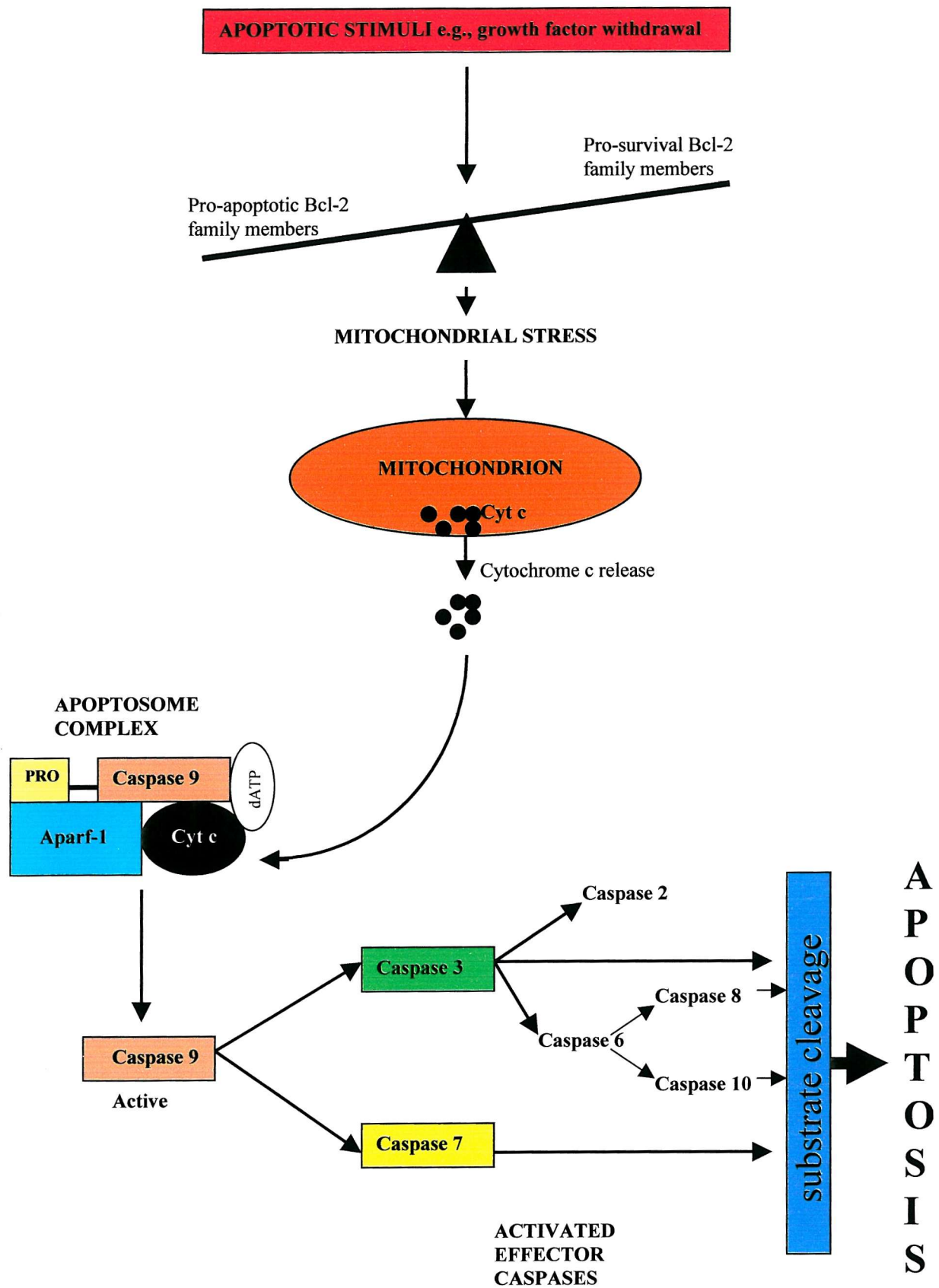
**Figure 1.5** A general overview of the apoptotic pathway leading to Caspase activation and substrate cleavage.

## 1.8.3 PI3-kinase, PKB and cell survival

Recently it has been the turn of the PI3-kinase/PKB cascade to come into the limelight of investigation involving apoptosis. This signalling pathway has been implicated in the ability of tropic factors to promote cell survival. Several targets of the PI3-kinase/PKB signalling pathway have already been identified as key components of the intrinsic cell death machinery, namely the Bcl-2 family member BAD, caspase 9, the forkhead transcription factors, IKK, the kinase which regulates the NF-κB transcription factor and more recently, GSK-3 (Figure 1.6).

PI3-kinase has the ability to suppress apoptosis and promote cell survival in a wide variety of cultured cells ranging from fibroblasts to neurones (Yao and Cooper, 1995; Dudek et al., 1997; Kauffmann-Zeh et al., 1997; Khwaja et al., 1997; Philpott et al., 1997; Kulik et al., 1997; Kennedy et al., 1997; Stambolic et al., 1998; Weiner and Chun, 1999; Fruman et al., 1999). If apoptosis is induced in cells by either removing growth factors or by the use of toxic stimuli, PI3-kinase activity is sufficient to block apoptosis and promote cell survival. In experiments where serum starved cells are transfected with constitutively active PI3-kinase, with receptor mutants in which PI3-kinase is exclusively activated in response to receptor ligation, or with constitutively active Ras mutants that bind to and activate only PI3-kinase, in each case these cells are resistant to apoptosis (Yao and Cooper, 1995; Kauffmann-Zeh et al., 1997; Khwaja et al., 1997; Philpott et al., 1997; ; Kulik et al., 1997; Kennedy et al., 1997).

The observation that PKB is a target of PI3-kinase alongside the evidence that PI3-kinase mediates growth factor-dependent cell survival suggested that PKB might be the critical regulator of cell survival. Experiments to test this theory have made use of both constitutively

active mutants of PKB and dominant negative mutants. A large number of studies have shown that transfection of cells with constitutively active PKB (and in some cases wild-type) blocks apoptosis induced by apoptotic stimuli including growth factor removal, UV irradiation, matrix detachment, DNA damage, and treatment of cells with anti-Fas antibody (Dudek et al., 1997; Kauffmann-Zeh et al., 1997; Kennedy et al., 1997; Kulik et al., 1997; Khwaja et al., 1997; Philpott et al., 1997; Songyang et al., 1997; Xiong and Parsons, 1997; Eves et al., 1998; Gerber et al., 1998; Kulik and Weber, 1998; Hausler et al., 1998). Four different dominant negative mutants have also been used to study PKB's role in growth factor mediated survival. Expression of a catalytically inactive PKB (Dudek et al., 1997) or a mutant PKB consisting only of its PH domain (Songyang et al., 1997) both blocked the ability of a variety of growth factors to prevent apoptosis. Recently two other dominant negative mutants have been described, one a wild-type PKB fused to the Ras CAAX membrane targeting domain, and the other a PKB mutant with both the PDK phosphorylation sites and the ATP binding site mutated to alanine (PKB-AAA). Both have been used demonstrated to effectively block cellular survival (Rohn et al., 1998, Stambolic et al., 1998).

The use of mutant forms of PKB has provided us with much evidence to suggest that PKB serves a crucial role as a mediator of cell survival. This evidence has also been backed up by indirect biological evidence. Treatment of cells with ceramide, a lipid intermediate involved in cell death signals (Perry, 1999), inhibits PKB activity by promoting Ser473 dephosphorylation (Schubert et al., 2000; Schmitz-Peiffer et al., 1999; Zhou et al., 1998). In addition, during the early stages of apoptosis, it has been shown that PKB undergoes selective proteolysis by Caspase 9 (Widmann et al., 1998). Genetic studies from Drosophila have also provided evidence for PKB involvement in cell survival. Flies defective in dAKT1 show significant ectopic apoptosis during embryogenesis (Staveley et al., 1998).

## 1.9 Downstram targets of PKB invoved in apoptosis (Refer to Figure 1.6).

## 1.9.1 BAD

The first potential apoptotic target of PKB to be discovered was BAD. BAD is a pro-apoptotic member of the Bcl-2 family and was found to bind to pro-survival Bcl-2 members such as Bcl-$X_L$ via its BH3 domain (Yang et al., 1995). This interaction between BAD and Bcl-$X_L$ blocks Bcl-$X_L$-dependent cell survival, inducing apoptosis (Yang et al., 1995; Ottilie et al., 1997). Growth factor induced phosphorylation of BAD occurs at two sites, Ser112 and Ser136 and phosphorylation of BAD at either one of these sites causes BAD to dissociate from Bcl-XL and associate with cytoplasmic 14-3-3 proteins (Zha et al., 1996). These 14-3-3 proteins are adapter proteins that interact with a variety of signalling molecules in a phosphoylation-dependent manner. This process may protect BAD from dephosphorylation or prevent it from reaching its targets in the mitochodria. Thus BAD phosphorylation disrupts its ability to bind Bcl-$X_L$ and inactivate Bcl-$X_L$. The Ser112 and Ser136 phosphorylation sites both lie within the PKB consensus phosphorylation sequence, however it was found that PKB preferentially phosphorylated the Ser136 site in vitro (Datta et al 1997). PKB activity was then found to be both necessary and sufficient for Ser136 phosphorylation in vivo since co-transfection of constitutively active PKB with BAD resulted in increased levels of BAD Ser136 phosphorylation (Datta et al., 1997; Del Peso et al., 1997; Blume-Jenson et al., 1998). Transfection of 3T3 cells with constitutively active PKB is enough to induce the phosphorylation of endogenous BAD at Ser136 (Datta et al., 1997). In addition, when BAD and PKB are over-expressed together, they co-immunoprecipitate and interact in GST-pulldown experiments (Datta et al., 1997; Blume-Jenson, 1998).

A number of survival factors induce phosphorylation of endogenous and transfected BAD and in cells exposed to these factors there is a concurrent increase in PKB activity (Zha et al., 1996; Datta et al., 1997; Blume-Jensen et al., 1998; Scheid and Duronio, 1998). On the other hand, it has been shown that stress induced inhibition of PI3-kinase activity not only inhibits PKB activity but also BAD phosphorylation (Zundel and Giaccia, 1998). In PTEN-deficient tumour lines there is an elevation of 3'-phosphorylated phosphoinositides and as a consequence, an elevation in PKB activity. This results in a high level of BAD phosphorylation which can be reverted by re-introducing PTEN back to the cells (Davis et al., 1998; Myers et al., 1998).

All this evidence therefore suggests an important role for BAD in PKB survival signalling. However, it has also been discovered that BAD is not expressed in some tissues where PKB promotes survival (Yang et al., 1995), therefore BAD is not the only possible mechanism by which PKB could block apoptosis.

### 1.9.2 Caspase 9

A second possible target of PKB is caspase 9 (Cardone et al., 1998). It was found that caspases in lysates from cell lines overexpressing constitutively active PKB were not activated effectively by the addition of cytochrome c to the lysates. Therefore the active PKB was inactivating caspase 9 downstream of cytochrome c release and formation of the apoptosome. Human caspase 9 has two potential PKB phosphorylation sites, Ser183 and Ser196. When overexpressed caspase 9 was immunoprecipitated from ^{32}P-labelled cells it was revealed that PKB phosphorylated caspase 9 at Ser196 (Cardone et al., 1998). When caspase 9 is phosphorylated by PKB, its protease activity is reduced.

PKB does not act as a generalized caspase inhibitor, constitutively active PKB is incapable of either phosphorylating caspase 3 or 8 in vitro, or blocking caspase 8-induced death (Cardone et al., 1998; Rohn et al., 1998). The phosphorylation of caspase 9 shows how PKB could potentially regulate apoptosis both at the level of mitochondria and downstream of the mitochondria.

## 1.9.3 The forkhead transcription factors.

Within 30 minutes of activation by growth factors, both PKB$\alpha$ and $\beta$ detach from the inner surface of the plasma membrane, where they are initally activated, and re-localise to the nucleus (Andjelkovic et al., 1997; Meier et al., 1997). In the nucleus, PKB isoforms have been hypothesised to phosphorylate and modulate the activity of transcription factors. Genetic studies in *C. elegans* have so far yielded only one definite substrate of PKB, namely the forkhead transcription factor DAF-16 (Paradis et al., 1998). Studies in *C. elegans* suggest that PKB negatively regulates DAF-16. Three homologues of DAF-16 have been identified in human and mouse and termed FKHR, FKHRL and AFX (Davis et al., 1995; Borkhardt et al., 1997; Anderson et al., 1998). DAF-16 and its three mammalian homologues are known as the forkhead family transcription factors and share a core domain of 100 amino acids, called the forkhead domain, that mediates their interaction with DNA. The carboxy-terminal domain of AFX and the related Forkhead isoforms correspond to the transcriptional activation domains of these transcriptional regulators (Kops et al., 1999). DAF-16 and its three mammalian homologues contain three consensus sites that can be phosphorylated in vitro by PKB and in co-transfection experiments. This phosphorylation serves to retain the transcription factors in the cytoplasm and to inhibit their activity via binding to 14-3-3 proteins (Biggs et al., 1999; Brunet et al., 1999; Kops et al., 1999; Rena et al., 1999).

Therefore, PKB-mediated phosphorylation may modulate the function of forkhead family members primarily through regulation of their sub-cellular localisation. Under conditions during which PKB is activated, FKHR and FKHRL are phosphorylated and retained in the cytoplasm. Under conditions were PKB is inactive, these forkhead family members are not phosphorylated and are re-localised to the nucleus. PKB-mediated sequestration of forkhead family members in the cytoplasm has functional consequences for forkhead-dependent transcription (Biggs et al., 1999; Brunet et al., 1999; Kops et al., 1999; Tang et al., 1999). In the absence of phosphorylation, forkhead family members that are localised to the nucleus bind to specific DNA elements. Electrophoretic mobility shift assays indicate that FKHR, FKHRL and AFX bind to the Insulin Response Sequence (IRS) within the IGF-1 binding protein (IGFBP1) promoter (Brunet et al., 1999; Kops et al., 1999; Tang et al., 1999). In addition, FKHRL binds a cluster of forkhead binding sites present in the promoter of the FasL gene (Brunet et al., 1999). Once bound to the promoters of target genes the forkhead family members all act as potent activators of transcription. However, this transactivating function of the forkhead family members is repressed either by stimulation of the cell with the survival factor IGF-1 or by over-expressing PI3-kinase or PKB resulting in down-regulation of the target genes.

Recent work has hinted towards an important role for the forkhead transcription factors in apoptosis. Non-phosphorylatable mutants of FKHR or FKHRL are potent transcription activators and trigger apoptosis in many cell types (Brunet et al., 1999; Tang et al., 1999). This apoptotic effect of FKHR was linked directly to its transcriptional activity since a mutation in the DNA-binding domain of FKHR abolishes its ability to induce apoptosis (Tang et al., 1999). This evidence showed that the forkhead family members up-regulate genes involved in causing cell death, but which ones? An important step towards answering

this question came with the finding that FKHRL binds to the promoter of the cytokine FasL gene and induces expression of a reporter gene driven by the FasL promoter (Brunet et al., 1999). The FasL gene has been shown to have an important role in survival factor withdrawal induced apoptosis since the FasL gene is upregulated in neurons upon survival factor withdrawal. Also, blocking FasL signalling reduced apoptosis triggered by survival factor withdrawal in cerebellar granule neurones (Le-Niculescu et al., 1999). The induction of apoptosis by the FKHRL phosphorylation site mutant can be reverted by treating the cells with a soluble form of Fas which binds newly synthesised FasL preventing it reaching its receptor (Brunet et al., 1999). Apoptosis may also be reverted when the mutant is expressed in Jurkat cells lacking components of the FasL signalling pathway (Fas and FADD).

These observations lead to the hypothesis that upon survival factor withdrawal, PKB is inactive and the forkhead family members are unphosphorylated and able to induce FasL gene transcription. FasL then binds to its cell surface receptors triggering a cascade of events leading to apoptosis. So PKB may promote survival by phosphorylating and sequestrating the forkhead transcription factors in the cytoplasm, thereby preventing them from inducing the transcription of death genes such as the FasL gene.

### 1.9.4 IKK and NF-κB

NF-κB is another transcription factor that may be indirectly regulated by PKB. NF-κB is responsible for the up-regulation of survival gene expression such as the pro-survival Bcl-2 family member Bfl-1/A1 (Zong et al., 1999). It is a ubiquitous, heterodimeric transcription factor that is sequestered in the cytoplasm by proteins of the IκB family (Mercurio and Manning, 1999). Phosphorylation of IκB targets it for proteosome-mediated degradation and

so degradation of IκB frees NF-κB, allowing its to translocate to the nucleus and activate its target genes. IκB is phosphorylated by two kinases IKKα and IKKβ and these kinases are phosphorylated and regulated by distinct upstream kinases including PKB. PKB has been shown to enhance the degradation of the IκBs and to induce NF-κB mediated activation of NF-κB-responsive model promoters (Kane et al., 1999). In addition, PKB is both necessary and sufficient for the ability of both PDGF and TNF to induce NF-κB activity (Khwaja, 1999; Ozes et al., 1999; Romashkova and Makarov et al., 1999). PKB may regulate NF-κB either by direct phosphorylation of the IKK complex or by phosphorylation of upstream kinases of the IKK complex. Evidence to suggest a direct role for PKB is that PKB can associate with the IKK complex in vivo, and can phosphorylate IKKα at Thr23 (Ozes et al., 1999). Although the exact mechanism by which PKB regulates NF-κB remains to be discovered, PKB appears to be a crucial component of the survival signalling pathway to NF-κB. In this way PKB could not only promote survival via post-translational regulation of the Bcl-2 family and caspase 9 but also via transcriptional regulation of their genes.

## 1.9.5 GSK3.

PKB may activate glycogen synthesis through the inactivation of GSK3, leading to activation of glycogen synthase, the enzyme responsible for converting glucose to glycogen (Cross et al., 1995; Van Weeren et al., 1998). PKB phosphorylates Ser21 in GSK3α and Ser9 in GSK3β and this serves to negatively regulate GSK3 activity thus maintaining glycogen synthase in a relatively de-phosphorylated and active state (Cross et al., 1995; Shaw et al., 1997; Markuns et al., 1999). Recently GSK3 has been implicated as a mediator of the PI3-kinase survival signal in Rat-1 and PC12 cells (Pap and Cooper, 1998). Transfection of these

cells with constitutively active GSK3 induces apoptosis even in the presence of growth factors, while conversely, transfection with kinase-dead GSK3 blocks apoptosis. PKB phosphorylates and inactivates GSK3 that in turn may regulate a number of key substrates involved in cellular metabolism including glycogen synthase and the GLUT4 glucose transporter. Therefore, PKB may simply promote survival by inactivating GSK3 and thereby causing a general increase in the cells' metabolic processes. A second theory is that PKB inactivation of GSK3 could inhibit the ability of GSK3 to signal as part of the wingless pathway. Disruption of this pathway has been implicated in the dysregulation of apoptosis (Zhang et al., 1998). However, it is not clear if the population of GSK3 molecules regulated by PKB is the same as that involved in wingless signalling. In addition, GSK3 negatively regulates many transcription factors, which may prove crucial in apoptotic signalling. For example, GSK3β phosphorylates β-catenin associated with the product of adenomatous polyposis coli gene and axin protein complex, thereby promoting its degradation by the proteosome. PKB mediated inhibition of GSK3β activity may lead to stabilization of β-catenin. β-catenin can then translocate to the nucleus, form a complex with the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of high-mobility-group transcription factors and increase their activity (Miller and Moon, 1996). Recently it has been reported that GSK-3 phosphorylates Tau, a neuronal microtubule-associated protein (Binder et al., 1985; Hong and Lee, 1997). Hyperphosphorylated Tau is a major component of paired helical filaments (PHF's), the building block of neurofibrillary lesions in Alzheimer's disease, typified by neuronal apoptosis. Therefore it has been hypothesised that activated GSK-3 could cause apoptosis in neuronal cells by this mechanism (Hong and Lee, 1997).

**Figure 1.6    Putative    downstream    substrates    of    PKB    involved    with    cell survival/apoptosis.**

PKB may mediate survival signalling through the phosphorylation of multiple putative substrates involved in apoptosis. PKB may phosphorylate BAD resulting in its dissociation from pro-survival Bcl-2 and association with the cytoplasmic 14-3-3 proteins. This could relieve the inhibition of BAD on Bcl-2. PKB may also negatively regulate the transcriptional activity of the forkhead family members that have been postulated to enhance the expression of certain death factor genes (e.g. FasL ligand). PKB has also been postulated to enhance the transcriptional activaity of NF-κB by phosphorylating and activating the kinases which phosphorylate IκB. This results in the degradation of Iκ-B and activation of NF-κB thought to enhance the transcription of cell survival genes (e.g. the Bcl-2 family member, Bfl-1/A1). PKB could also phosphorylate and inactivate Caspase 9 preventing the apoptotic caspase cascade. Inhibition of GSK3 by PKB may promote glucose metabloism, gene expression and protein translation. GSK-3 has also been implicated in the regulation of adenomatous polyposis coli and β-catenin, which modulates cell adhesion and regulates gene expression.
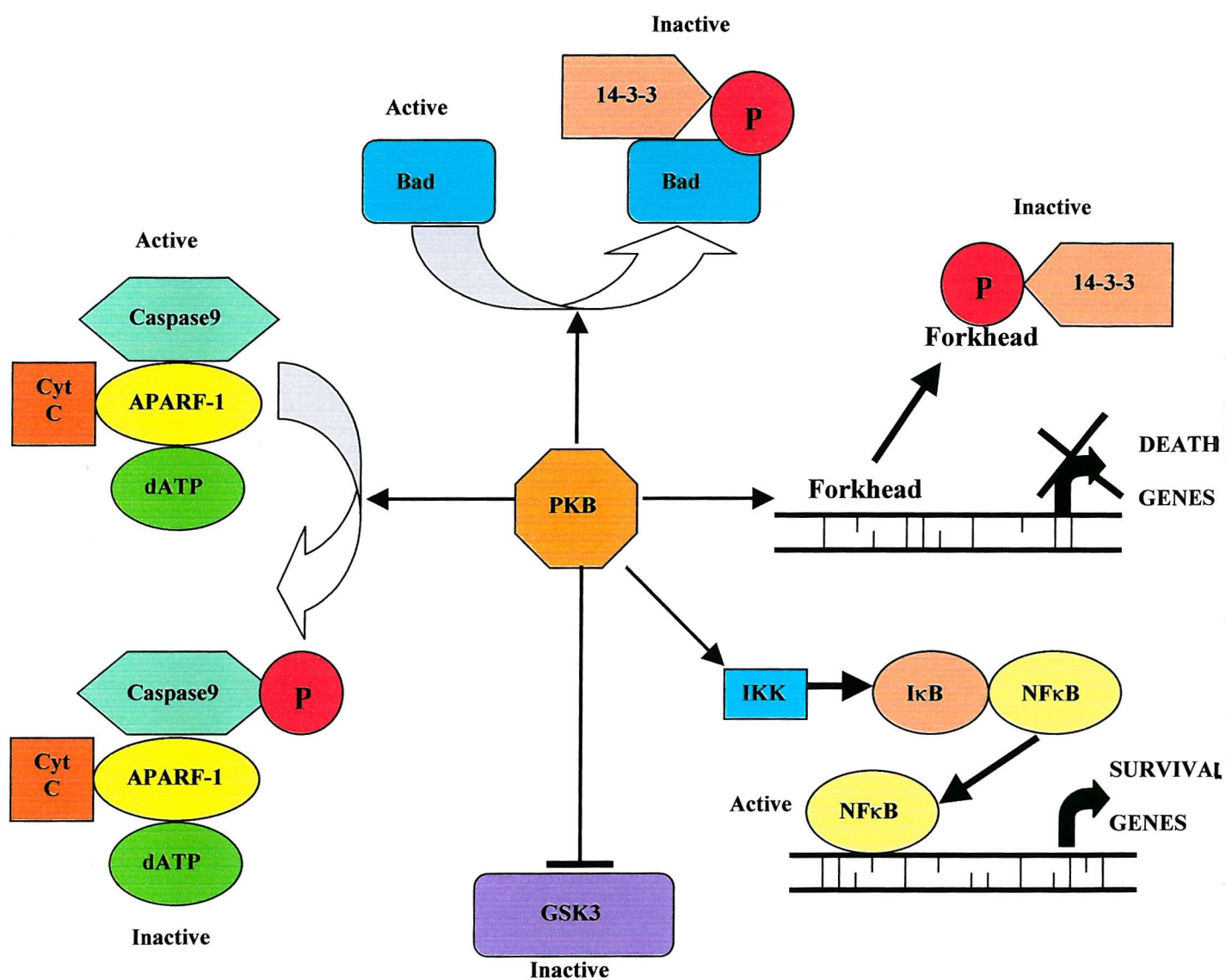
**Figure 1.6** Putative downstream substrates of PKB involved with cell survival/apoptosis.

## 1.10 PKB and cancer

PKB was first identified as the cellular homologue of the retroviral oncogene v-akt and so was implicated as a potential proto-oncogene in mammalian cells. In has been reported that all tumour cell lines in which PTEN is inactive exhibit elevated PKB activity (Cantley et al., 1999). Not only this, but PKB has been found to be over-expressed in a number of different human cancers. PKBα amplification was reported in primary gastric adenocarcinoma (Staal, 1987). PKBβ has been found to be amplified and over-expressed in pancreatic (Cheng et al., 1996; Miwa et al., 1996; Ruggeri et al., 1998), ovarian and breast cancers (Bellacosa et al., 1995; Thompson et al., 1996). PKBγ is up regulated in estrogen receptor-deficient breast cancer and in androgen-independent prostate cell lines (Nakatani et al., 1999). This evidence, together with the well documented antiapoptotic effects of PKB described earlier suggest a role for inappropriate PKB activation in the genesis of cancer. The activation of PKB may be oncogenic by preventing normal programmed cell death, thereby enabling accumulation of more oncogenic mutations in the cells.

## 1.11 PKB and Diabetes

Type 2 (Non-Insulin dependant) Diabetes is characterised by: (1) resistance to insulin action on glucose uptake in mainly skeletal muscle and adipocytes; (2) impaired insulin action to inhibit hepatic glucose production; and (3) dysregulated insulin secretion (DeFonzo, 1997). Since PKB mediates many of the metabolic actions of insulin it is not hard to imagine that PKB activity has profound implications for diabetes. Recently it has been reported that insulin-stimulated PKB activity is reduced in the skeletal muscle and adipocytes of Type 2 diabetics (Krook et al., 1998; Rondinone et al., 1999), and diabetes-prone mice exhibit

elevated activity of GSK3 (Eldar-Finkelmann et al., 1999). However, so far no mutations have been identified in any of the PKB isoforms in patients suffering type 2 diabetes (Hansen et al., 1999).

## 1.12 PKB and neurodegenerative disease.

A wide variety of neurodegenerative diseases are characterised by the loss of specific neuronal cells (Isacson, 1993; Heintz, 1993). Such diseases include Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), retinitis pigmentosa, spinal muscular atrophy, and various forms of cerebellar degeneration. In most cases apoptosis appears to be the mechanism responsible for the neuronal cell death. Oxidative stress, calcium toxicity, mitochondrial defects, excitory toxicity, and deficiency of survival (growth) factors have all been proposed as contributors to the pathogenesis of these diseases (Choi, 1992; Ziv et al., 1994). Neurons, as for most cells, require the presence of growth factors in order to survive. It is the Nerve Growth Factor (NGF) which is fundamental in the survival of nerve cells (Levi-Montalcini, 1987) and it was discovered that the PI 3-kinase/PKB pathway mediates the survival signal of NGF (Philpott et al., 1997). In addition, PKB has been found to protect neurons against apoptosis by acting downstream of IGF-1 (Dudek et al., 1997). Indeed, as discussed previously, activated PKB supports the survival of neurones in the absence of growth factors, whereas dominant negative PKB inhibits neuronal cell survival, even in the presence of survival factors (Philpott et al., 1997; Dudek et al., 1997). Therefore it is conceivable that any biochemical or genetic event resulting in the inactivation of PKB may lead to the onset of apoptosis and as a consequence, neurodegenerative disease, in neuronal cells.

## 1.13 Summary and aims of this project

PKB is one of the most important mediators of cell survival signalling pathways to have been discovered in recent years. Although a great deal of research has already been carried out concerning the activation and downstream effects of PKB there are still many questions that remain unanswered. For instance, what is the significance in the presence of three isoforms of PKB? It is not yet known if these separate isoforms perform individual tasks, or if they are present simply to take over in the event of a decrease in the levels of the other isoforms. Although the mechanism of PKB activation has almost been resolved, we are still in the dark concerning several upstream activators such as the identity of PDK2 and the mechanism of PI3-kinase-independent activation of PKB. Equally important is the need to identify the exact target molecules of PKB and to elucidate its many functions in the cell including metabolism and blocking apoptosis. The ability of PKB to block apoptosis is perhaps the most important discovery yet and opens a wide range of possibilities concerning the involvement of PKB in cell cycle progression and tumorigenesis.

Cancer, diabetes and neurodegenerative diseases are some of the most well known diseases and are responsible for millions of deaths each year. Disorders in the cell signalling pathways associated with these diseases suggest that PKB may indeed be an important therapeutic target. Specific inhibitors of PKB could be used for cancer therapy, whereas specific activators might be used to treat diabetes and neurodegenerative diseases that result from inappropriate cell death.

The main aim of this project is to use molecular biological techniques to investigate the role of the third isoform of PKB, PKBγ, in cell survival signalling and to try and identify further downstream targets of PKBγ. Hopefully this information will add to the ever-increasing knowledge concerning PKB and eventually aid in the search for specific therapeutic cures for diseases such as cancer, diabetes and Alzheimer's disease.

# CHAPTER 2: Materials and Methods.

## 2.1 Materials.

### 2.1.1 Chemicals.

Chemicals were from Sigma Chemical Company, Poole UK unless otherwise stated. Nitrocellulose (0.45μm) was from Sartorius, Epsom, Surrey, UK. [$\Upsilon^{32}$P]ATP, Hyperfilm-βmax and ECL solutions were from Amersham International PLC, Buckinghamshire, England. All molecular biology reagents, restriction enzymes and Polymerase enzymes were purchased from Promega, Southampton, England.

### 2.1.2 Water.

Deionised, autoclaved water was used for all techniques unless otherwise stated. For RNA work water was further treated with Diethyl pyrocarbonate (DEPC). 0.01% (v/v) DEPC was added to the water then the solution was allowed to stand overnight prior to autoclaving. For molecular biology nuclease-free water purchased from Promega was used.

### 2.1.3 Sterilisation.

Heat stable materials were sterilised by autoclaving at 15 psi for 20mins. Heat labile solutions were sterilised by filtration through a 0.22μm filter (Millipore).

## 2.2 Molecular biological techniques.

### 2.2.1 Bacterial culture media.

### Antibiotics.

Antibiotics were added to culture media to select for bacteria containing plasmids encoding antibiotic resistance. Ampicillin was made up as a stock solution of 100mg/ml in AnalaR water, filter sterilised and stored in aliquots at -20°C. Kanamycin was made up as a stock solution of 50mg/ml. Routinely ampicillin was used at a working concentration of 100µg/ml and Kanamycin was used at 30µg/ml.

### Luria-Bertani (LB) media.

LB media is an all-purpose bacterial culture medium. 500ml of medium was prepared by dissolving 2.5g NaCl, 5g Bacto-tryptone and 2.5g Bacto-yeast extract into deionised water then autoclaved.

### LB plates with ampicillin.

For solid medium (LB Agar) 1.5% (w/v) agar was added prior to autoclaving. The solution was allowed to cool (50°C) and ampicillin (50mg/ml) was added to a final concentration of 100µg/ml. 30ml of media was then poured aseptically onto 85mm petri dishes and then the agar was allowed to harden. Plates were stored at 4°C for up to a month.

## LB plates with ampicillin/IPTG/X-gal.

As above except 100μl of 100mM IPTG and 20μl of 50mg/ml X-Gal were spread over the surface of a LB/ampicillin plate and allowed to absorb for 30mins at 37°C prior to use.

## 2.2.2 Isolation of total RNA from tissue.

Total RNA was isolated from rat brain tissue using the RNAzol reagent (Biogenesis) and following the manufacturer's instructions. Briefly, the brain from one sacrificed rat was homogenised (Ultra turrex) in the presence of 10mls RNAzol. The homogenate was transfered to a sterile 50ml polypropylene tube and 1ml choroform (RNA grade) was added. The tube was then covered and shaken vigorously for 20secs, then incubated on ice for 5mins. The extract was centrifuged (15mins, 10 000rpm at 4°C), the upper colourless (aqueous) layer was removed and placed into a clean 50ml tube. To precipitate the RNA, 10mls of isopropanol was added and the tube was shaken then incubated on ice for 20mins. After incubation, the sample was aliquoted into 1.9ml Eppendorf tubes and centrifuged (15mins, 14 000rpm at 4°C) to collect the RNA as a pellet. The supernatant was discarded and the pellets were then washed with 70% ethanol (in DEPC-treated $H_2O$). The samples were re-centrifuged (8mins, 14 000rpm, at 4°C) and the supernatant removed to allow the RNA pellets to dry (5-10mins). Finally the total RNA was redissolved in 500μl of DEPC-treated $H_2O$ and assayed for concentration and purity by gel electrophoresis.

## 2.2.3 Generation of single-stranded cDNA library from total RNA.

The total RNA ($4\mu g/\mu l$) isolated from rat brain tissue was heated to 70°C for 5mins to remove all secondary structure then stored on ice immediately. For the generation of first-strand cDNA, the following components were added to $4\mu g$ of total RNA: $5\mu l$ 5x first strand buffer, $5\mu l$ 10mM dNTP mix, $1\mu l$ oligo (dT)$_{15}$ primer ($500\mu g/ml$), $3\mu l$ Random primers ($500\mu g/ml$) and $1.5\mu l$ RNAsin (20u). The reaction was then made up to $23\mu l$ with nuclease-free H$_2$O. $1\mu L$ (200u) of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) was then added and the reaction incubated at 37°C for 90mins. Following incubation, the reaction was heated to 70°C for 15mins to terminate the M-MLV RT, then placed on ice. To degrade the template RNA $1\mu l$ (2u) of RNase H was added and the reaction incubated at 37°C for 20mins. Finally the single stranded cDNA library was stored at -70°C until required for PCR.

## 2.2.4 The Polymerase Chain Reaction (PCR).

PCR primers were obtained from Gibco and re-hydrated in nuclease-free H$_2$O, then stored at –20°C.

PCR reactions were set up using Pfu polymerase (Promega) and the single-stranded rat cDNA library as template DNA, unless otherwise stated. In a sterile 0.5ml PCR tube the following components were mixed: $5\mu l$ 10x Pfu buffer, $1\mu l$ 10mM dNTP mix, $1\mu l$ $50\mu M$ forward primer, $1\mu l$ $50\mu M$ reverse primer, $2\mu l$ template DNA and made up to $39.5\mu l$ with nuclease-free H$_2$O. $0.5\mu l$ (1.25u) of Pfu polymerase enzyme was added and the reaction mixed by pipetting then placed into a programmed thermal heating block (Hybaid). The reactions were

cycled once between 95°C (2mins), 60°C (30s) and 72°C (4mins); 35 times between 95°C (30s), 60°C (30s) and 72°C (4mins); and finally, once between 95°C (30s), 60°C (30s) and 72°C (5mins).

Following PCR the reactions were analysed by agarose gel electrophoresis. A 10μl sample was loaded onto a 1% agarose gel and electrophoresis performed at 100V for 1hr with a 1Kb DNA ladder (Gibco BRL) as a marker.

## 2.2.5 Site-directed mutagenesis of rat PKBγ by polymerase chain reaction.

Site-directed mutagenesis was carried out according to the method described by Higuchi (1990). Mutagenesis was achieved in three PCR reactions. Reaction 1 involved the gene-specific forward primer and a mutating reverse primer which mismatched to the sequence to be changed. Reaction 2 involved a mutating forward primer and a gene-specific reverse primer. Each of these reactions was carried out as in section **2.2.4** using rat brain cDNA as template and the same PCR cycling conditions.

The two PCR products were gel-purified and combined in equal molar ratios functioning as template DNA in the third PCR reaction. This was performed using the gene-specific forward and reverse primers to produce the full length PKBγ mutant.

**Table 2.1 Primers used in PCR reactions.**

| FORWARD PRIMERS | FORWARD PRIMER SEQUENCE (5'-3') | REVERSE PRIMERS | REVERSE PRIMER SEQUENCE (5'-3') |
|---|---|---|---|
| rat wtPKBγ forward primer | atgagcgatgttaccatcgttaaag | rat wtPKBγ reverse primer | ggatcgattatagtggacacttttcaggtg gtgttattg |
| rat HA forward primer | ggactagtgccaccatgtacccatacgatgt gccagattacgccagcgatgttaccatcgtt aaag | rat HA reverse primer 1 | tagtggacacttttcaggtggtgttattg |
| rat myrPKBγ forward primer | ggctgtgtctgcagctcaaaccctgaagatg acagcgatgttaccatcgttaaag | rat HA reverse primer 2 | tggcacatcgtatgggtatagtggacactt ttcagg |
| rat Spe/myr forward primer | ggactagtgccaccatgggctgtgtctgca gctca | rat HA reverse primer 3 | ggatcgattaggcgtaatctggcacatcg tatgggta |
| rat kpn1 forward primer | ggaggtaccatgagcgatgttaccatcgtta aag | Xba1 HA reverse primer | cgatctagattaggcgtaatctggcacatc gtatgggta |
| T305D forward primer | gcagctaccatgaaggacttctgtggtacac ca | T305D reverse primer | tggtgtaccacagaagtccttcatggtag ctgc |
| mouse wtPKBγ forward primer | ggaggtaccatgagcgatgttaccattgtga aa | mouse wtPKBγ reverse primer | ttcccgtccgcttgcagagtaggagaact gagg |
| mouse 1/2myrPKBγ-HA forward primer | tcaaaccctgaagatgacatgagcgatgtta ccattgtg | mouse wtPKBγ-1/2HA reverse primer | tggcacatcgtatgggtattcccgtccgct tgcaga |
| mouse Kpn1 myr forward primer | ggaggtaccatgggctgtgtctgcagctca aaccctgaagatgac | | |
| mouse wtPKBα forward primer | acgggtaccatgaacgacgtagccattgtg aag | mouse wtPKBα reverse primer | ggctgtgccactggctgagtaggagaac tgggg |
| mouse 1/2myrPKBα-HA forward primer | tcaaaccctgaagatgacatgaacgacgta gccattgtg | mouse wtPKBα-1/2HA reverse primer | tggcacatcgtatgggtaggctgtgccac tggctga |
| T305A forward primer | gcagctaccatgaaggctttctgtggtacac ca | T305A reverse primer | tggtgtaccacagaaagccttcatggtag ctgc |
| K177A forward primer | aaatactacgctatggctattctgaagaaag aa | K177A reverse primer | ttctttcttcagaatagccatagcgtagta |

## 2.2.6 Agarose gel electrophoresis.

Agarose gel electrophoresis was used for separation and characterisation of DNA, as well as a

method for purifying specific DNA fragments by the Qiaquick gel purification kit (Qiagen).

1% and 1.5% (w/v) agarose gels containing 0.5µg/ml ethidium bromide in Tris-Acetate-EDTA (TAE) buffer were set directly into electrophoresis trays with plastic combs inserted to form loading wells (Bio-rad). Samples were loaded with 6x agarose gel loading dye and electrophoresis performed in TAE buffer for 1hr at 100V. One well containing 1µg of a 1kb DNA marker (Gibco) was used to assess the size of the sample DNA. DNA was visualised by UV light.

## 2.2.7 Gel purification of DNA fragments.

DNA fragments were purified from 1% agarose gels using the Qiaquick gel purification kit (Qiagen). The DNA band of interest was excised from the agarose gel and purified according to the manufacturer guidelines.

## 2.2.8 DNA Ligation.

### 2.2.8.1 Ligation of PCR products into the pGEM-T Easy vector.

**A-tailing reaction.**

PCR products were purified from a 1% agarose gel using the Qiaquick gel purification kit (Qiagen) to remove the PCR reaction components as well as any non-specific PCR products such as primer dimers. Since Pfu Polymerase produces blunt ended PCR products, the DNA must be tailed with a single A overhang before ligation into the pGEM-T Easy vector by T-A cloning. To achieve this the gel purified PCR product has to be incubated in the presence of Taq polymerase which catalyses the addition of a singe A to each end of the DNA. The following A-tailing reaction was set up: 1µl 10x Taq buffer, 1µl 25mM MgCl$_2$, 2µl 1mM

dATP, 2μl gel-purified PCR product 1μl Taq Polymerase and made up to 10μl with nuclease-free H₂O. This reaction was incubated for 30mins at 70°C, then stored at -20°C.

## Ligation reactions.

For ligation of a PCR product into the pGEM-T Easy vector the ratio of A-tailed insert was titrated against vector (1:1, 2:1, and 5:1). For a 1:1 ligation reaction the following components were added: 1μl 10x ligase buffer, 1μl pGEM-T Easy vector, 1μl A-tailed PCR product, 1μl T4 DNA ligase enzyme and made up to 10μl with nuclease-free H₂O. The reactions were incubated overnight at 4°C.

## 2.2.8.2 Ligation of PCR products into the pcDNA3.1 expression vector.

## Dephosphorylation of vector DNA.

The terminal phosphate at the 5'end of vector DNA cut with restriction enzymes was removed by the addition of alkaline phosphatase (AP) from calf intestine to minimise ligation of vector DNA to itself. After restriction digest of the vector DNA, the total volume of the reaction was made up to 40μl with AP buffer and water. Four units of AP were added and the reaction incubated at 37°C for 30mins. The AP was inactivated by the addition of 1% SDS, 1xSTE in a final volume of 75μl and incubated at 65°C for 10mins. The DNA was phenol:chloroform extracted and ethanol precipitated.

## Ligation reactions.

The ligation reactions were set up as described above (2.2.5.1).

## 2.2.9 Transformation of Ca$^{2+}$ competent *E.coli.*

On ice, 2μl of ligation reaction was added to a pre-chilled 1.5ml eppendorf tube and 50μl of Ca$^{2+}$ competent JM109 *E.coli* cells(1x 10$^8$ cfu/μg DNA) were added. The tube was mixed by flicking then incubated in ice for 45mins. This allows the DNA to precipitate onto the surface of the bacteria. The cells were heat shocked at 42°C for 50secs then returned immediately onto ice for a further 4mins to allow them to recover. 950μl of LB media (w/o antibiotics) was added to the cells and incubated for 1.5hrs with shaking. Finally the cells were pelleted by centrifugation (15secs, 14 000rpm), 800μl of supernatant media was removed and the cells resuspended into the remaining 200μl. 100μl of this cell suspension was spread onto duplicate LB plates containing the appropriate antibiotic for selection as well as IPTG and X-Gal (if required for blue/white screening). Plates were then incubated overnight at 37°C inverted.

## 2.2.10 Plasmid preparation.

Unless stated all plasmids were maintained in cultures of *E.coli* strain JM109 frozen at -70°C in 25% glycerol in LB media. Cultures from frozen stocks were streaked out onto LB plates containing the appropriate antibiotic and cultured overnight at 37°C. A single colony was picked from this culture and used to innoculate 10ml of LB media containing antibiotic (small scale prep) or 500ml of media (large scale prep). The cultures were then allowed to grow up overnight at 37°C with continuous agitation.

For small scale plasmid preparation (mini-preps) the Wizard-SV kit (Promega) was used according to the manufacturers instructions. For large scale preparations (maxi-preps) the Qiagen maxi-prep kit was used. DNA was resuspended in 10mM Tris-HCl pH7.6 to $1\mu g/\mu l$.

## 2.2.11 Phenol chloroform extraction and ethanol precipitation.

DNA was extracted from aqueous solutions and restriction digest mixtures by addition of an equal volume of phenol:chloroform (1:1), through mixing and centrifugation at 14 000g for 5mins. The aqueous top layer was removed and transferred to a fresh tube and a 1/10 volume of 3M Na acetate added. The DNA was precipitated by adding 2.5 volumes of ethanol and incubated at $-20^{\circ}C$ for 10mins. DNA was recovered by centrifugation at 14 000g for 15mins, washed once in 70% (v/v) ethanol and allowed to air-dry for 5mins prior to resuspension in the appropriate volume of 10mM Tris-HCl pH7.6 buffer.

## 2.2.12 Quantitative analysis of DNA.

Plasmid preps were dissolved into nuclease-free water and DNA concentration was obtained by reading optical density at 260nm using quartz capillary tubes and the Gene Quant (Pharmacia Biotech) DNA/RNA calculator.

## 2.2.13 Restriction digests of DNA.

Diagnostic digestions were used to test the integrity and purity of the plasmid. $1\mu g$ of plasmid was made up to $17.3\mu l$ with nuclease-free water and $2\mu l$ of the appropriate 10x

buffer, 0.2μl acetylated BSA and 0.5μl of restriction enzyme were added. The reaction was incubated for 1hr at 37oC then stopped by the addition of 4μl 6x loading dye. The digest was then loaded onto a 1% agarose gel and separated according to size by electrophoresis at 100V for 1hr.

## 2.3 Cell culture.

### 2.3.1 Materials for cell culture.

Madin-Darby canine epithelial kidney (MDCK) cells were a gift from Dr. M. East (Southampton). Sterile multi-well plates, centrifuge tubes and flasks were from Iwaki. Sterile cell scrapers were from Costar, graduated sterile 3ml pipettes from Sterilin, and cryo-tubes were from Nunc. Sterile filters were from Sartorius.

Dulbecco's modified Eagles medium (DMEM, $1g.l^{-1}$ glucose without sodium pyruvate), glutamine and trypsin-EDTA solution (0.05% (w/v) trypsin, 0.02% (w/v) EDTA in Puck's saline A) were from GIBCO-BRL. FBS, BSA (Tissue culture grade), dimethylsulphoxide (DMSO) and PBS (137mM NaCl, 8.1mM $Na_2HPO_4$, 1.47mM $KH2PO_4$, 2.68mM KCL, pH 7.4) were from Sigma.

### 2.3.2 Conditions for cell culture.

Sterile conditions were used at all times for cell culture work. All procedures were carried out within a cell culture cabinet (Gelman, class II) and cell culture media and solutions were sterilzed by filtering (0.22μm) before adding to cells. All non-sterile equipment or solutions

were autoclaved (121°C, 15lbs.in$^{-2}$, 20min). Cells were grown in a humidified incubator at 37°C in the presence of 5% $CO_2$ (New Brunswick Scientific).

MDCK cells were grown in 15mls DMEM medium containing 10% (v/v) FBS, 2mM glutamine only. When cell cultures reached 90%-100% confluency the cells were either sub-cultured, prepared for liquid $N_2$ storage or used experimentally. Growth medium was changed three times a week.

### 2.3.3 Cell sub-culture.

For all cells, the growth medium from one 75cm$^2$ flask was removed and the cells washed twice with PBS. 2-3ml of trypsin-EDTA solution was added and the flask was incubated at 37°C for 5 mins to release the cells. Following incubation 10mls of pre-heated growth medium was added to neutralise the trypsin, then the cells were collected by centrifugation (5mins, 1500rpm, Centurion 4000series). Cells were then resuspended in 10mls of growth media and either counted then seeded into multi-well plates for experiments or into 75cm$^2$ flasks for further passage.

### 2.3.4 Cell storage and resusitation.

One 75cm$^2$ flask grown to confluence was trypsinised as described in section **2.3.3**. The cell suspension was centrifuged (5mins, 1500rpm, Centurion 4000series) to collect a pellet. The supernatant was discarded and the cells resuspended in 10mls cryo-protectant medium (10% (v/v) DMSO in growth medium). 1ml of this suspension was placed into cryotubes and slowly frozen to -70°C, then transferred to liquid $N_2$ for long term storage.

To resuscitate the cells, a cryo-tube was allowed to thaw at 37°C and the cells were collected by centrifugation (5mins, 1500rpm). The supernatant was discarded and the pellet re-suspended in 1ml of pre-heated growth medium. This suspension was then added to a 75cm$^2$ flask containing growth medium for culture.

### 2.3.5 Transfections of MDCK cells with mammaliam expression vectors.

### 2.3.5.1 DNA for transfections.

The plasmid DNA used for transfection was made from a large-scale preparation and suspended in Tris buffer (10mM Tris/HCl pH 7.6) to 1µg/µl under sterile conditions.

### 2.3.5.2 Transient DNA transfection using the Lipofectamine$^{TM}$ Transfection reagent from Gibco.

Transfections were carried out in accordance with the manufacturer guidelines. 2 x10$^5$ MDCK cells were plated out onto 35mm 6 well tissue culture grade plates in 2ml of growth media, then incubated overnight. On day 2, a DNA/lipid complex was prepared by adding 2µg plasmid DNA to 10µl Lipofectamine in the presence of serum-free DMEM. This complex was incubated for 30mins at room temperature, in the meantime the cells were washed twice with 1ml serum-free medium. Following incubation, serum-free DMEM was added to the DNA-lipid solution and this was added to the cells dropwise. The media on the plate was then swirled gently to allow even distribution of the complexes. The transfections were incubated at 37°C/ 5% CO$_2$ for 6hrs after which time the media on the cells was removed and replaced with fresh media to prevent cyto-toxicity from the Lipofectamine

reagent. In addition to transfection of experimental plasmids, control transfections were routinely carried out using empty vector only. Transiently transfected cells were used for experiments 48hrs post-transfection unless otherwise stated.

## 2.3.5.3 Transient DNA transfection using the Effectene Transfection reagent from QIAGEN.

Transfections were carried out in accordance with the manufacturer guidelines. $2 \times 10^5$ MDCK cells were plated out onto 35mm 6 well tissue culture grade plates in 2ml of growth media, then incubated overnight. The following day 0.4µg of plasmid DNA was added to 100µl of buffer EC then the DNA was condensed by adding 3.2µl Enhancer solution, mixed by vortexing for 1sec then incubated for 5mins at room temperature. To form the DNA/lipid complex, 10µl of Effectene reagent was added and the solution mixed by pipetting up and down 5 times. This DNA/lipid mixture was incubated for 10mins at room temperature. Meanwhile, the media was removed and the cells washed twice with PBS. Then 600µl of fresh complete growth media was added to each well. 1600µl of complete growth media was added to the DNA/lipid complex and mixed by pipetting up and down twice, this mixture was then added to the media on the cells. The plate was gently swirled to evenly distribute the complexes then returned to the incubator for 48 hours. Note that there was no need to change the media during the transfection. As before, empty vector only transfections were routinely carried out as a control.

### 2.3.5.4 Stable DNA transfection.

MDCK cells were transfected as for transient transfections. 48hrs post-transfection the cells were replated onto a 10cm tissue culture grade dish and selected by supplementing the growth media with 500µg/ml G418 (Geneticin, Gibco). After selection in G418 for 14 days, individual resistant colonies were isolated using cloning rings (Sigma) and expanded for analysis. Expression of recombinant proteins was assessed by western blotting of cell lysates. Clones expressing the protein of interest were grown in growth media containing G418.

### 2.3.6 Agonist stimulation of cells.

To render cells quiescent the growth media was removed and the cells washed 3-5 times with serum-free DMEM. This same media was then added to the cells and incubated for 20-24hrs. Cells were then incubated with or without 25% FBS in DMEM for 10mins at 37°C. Following stimulation, cells were removed to ice, washed in ice-cold PBS (3x), then harvested as described below.

### 2.3.7 Cell extraction: SDS-Detergent lysates.

SDS-detergent lysates were used only for Western blotting. Whole cell lysates were prepared by washing the cells (3x) in ice-cold PBS then adding lysis buffer (62.5mM Tris/HCl (pH 7.4), 0.1% (v/v) SDS). Extraction was aided by using cell scrapers and the extract was homogenised by passig through a 1ml syringe with a fine gauge needle (10x). An aliquot of this extract was removed for protein concentration determination and the remainder prepared for SDS-PAGE then stored at -70°C.

## 2.3.8 Cell Lysates for GSK3 kinase assay

For GSK3 kinase assay, crude cell extracts were prepared using a freshly made cell lysis buffer containing a cocktail of protease/protein kinase/protein phosphatase inhibitors to prevent protein degradation and inhibit certain cellular proteins (i.e. other kinases or phosphatases). Cells were first washed 3x with ice-cold PBS then extracted by scraping into the appropriate volume of cell lysis buffer (50mM Tris (pH 7.5), 150mM sodium chloride, 0.25% (v/v) sodium deoxycholate, 1% (v/v) NP40, 50mM $\beta$-Glycerophosphate (pH 7.5), 1mM Benzamidine, 1mM DTT, 1mM Sodium orthovanadate, 1mM PMSF, 2mM EDTA, 10mM Sodium Fluoride, 1.5mM EGTA, 1$\mu$g/ml Pepstatin, 1$\mu$g/ml Antipain, 1$\mu$g/ml Leupeptin, 20$\mu$M PKC inhibitor peptide (Calbiochem), 2$\mu$M PKA inhibitor peptide (Sigma), 20$\mu$M R24571 (Calmodulin dependent protein kinase II inhibitor compound – Calbiochem), 1$\mu$M Microcystin (Calbiochem). Lysates were allowed to mix by gentle rotation at 4$^{\circ}$C for 20mins. Cell lysates were then cleared by centrifugation at 14, 000g for 10mins at 4$^{\circ}$C. Once cleared the cell lysate could be used for GSK3 kinase assay, protein concentration determination and western blotting.

## 2.3.9 GSK-3 kinase assay.

Glycogen synthase kinase-3 (GSK-3) kinase activity was measured from serum-starved cells and cells stimulated with serum (25% FBS). Due to the use of protein kinase/phosphatase inhibitors in the extraction buffer, crude cell lysates could be assayed for GSK-3 activity without the requirement for further processing (e.g. immunoprecipitation of the GSK-3

isoforms). The method described below was developed by Neil Jones whilst working in our lab.

10μl of assay dilution buffer ADB (20mM MOPS (pH 7.2), 25mM β-Glycerophosphate, 5mM EGTA, 1mM Sodium orthovanadate, 1mM DTT) was mixed with 10μl of inhibitor cocktail solution (20μM PKC inhibitor peptide, 2μM PKA inhibitor peptide, 20μM R24571, all in ADB) and placed on ice. To this was added 10μl of either 1mM GSK-3 Phospho-peptide or 1mM control peptide. 10μl of crude cell lysate was then added to each reaction on ice.

The kinase reaction was commenced by the addition of 10μl of [γ-$^{32}$P] ATP stock solution mixture (75mM magnesium chloride, 500μM ATP, 1μCi [γ-32P]ATP (~3000 Ci/mmol) all in ADB). The kinase reactions were allowed to proceed for 10mins at 30°C, then stopped by pipetting duplicate 20μl aliquots onto two 4cm x 1cm strips of P81 phosphocellulose paper (Whatman). After allowing the radiolabelled peptide to bind to the P81 paper for 1min, the strips were immersed in 1% (v/v) phosphoric acid solution and washed for 5mins. The strips were washed twice more in fresh 1% (v/v) phosphoric acid solution before being washed once in acetone (5mins).

The strips were allowed to air-dry before being folded and placed in a scintillation vial containing 3ml Liquid Scintillation cocktail (Optiphase, Hi-safe). Counting on a Beckman Liquid Scintillation Counter for 2mins then quantitated the radioactivity bound to the paper. The amount of radioactivity incorporated into the peptide was then calculated and corrected for protein concentrations. The final result was expressed as a percentage of kinase activity.

In all GSK-3 kinase activity experiments suitable blanks were used and these were subtracted in the final calculations. For each cell lysate examined, reactions were carried out using both the specific phospho-peptide and the non-phosphorylated control peptide. The value obtained for the control peptide was subtracted from the value obtained for the phospho-peptide to give the specific GSK-3 activity. Lysis buffer only controls were carried out with both peptides and the values for these also used to account for non-specific binding of $[\gamma\text{-}^{32}P]$ ATP and its breakdown products to the P81 phosphocellulose paper.

## 2.4 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

### 2.4.1 Materials and solutions for SDS-PAGE.

SDS-PAGE was performed according to the method of Laemmli *et al* (1970). The mini-gel equipment was from Bio-Rad (5cm x 8cm in size) and band-shift gels with longer resolving gel were from Hoeffer (8cm x 8cm). A concentrated stock acrylamide solution (30% (w/v) Acrylamide/ 0.8% (w/v) *N,N'*-methylene-bis-acrylamide; National Diagnostics Ltd, Mannville, New Jersey, USA) was used to prepare mini-gels. A low bis-acrylamide stock (30% (w/v) Acrylamide/ 0.36% N,N'-methylene-bis-acrylamide) was required for the band-shift gels. A two times concentrated stock of resolving gel buffer (0.75M Tris/HCl (pH 8.8), 0.2% (w/v) SDS) and stacking gel buffer (0.25M Tris/HCl (pH 6.8), 0.25% (w/v) SDS) was prepared in advance. The acrylamide solution was diluted according to the percentage gel required. The acrylamide concentration of the stacking gel was 4% for all gels.

## 2.4.2 Gel preparation.

Gel plates were set up according to the manufacturers guidelines and the apparatus checked for leaks using water. Polymerization of acrylamide and bis-acrylamide was catalyzed by the addition of 0.2% (v/v) TEMED and 0.1% (w/v) ammonium persulphate. In order to ensure that the top of the resolving gel was level, it was overlaid with water directly after pouring. This was removed prior to pouring the stacking gel. Gels were routinely used immediately after they had set, however gels could also be stored overnight at $4^{o}C$ without adverse affect.

Before loading the samples, the gels were positioned in an electrophoresis tank filled with running buffer (25mM Tris, 192mM glycine, 0.1 (w/v) SDS).

## 2.4.3 Sample preparation and electrophoresis.

Protein samples were prepared by adding one quarter volume of 5x concentrated Laemmli sample buffer then boiling (5mins, $100^{o}C$). Samples were then loaded onto the gel and electrophoresis performed at a constant current of 30mA until the dye front (Pyronin Y) just ran off the bottom of the gel. Following electrophoresis, gels were then removed from the apparatus and either stained (0.25% (w/v) Coomassie Brilliant Blue, 50% (w/v) TCA) and destained (45% (v/v) methanol, 10% (v/v) acetic acid) or prepared for western blotting (see section **2.5.2**).

## 2.5 Immuno-detection procedures.

### 2.5.1 Commercial antibodies.

The polyclonal rabbit anti-Haemaggluttinin (HA) Tag antibody was obtained from Upstate Biotechnology Incorporated (UBI).

### 2.5.2 Western blotting procedure.

Western (Immuno) blotting was performed according to the method described by Towbin *et al.* (1979). Nitrocellulose membrane ($0.45\mu m$) was used and required pre-soaking in transfer buffer (25mM Tris, 192mM glycine, 20% methanol, pH 8.3) prior to use.

Following SDS-PAGE, proteins were transferred from the gel to membrane by means of a 'transfer sandwich'. Six pieces of 3MM Whatman paper were preequilibrated along with the gel and membrane in transfer buffer. The gel and membrane were then placed together between the pre-soaked 3MM paper (3 pieces on each side). Proteins were then transferred using a Hoeffer Semi-Phor™ transfer apparatus (72mA, 90mins). Following transfer membranes were blocked in the appropriate blocking buffer. The transferred gel was stained (0.25% (w/v) Coomassie Brilliant Blue, 50% (w/v) TCA) and destained (45% (v/v) methanol, 10% (v/v) acetic acid) in order to determine whether transfer had been successful.

All antibody incubations were performed by constant rocking of the membrane in each solution as described below. In all cases the secondary antibody was visualised using an 'enhanced chemilluminescence' technique (ECL, Amersham). The membrane was incubated

in the ECL reagents (30secs, room temp), then placed onto a glass plate and wrapped in cling-film. The membrane was then placed into an autoradiography cassette (Genetic Research Instruments, Ltd) fitted with an intensifying screen (Dupont cronex lighting plus), and the light signal emitted was detected on photographic film (Hyperfilm, Amersham). The membranes were stained with amido black (45% (v/v) methanol, 10% (v/v) acetic acid, 0.1% (w/v) Napthol blue black; 3mins, 30ml). The protein bands were then visualised by destaining (45% (v/v) methanol, 10% (v/v) acetic acid).

### 2.5.3 Immunoblotting protocol for anti-HA antibody.

Membranes were blocked for 20 mins at room temp in 'blocking buffer' (TBS-T containing 3% (w/v) non-fat dried milk). Membranes were then incubated overnight at $4^{\circ}C$ in blocking buffer containing the anti-HA primary antibody (1:2000 dilution). Following primary antibody incubation, the membrane was washed in AnalaR $H_2O$ (2x 1min) then incubated for 90 mins at room temp with blocking buffer containing a goat anti-rabbit antibody conjugated to horse-radish peroxidase (GAR-HRP; 1:3000 dilution). Further washing of membranes with TBS (5min) and AnalaR $H_2O$ (5x 5min) was then performed.

### 2.6 Apoptosis assays.

### 2.6.1 Serum-starving to induce apoptosis.

$1x10^6$ cells were plated out onto 10cm tissue culture grade plates. The next day the media was removed and the cells washed 3-5 times with 3mls DMEM containing glutamine only. Finally 6mls of DMEM containing glutamine only was added and the cells returned to the incubator

for the indicated times. Cells were harvested for DNA laddering by scraping into the media in which they had been incubated so that floating apoptotic bodies were combined with attached cells.

## 2.6.2 DNA fragmentation assessed by agarose gel electrophoresis (DNA laddering).

Following incubation of cells under appropriate conditions, samples containing $1 \times 10^6$ cells were harvested, transferred to 1.5ml sterile, Eppendorf tubes and resuspended in no more than 10μl of supernatant. 20μl of TE-SLS buffer (50mM Tris-HCl pH 8.0, 10mM EDTA, 0.5% (v/v) sodium lauroyl sarcosine (SLS, Sigma), 0.5mg/ml Proteinase K) was added and the samples incubated at 50°C for 1hr. 10μl of 0.5mg/ml RNase A (Sigma) was added and the samples incubated for a further 1hr at 50°C. The temperature of the samples was then increased to 70°C for addition of 10μl of loading buffer (10mM EDTA, 1% (w/v) low melting point agarose, 0.25% (w/v) bromophenol blue, 40% (w/v) sucrose). 30μl of each sample was then loaded into dry wells of 1.5% (w/v) agarose gels containing 0.5μg/ml ethidium bromide and run for 1hr at 100V in TAE buffer. DNA was visualised by UV light.

## 2.6.3 The CellTitre 96 AQueous One Solution Cell Poliferation Assay (Promega) – the MTS method.

The CellTitre 96 AQueous One Solution cell proliferation assay (Promega) is a useful way of measuring cell survival following apoptosis. $1 \times 10^6$ cells were plated out onto 10cm tissue culture plates and serum starved the following day. Following 48 hours of serum starvation, the cells were harvested by trypsinization and combined with floating cells in the media. The

cells were then accurately counted using a haemocytometer and resuspended to a final concentration of 1 x$10^5$/ml in DMEM media containing 10% FBS. 50µl of this cell suspension (5000 cells) were then added to 50µl of DMEM media containing 10% FBS in quadruplicate wells of a 96 well tissue culture plate. To measure any background absorbance, a quadruplicate set of control wells (without cells) containing the same volume of media was used as a control. The values obtained for this control was subtracted from the experimental absorbances to obtain corrected absorbance values. The plate was then incubated at 37°C/5% $CO_2$ for 4 hours to allow the cells to recover and start to grow. Following this incubation, 20µl / well of CellTitre 96AQueous One Solution reagent was added and the plate returned to the incubator for a further 2 hours to allow the colour to develop. Finally, the absorbance of each sample was read at 490nm using a 96 well plate reader.

## 2.6.4 The ApoAlert Annexin V kit (Clontech).

1x$10^5$ MDCK cells were plated out onto glass coverslips (Astel Scientific, 13mm) / well of a 12 well plate. The next day the cells were either allowed to grow or induced to undergo apoptosis for 48 hours. Following this time, the media was removed and the cells washed once with PBS, then once with 1x binding buffer. 200µl of 1x binding buffer was added to each sample followed by 5µl of Annexin V (green) and 10µl of Propidium iodide (red). The samples were incubated for 15 minutes at room temperature (in dark) then the stain was removed and 2% formaldehyde added for 5mins to fix the cells. The samples were mounted onto a glass slide in Citifluor/PBS/glycerol solution (Citifluor Ltd.) for immunofluorescence laser confocal microscopy.

## 2.7 Immunofluorescence laser scanning confocal microscopy.


Certain flurophores which are capable of absorbing light of one wavelength and emmitting light of a different wavelength are useful in analysing cells. A confocal microscope is capable of transmitting exciting light through the lens onto a specimen and then collecting and visualising the emitted light from the specimen. Immunofluorescence laser scanning confocal microscopy was used to visualise Fluorescein isothiocyanate (FITC)-labelled Annexin V protein bound to externalised phosphatidylserine molecules at the plasma membrane of apoptotic cells. The excitation wavelength of FITC is at 495nm with emission at 525nm.


Annexin V stained samples were mounted in Citifluor/PBS/glycerol (Citifluor Ltd.) and examined using an MRC-600 imaging system (Bio-rad) equipped with a krypton-argon laser and a Nikon lens (magnification x10, x40). Images were produced using COMOS software.

# CHAPTER 3

# STABLE CELL LINES EXPRESSING CONSTITUTIVELY ACTIVE MUTANTS OF RAT PKBγ, MOUSE PKBγ AND MOUSE PKBα

# CHAPTER 3: Stable Cell Lines Expressing constitutively active mutants of rat PKBγ, mouse PKBγ and mouse PKBα.

## 3.1 Introduction.

### 3.1.1 PKBγ, its structure and activation.

Much research has already been carried out on the PKBα isoform. However, there is still a notable lack of information concerning PKBβ, and even less concerning the third isoform, PKBγ. In 1995 Konishi and colleagues reported the cloning of PKBγ from a rat brain cDNA library (Konishi *et al.*, 1995). As with PKBα and β, rat PKBγ possesses an N-terminal PH domain and a residue equivalent to $Thr^{308}$ ($Thr^{305}$ in rat PKBγ) which is phosphorylated in response to growth factors. However, intriguingly, rat PKBγ is truncated with 23 amino acids missing from the C-terminal and lacks the regulatory serine phosphorylation site ($Ser^{473}$ in PKBα). Until very recently only one other study was reported concerning PKBγ and its activation (Walker *et al.*, 1998). Then in 1999 two groups working independently cloned the human form of PKBγ and found that it differed from the rat form in that it contains the regulatory serine phosphorylation site at the C-terminal (Brodbeck *et al.*, 1999; Nakatani *et al.*, 1999). As Figue 3.1 illustrates, rat PKBγ is 83% identical to PKBα and has an open reading frame of 1365bp encoding a sequence of 454 amino acids with the molecular weight of 53KDa. Human PKBγ is 83% identical to PKBα, 99% identical to rat PKBγ (two changes in 451 amino acids and a different C-terminal) and 99% identical to mouse PKBγ (2 amino acid changes in 479). Human PKBγ has an open reading frame of 1437bp encoding a sequence of 479 amino acids with a molecular weight of 57KDa.

```
HumanAKT3    MSDVTIVKEGWVQKRGEYIKNWRPRYFLLKTDGSFIGYKEKPQDVDLP-YPLNNFSVAKC 59
Mouseakt3    MSDVTIVKEGWVQKRGEYIKNWRPRYFLLKTDGSFIGYKEKPQDVDLP-YPLNNFSVAKC 59
Ratakt3      MSDVTIVKEDWVQKRGEYIKNWRPRYFLLKTDGSFIGYKEKPQDVDLP-YPLNNFSVAKC 59
HUMAkt1      MSDVAIVKEGWLHKRGEYIKTWRPRYFLLKNDGTFIGYKERPQDVDQREAPLNNFSVAQC 60
             ****:****.*::******* .*********.**:******.*****   ********:*

HumanAKT3    QLMKTERPKPNTFIIRCLQWTTVIERTFHVDTPEEREEWTEAIQAVADRLQRQEEERMNC 119
Mouseakt3    QLMKTERPKPNTFIIRCLQWTTVIERTFHVDTPEEREEWTEAIQAVADRLQRQEEERMNC 119
Ratakt3      QLMKTERPKPNTFIIRCLQWTTVIERTFHVDTPEEREEWTEAIQAVADRLQRQEEERMNC 119
HUMAkt1      QLMKTERPRPNTFIIRCLQWTTVIERTFHVETPEEREEWTTAIQTVADGLKKQEEEEMDF 120
             ********:*******************:********* ***:*** *::****.*:

HumanAKT3    SPTSQIDNIGEEEMDASTTHHKR-KTMNDFDYLKLLGKGTFGKVILVREKASGKYYAMKI 178
Mouseakt3    SPTSQIDNIGEEEMDASTTHHKR-KTMNDFDYLKLLGKGTFGKVILVREKASGKYYAMKI 178
Ratakt3      SPTSQIDNIGEEEMDASTTHHKR-KTMNDFDYLKLLGKGTFGKVILVREKASGKYYAMKI 178
HUMAkt1      RSGSPSDNSGAEEMEVSLAKPKHRVTMNEFEYLKLLGKGTFGKVILVKEKATGRYYAMKI 180
              . *   ** * ***:.* :: *:  ***:*:*************:***.*:******

HumanAKT3    LKKEVIIAKDEVAHTLTESRVLKNTRHPFLTSLKYSFQTKDRLCFVMEYVNGGELFFHLS 238
Mouseakt3    LKKEVIIAKDEVAHTLTESRVLKNTRHPFLTSLKYSFQTKDRLCFVMEYVNGGELFFHLS 238
Ratakt3      LKKEVIIAKDEVAHTLTESRVLKNTRHPFLTSLKYSFQTKDRLCFVMEYVNGGELFFHLS 238
HUMAkt1      LKKEVIVAKDEVAHTLTENRVLQNSRHPFLTALKYSFQTHDRLCFVMEYANGGELFFHLS 240
             ******:***********.***:*:******:*******:*********.**********

HumanAKT3    RERVFSEDRTRFYGAEIVSALDYLHSG-KIVYRDLKLENLMLDKDGHIKITDFGLCKEGI 297
Mouseakt3    RERVFSEDRTRFYGAEIVSALDYLHSG-KIVYRDLKLENLMLDKDGHIKITDFGLCKEGI 297
Ratakt3      RERVFSEDRTRFYGAEIVSALDYLHSG-KIVYRDLKLENLMLDKDGHIKITDFGLCKEGI 297
HUMAkt1      RERVFSEDRARFYGAEIVSALDYLHSEKNVVYRDLKLENLMLDKDGHIKITDFGLCKEGI 300
             *********:****************   ::*****************************

HumanAKT3    TDAATMKTFCGTPEYLAPEVLEDNDYGRAVDWWGLGVVMYEMMCGRLPFYNQDHEKLFEL 357
Mouseakt3    TDAATMKTFCGTPEYLAPEVLEDNDYGRAVDWWGLGVVMYEMMCGRLPFYNQDHEKLFEL 357
Ratakt3      TDAATMKTFCGTPEYLAPEVLEDNDYGRAVDWWGLGVVMYEMMCGRLPFYNQDHEKLFEL 357
HUMAkt1      KDGATMKTFCGTPEYLAPEVLEDNDYGRAVDWWGLGVVMYEMMCGRLPFYNQDHEKLFEL 360
             .*.*********************************************************

HumanAKT3    ILMEDIKFPRTLSSDAKSLLSGLLIKDPNKRLGGGPDDAKEIMRHSFFSGVNWQDVYDKK 417
Mouseakt3    ILMEDIKFPRTLSSDAKSLLSGLLIKDPNKRLGGGPDDAKEIMRHSFFSGVNWQDVYDKK 417
Ratakt3      ILMEDIKFPRTLSSDAKSLLSGLLIKDPNKRLGGGPDDPKEIMRHSFFSGVNWQDVYDKK 417
HUMAkt1      ILMEEIRFPRTLGPEAKSLLSGLLKKDPKQRLGGGSEDAKEIMQHRFFAGIVWQHVYEKK 420
             ****:*:******..:********* ***::*****.*.****:* **:*: **.**:**

HumanAKT3    LVPPFKPQVTSETDTRYFDEEFTAQTITITPPEKYDEDGMDCMDNERRPHFPQFSYSASG 477
Mouseakt3    LVPPFKPQVTSETDTRYFDEEFTAQTITITPPEKYDDDGMDGMDNERRPHFPQFSYSASG 477
Ratakt3      LVPPFKPQVTSETDTRYFDEEFTAQTITITPPEKCPL---------------------- 454
HUMAkt1      LSPPFKPQVTSETDTRYFDEEFTAQMITITPPD--QDDSMECVDSERRPHFPQFSYSASS 478
             * ********************** ******:

HumanAKT3    RE 479
Mouseakt3    RE 479
Ratakt3      --
HUMAkt1      TA 480
```

**Figure 3.1    Multiple sequence alignments of PKBγ with PKBα.**

Multiple sequence alignment of PKBγ from human (HumanAKT3), mouse (mouseakt3) and rat (ratakt3) compared to human PKBα (HUMakt1) using the ClustalW alignment device from EBI. The two key regulatory phosphorylation sites of PKB are shown highlighted in red.

Rat PKBγ is activated in response to IGF-1 in 293 cells, insulin in rat L6 myotubes and NGF in PC12 cells (Walker *et al.*, 1998; Andelkovic et al., 1998). As for the other isoforms, activation of rat PKBγ is thought to be dependent on PDK1 in the presence of PI(3,4,5)P$_3$ and/or PI (3,4)P$_2$ and phosphorylation occurs on Thr$^{305}$ (Walker *et al.*, 1998).

Human PKBγ is activated by insulin and pervanadate in 293 cells (Brodbeck *et al.*, 1999) and insulin in Chinese Hamster Ovary cells overexpressing the insulin receptor (CHO-IR) (Nakatani *et al.*, 1999). Mouse PKBγ is activated by IGF-1 in 293 cells (Shaw *et al.*, 1997) and EGF in Swiss 3T3 cells (Shaw and Cohen, 1999). As expected, both human and mouse PKBγ undergo phosphorylation on the Thr$^{305}$ residue and also on the Ser$^{472}$ residue consistent with the other full length PKB isoforms.

So far there have been few studies published concerning either downstream targets or biological functions of PKBγ. With this in mind it is therefore of the utmost importance to investigate the potential role of PKBγ in cell survival signalling (see Chapter 5: The role of PKBγ in cell survival signalling and apoptosis).

## 3.1.2 Strategies for studying the downstream effects of PKBγ.

Several different approaches have already been employed to study PKBα. These include the use of membrane targeting to cause constitutive activation and expression of site directed active or dominant negative mutants. So far there has been no pharmacological inhibitor of PKB produced.

**Protein *N*-Myristoylation to constitutively activate PKB**

Protein *N*-myristoylation refers to the co-translational linkage of myristic acid (C14:0) via an amide bond to the $NH_2$-terminal Gly residues of a variety of eukaryotic cellular and viral proteins (Towler *et al.*, 1988; James *et al.*, 1990). The reaction is catalysed by myristoyl-CoA:protein *N*-myristoyltransferase (NMT) and the process is irreversible (James *et al.*, 1989). The result of this *N*-myristoylation is the targeting of the protein to the plasma membrane. PKB isoforms mutated to contain the myristoylation sequence at their N-terminal become constitutively activated (Bellacosa *et al.*, 1991; Andjelkovic et al., 1997) since membrane targeting allows close association with the constitutively active PDK1 and hence are phosphorylated at the $Thr^{308}$ and $Ser^{473}$ residues by PDK1 and PDK2. Such membrane targeting of PKBα has been used to suggest PKBα involvement in glucose metabolism (Kohn *et al.*, 1996), amino acid transport and protein synthesis (Hajduch *et al.*, 1998) and apoptosis (Franke *et al.*, 1995; Kulik *et al.*, 1997).

**Site directed Mutagenesis to produce constitutively active PKB or dominant negative PKB.**

A second very different approach to constitutively activate PKB is to employ the technique of site directed mutagenesis (Higuchi, 1990). Site directed mutagenesis enables us to change any base (or bases) in a known DNA sequence for a base (or bases) of our choice and consequently any amino acid in the protein sequence may also be changed for another. In this way it has been possible to produce a recombinant PKB with the two regulatory phosphorylation sites mutated from Thr308 and Ser473 to the acidic amino acid aspartate which has a negetive charge and in doing so to mimic the effects of phosphorylation. In this

way the mutant PKB construct is constitutively active even in the absence of any growth factors (Alessi et al., 1996). Conversely, a dominant negative mutant of PKBα has been described where the phosphorylation sites have been altered to the neutral amino acid alanine to produce an inactive PKB construct which cannot be phosphorylated by the PDK1 and PDK2 upstream kinases (Stambolic et al., 1998; Wang et al., 1999).

The main aim of this chapter was to successfully clone the truncated rat PKBγ, the full length mouse PKBγ and mouse PKBα (as a positive control) using the polymerase chain reaction. These genes were then used to produce constitutively active membrane targeted mutants of rat PKBγ, mouse PKBγ and mouse PKBα and site directed active and dominant negative mutants of rat PKBγ using the two techniques described above (Figure 3.2). The constitutively active mutants were then used to establish stably transfected Madin-Darby canine embryonic kidney (MDCK) cell lines in order to study PKBγ involvement in apoptosis. MDCK cells in particular were chosen for this study since they have proved a good model for inducing apoptosis either by serum starvation or by matrix detachment, a process referred to as aniokis (Frisch and Francis, 1994; Khawaja et al., 1997).

**Figure 3.2    Diagramatic representation of the PKB mutant constructs used in this study.**

The N-terminal myristoylation/palmitoylation sequence (myr/palm) for membrane targeting, PH domain (PH), glycine rich region (G) and the kinase domain are shown. The phosphorylation sites of the regulatory domain (R) are also indicated. All constructs, except for rat HA-wtPKBγ, have a C-terminal haemaglutinin tag (HA).
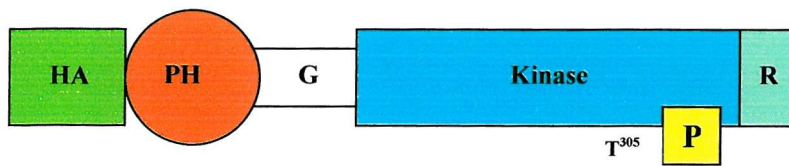
1. rat
   HA-PKBγ
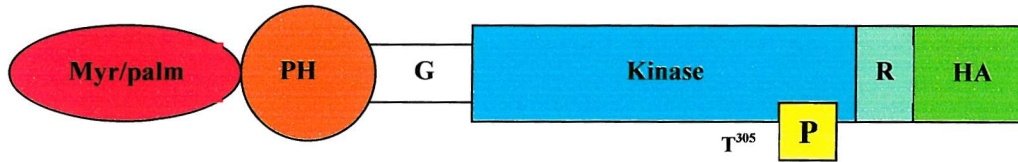
2. rat
   mvrPKBγ-HA

3. rat
   PKBγ (T305D)-HA
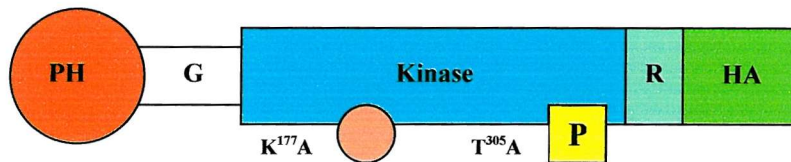
4. rat PKBγ
   (K179A,T305A)-HA
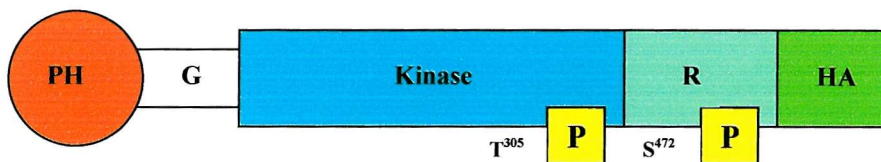
5. mouse
   PKBγ-HA

6. mouse
   myrPKBγ-HA
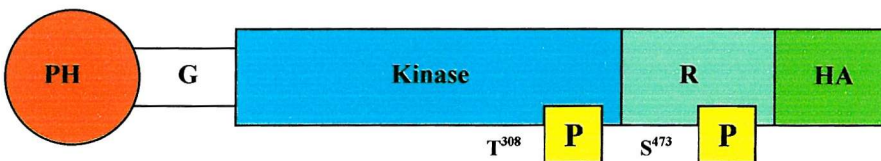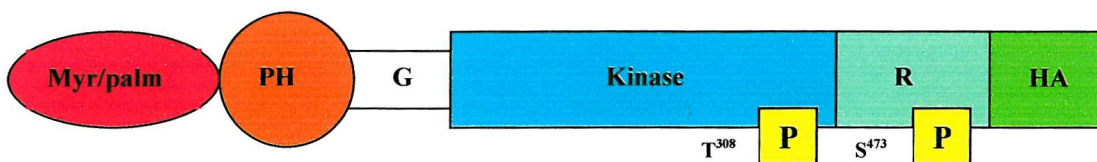
7. mouse
   PKBα-HA

8. mouse
   myrPKBα-HA

### 3.1.3 Using the Polymerase Chain Reaction (PCR) to clone genes.

The basic PCR reaction leads to the amplification of specific DNA sequences by an enormous factor (Mullis et al., 1987; Erlich et al., 1988). PCR involves two oligonucleotide primers that flank the DNA sequence that is to be amplified. The primers hybridize to opposite strands of the DNA after it has been denatured and are orientated so that DNA synthesis by the polymerase enzyme proceeds through the region between the two primers. The extension reactions create two double-stranded target regions, each of which can again be denatured ready for a second cycle of hybridization and extension. The third cycle produces two double-stranded molecules that comprise precisely the target region in double-stranded form. By repeated cycles of heat denaturation, primer hybridization and extension, there follows a rapid exponential accumulation of the specific target fragment of DNA.

This technique, initially used to amplify relatively small gene fragments was soon

adapted to amplify whole genes up to several Kilobases in length. This was achieved by adapting the original method in conjunction with the use of new 'proofreading' polymerase enzymes with exonuclease activity (eg. *Pfu* polymerase, Promega). The starting material for amplifying genes by PCR is messenger RNA (mRNA) isolated from tissue expressing the gene of interest. This mRNA is then used to produce single stranded complementary DNA (cDNA) using the enzyme reverse transcriptase and it is the cDNA that is the template material in the PCR reaction (Figure 3.3).
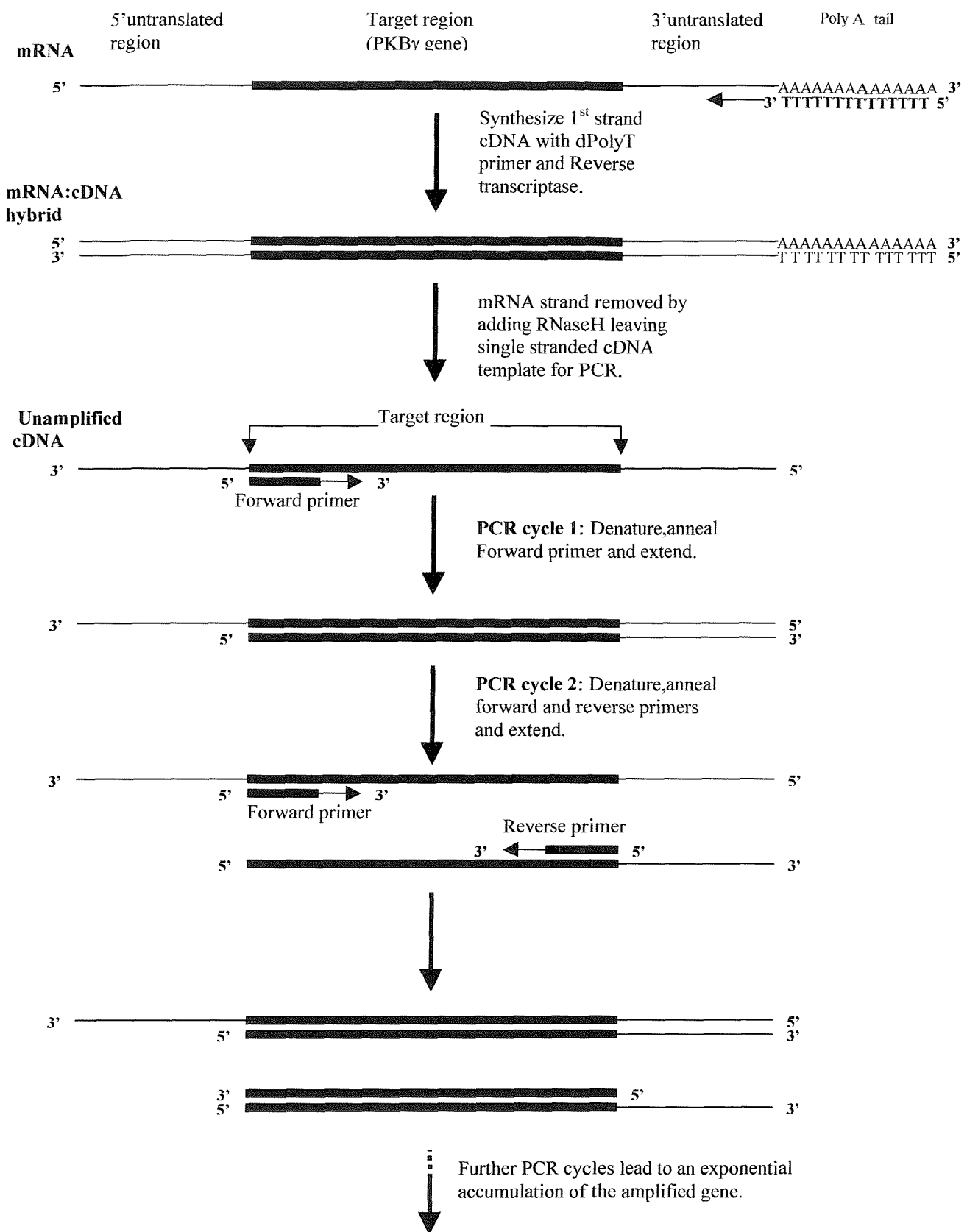
**Figure 3.3     The Polymerase Chain Reaction (PCR).**

Amplification of a specific gene by PCR following reverse transcription of messenger RNA (mRNA).

## 3.2 Cloning rat wild-type PKBγ by PCR.

Reverse transcription of isolated total RNA followed by the polymerase chain reaction leading to amplification of specific mRNA sequences in cDNA form, is a highly sensitive means of cloning a gene of interest. From previous studies rat PKBγ was found to be highly expressed in the brain and testis (Konishi et al., 1995). Therefore rat brain tissue was used as a source of PKBγ mRNA to make cDNA in order to clone it by PCR using the rat wtPKBγ forward and reverse primers. The first attempts using Taq polymerase (Promega) to amplify rat PKBγ by PCR were not successful, yielding no product. However, after changing to using the proof-reading enzyme Pfu polymerase (Promega) a 1.4kb fragment corresponding to the reported size of the PKBγ coding region was obtained (Figure 3.4).

### 3.2.1 Addition of a N-terminal HA tag and ligation into the pGEM T-Easy Vector.

A hemagglutinin-antigen (HA) epitope tag corresponding to the sequence YPYDVPDYA was incorporated onto the N-terminal of the PKBγ gene to produce rat HA-PKBγ. This was achieved by PCR mediated addition using the rat HA forward primer in a second PCR reaction (Figure 3.4). Pfu polymerase produces a blunt ended PCR product, so in order to ligate the HA-PKBγ construct into the pGEM-T Easy vector a single deoxyadenosine residue was added to each 3'end by incubating the PCR product with Taq polymerase. The resulting 'A-tailed' HA-PKBγ was then ligated into the vector by TA cloning. One of the benefits of using the pGEM-T Easy vector to clone PCR products is that the insertional inactivation of the β-galactosidase gene allows recombinant clones to be identified when plated out on indicator plates, a process called blue-white screening. Clones that contain the PCR product,

in most cases, produce white colonies. However, it must be noted that blue colonies can result from PCR fragments cloned in-frame with the Lac Z gene.

Calcium competent JM109 *E. coli* bacteria were transformed with the ligation reactions by heat shock and blue-white screening was used to identify clones containing the vector with insert. six ampicillin resistant, white bacterial colonies were picked for restriction digest analysis using the restriction enzyme EcoR1 sites for which lye on either side of the HA-PKBγ insert. All colonies, except colony 3, were found to contain the rat HA-PKBγ insert (Figure 3.6).

## 3.2.2 Subcloning rat HA-PKBγ into the pcDNA3.1 mammalian expression vector and sequencing by primer walking.

The rat HA-PKBγ was sub-cloned into the mammalian expression vector pcDNA3.1 (Invitrogen) (Figure 3.7) as an EcoR1 fragment. Following ligations and transformation of JM109 bacteria by heat shock, 6 ampicillin resistant bacterial colonies were picked for mini-prep and restriction digest analysis using the restriction enzymes Spe I and Xho I. The Spe I site is found at the 5'-end of the rat HA-PKBγ insert and the Xho I site is at the 3'-end in the vector. Therefore any 1.4Kb fragment will be the rat HA-PKBγ insert and this insert will be in the correct orientation within the vector. All colonies contained pcDNA.HA-PKBγ in the correct orientation (Figure 3.8). Glycerol stocks were made from colony 1.

The rat pcDNA.HA-PKBγ construct was sequenced by primer walking using the T7 forward primer as the initial primer (Figure 3.9). The sequencing results obtained differed at two positions from the published sequence (Konishi et al., 1995), changing Asn10 to Gly, and

Pro396 to Ala. The equivalent residues in PKBα and β are also Gly and Ala respectively.

This finding was in accordance with Walker and colleagues who also noted these changes

when cloning rat PKBγ (Walker et al., 1998).
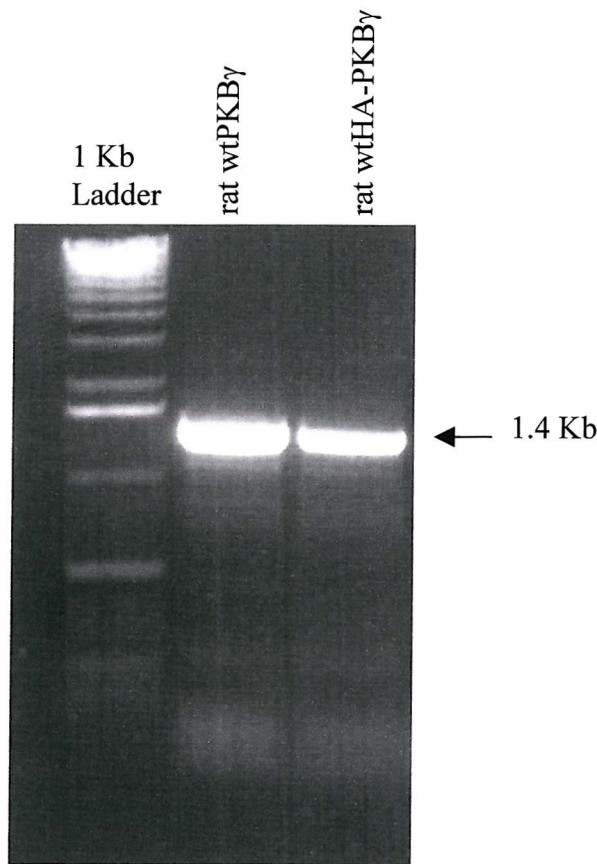
**Figure 3.4    The cloning of wild-type rat HA-PKBγ by PCR.**

Total RNA (2μg) extracted from a rat brain using the RNazol reagent (Biogenesis) was used in a 25μl reaction to produce a cDNA library representing all the expressed genes in the rat brain tissue. 2μl of cDNA was then used as template in a PCR reaction with the rat PKBγ gene-specific forward and reverse primers. The 1.4Kb PCR product from this reaction was then used as the template DNA in a second reaction along with the rat HA forward primer in order to add the HA tag onto the N-terminal of the rat PKBγ gene. The final PCR product was rat HA-PKBγ. For both reactions, PCR conditions after initial denaturing at 95°C for 2mins were: 95°C for 30s; annealing at 60°C for 30s; then extension at 72°C for 4mins. This was repeated for 30 cycles with a final extension at 72°C for 5mins. 10μl of each PCR reaction was loaded onto a 1% agarose gel and subjected to electrophoresis for 1 hour at 100V.

**Figure 3.5    The pGEM-T Easy vector.**

**A. Amp**[r] ampicillin resistance gene; **fi ori** f1 origin of replication in bacteria; **T7** promoter/priming site; **SP6** promoter/priming site; **Lac Z** gene coding for β-galactosidase; **MCS** multiple cloning site.

**B.** Ligation of an insert by TA cloning results in the inactivation of the Lac Z gene allowing for selection of clones by blue-white screening.

**A.**

pGEM-T Easy Vector (3018bp)

Amp$^r$

f1 ori

Lac Z

T

T

MCS

T7 ↓
*Apa I*
*Aat II*
*Sph I*
*Nco I*
*BstZ I*
*Not I*
*Sac II*
*EcoR I*

MCS

*Spe I*
*EcoR I*
*Not I*
*BstZ I*
*Pst I*
*Sal I*
*Nde I*
*Sac I*
*BstX I*
*Nsi I*
SP6 ↑

**B.**

pGEM-T.HA-PKBγ (4418bp)

Amp$^r$

f1 ori

Lac Z interupted

PKBγ
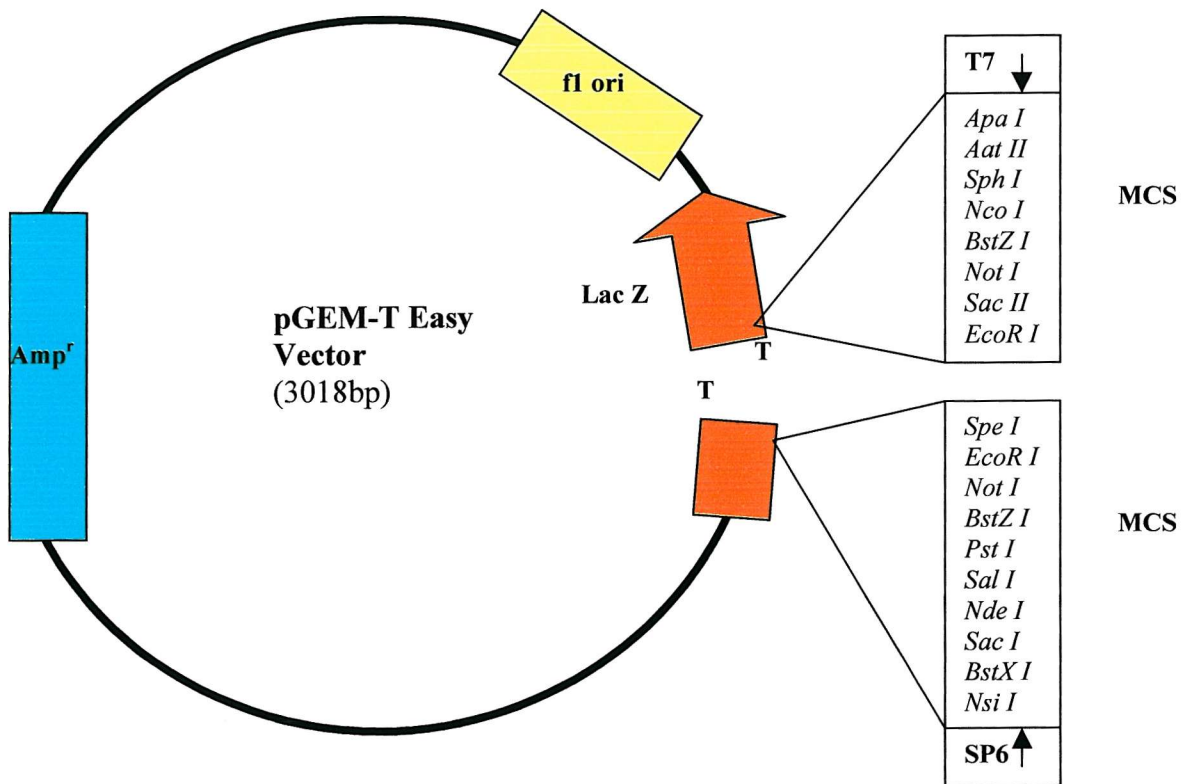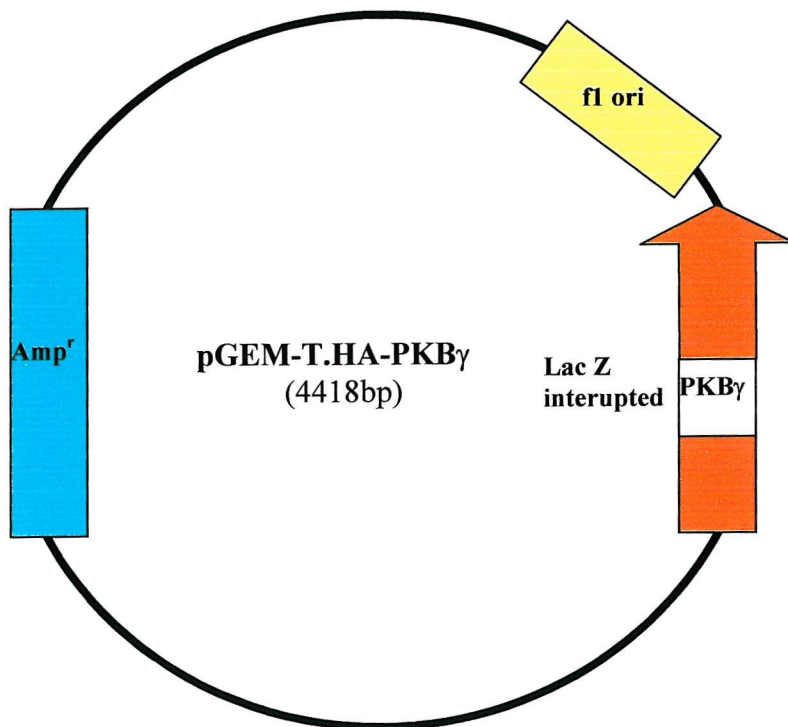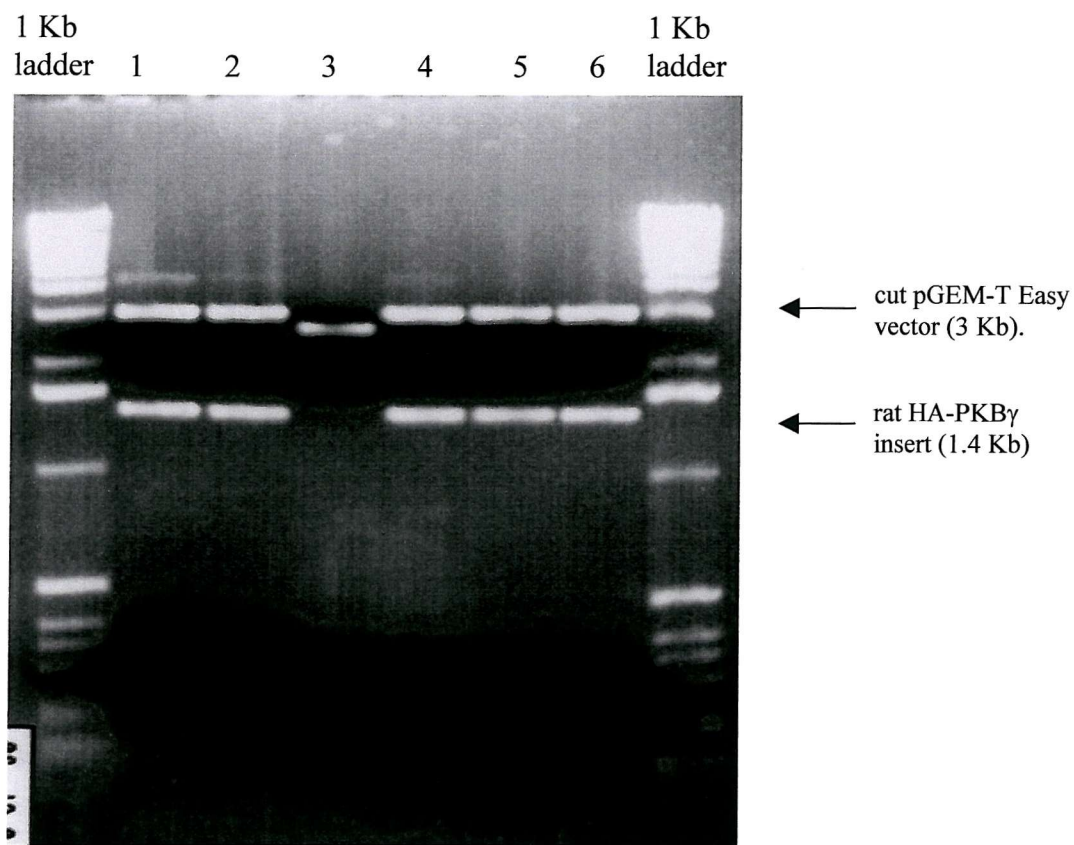
**Figure 3.6    Restriction digest analysis of pGEM-T.rat HA-PKBγ transformations.**

Following ligation of rat HA-PKBγ into the pGEM-T Easy vector by TA cloning, transformation of JM109 calcium competent *E. coli* and blue-white screening, 6 ampicillin resistant, white bacterial colonies were picked and their plasmid DNA mini-preped (Promega). Restriction digests were set up using 5μl of plasmid DNA and 0.5μl EcoR1 restriction enzyme. Colonies 1,2,4,5 and 6 all contain the rat HA-PKBγ insert.
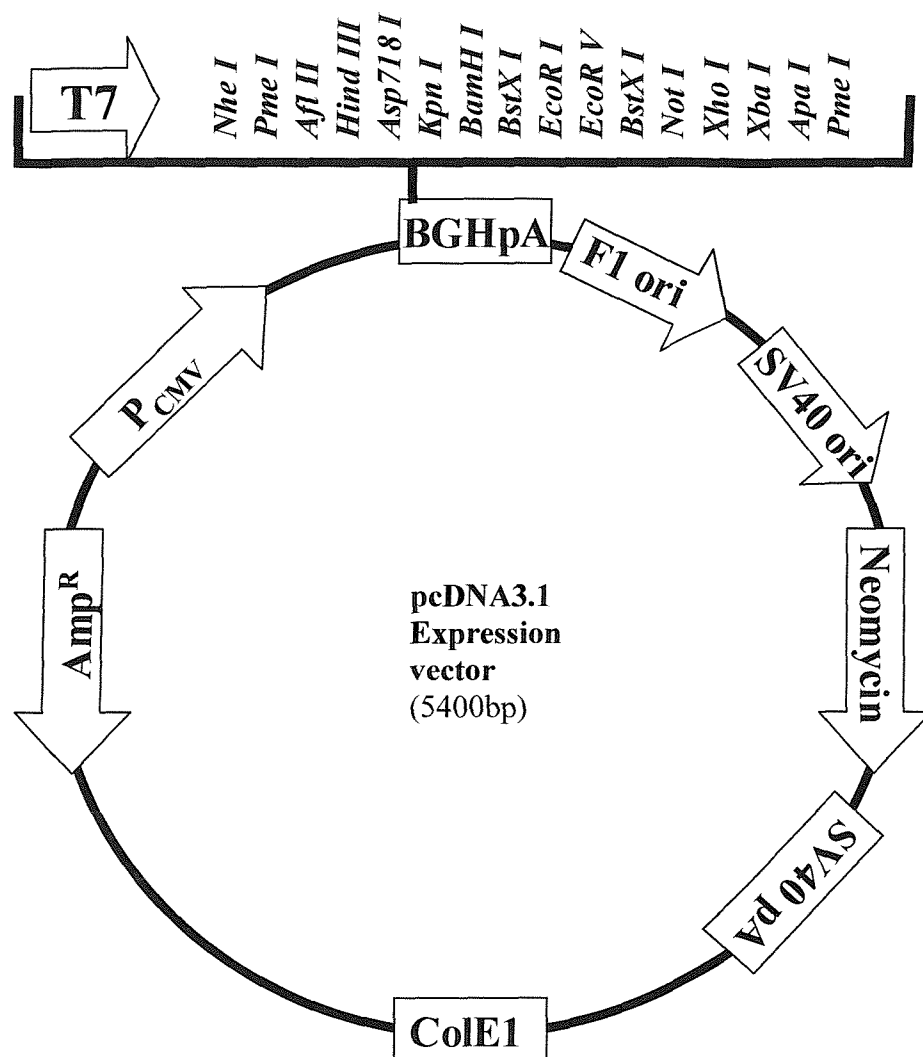
**Figure 3.7    pcDNA3.1 Expression vector legend.**

**pCMV** Cytomegalovirus promoter; **T7** promoter/priming site; **BGHpA** bovine growth hormone polyadenylation signal; **SV40 *ori*** SV40 origin of replication; **Neo**[R] Neomycin resistance; **SV40pA** SV40 polyadenylation signal; **Amp**[R] ampicillin resistance; **fi *ori*** f1 origin of replication in bacteria.
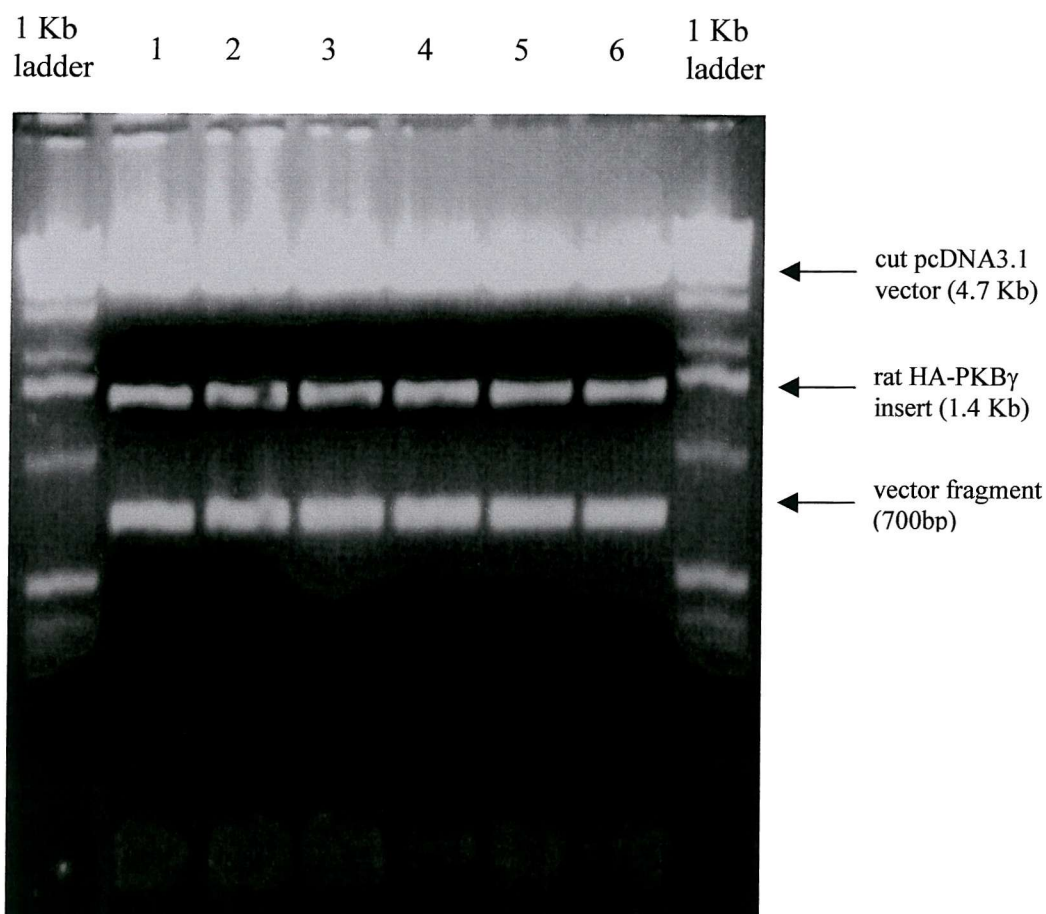
**Figure 3.8    Restriction digest analysis of pcDNA.rat HA-PKBγ transformations.**

Following ligation of rat HA-PKBγ into the pcDNA3.1 mammalian expression vector and transformation of JM109 calcium competent *E. coli*, 6 ampicillin-resistent bacterial colonies were picked and their plasmid DNA mini-preped (Promega). Restriction digests were set up using 5μl of plasmid DNA and 0.5μl each of Spe1 and Xho1 restriction enzymes. All 6 picked colonies were found to contain contain the rat HA-PKBγ insert in the correct orientation. Glycerol stocks were prepared from Colony 1.

ATGTACCCATACGATGTGCCAGATTACGCCAGCGATGTTACCATCGTTAAAGAAGGCTGGGTTCAGAAGAGGGGA

GAATATATAAAAAATTGGAGGCCAAGATACTTCCTTTTGAAGACAGACGGCTCATTCATAGGCTATAAAGAGAAA

CCTCAAGATGTGGACTTACCTTATCCCCTCAACAACTTCTCAGTGGCAAAATGTCAATTAATGAAAACAGAACGA

CCAAAGCCAAATACATTTATTATCAGATGTCTTCAGTGGACCACTGTTATAGAGAGAACATTTCATGTAGATACT

CCAGAGGAAAGAGAAGAATGGACAGAAGCTATCCAAGCTGTAGCCGATCGACTGCAGAGGCAAGAAGAGGAGAGG

ATGAATTGTAGTCCAACGTCACAGATTGATAATATTGGAGAGGAAGAGATGGATGCATCTACAACCCATCATAAA

AGAAAGACAATGAATGATTTTGACTATTTGAAACTGCTAGGTAAAGGCACTTTTGGGAAGGTTATTTTGGTTCGA

GAGAAGGCAAGTGGAAAATACTACGCTATGAAGATTCTGAAGAAAGAAGTCATTATCGCAAAGGATGAAGTGGCA

CATACTCTGACTGAGAGCAGAGTTCTAAAGAACACCAGACATCCATTTCTAACATCCTTGAAATATTCCTTCCAG

ACAAAAGACCGTTTGTGTTTTGTGATGGAATATGTTAATGGGGGAGAGCTGTTTTTCCATTTGTCGAGAGAGCGG

GTGTTCTCTGAGGACCGCACACGTTTCTATGGTGCAGAAATTGTCTCTGCTTTGGACTATCTACATTCTGGAAAG

ATTGTGTACCCGAGATCTCAAGTTGGAGAATTTGATGCTAGATAAGGATGGCCATATAAAAATTACGGATTTTGG

ACTTTGCAAAGAAGGGATCACAGATGCAGCTACCATGAAGACATTCTGTGGTACACCAGAGTATCTGGCACCAGA

GGTATTAGAAGATAATGACTATGGCCGAGCTGTGGACTGGTGGGGCCTAGTGTTGTCATGTATGAAATGATGTGT

GGGAGGTTGCCTTTCTACAACCAGGATCATGAGAAACTCTTTGAACTAATACTCATGGAAGACATCAAATTCCCC

CGAACACTCTCTTCAGATGCAAAGTCATTGCTTTCAGGGCTCTTGATAAAGGATCCAAATAAACGCCTTGGTGGA

GGGCCAGATGATCCAAAAGAGATAATGAGGCACAGTTTCTTTTCTGGAGTAAACTGGCAGGACGTATATGACAAA

AAGCTTGTACCTCCTTTTAAGCCTCAAGTAACATCTGAGACAGACACCAGATATTTCGATGAATTTACAGCTCAG

ACTATTACAATAACACCACCTGAAAAGTGTCCACTA**TAA**

## Figure 3.9    Full length sequencing of rat HA-PKBγ.

Full length sequencing 5'-3'of rat HA-PKBγ (sequencing by Oswell). The translational initiation and stop codons ATG and TAA respectively are highlighted. The N-terminal HA tag sequence (in red) encodes the amino acid sequence recognised by the anti-HA antibody (U.B.I).

### 3.3 Construction of constitutively active *N*-myristoylated rat PKBγ (rat myrPKBγ-HA).

### 3.3.1 PCR of rat myrPKBγ-HA.

To create the membrane targeted constitutively active rat PKBγ, a mouse Lck myristoylation/palmitoylation signal MGCVCSSNPEDD (Bijlmakers et al., 1997) was added to the N-terminal of rat wtPKBγ by PCR mediated addition using a series of 3 PCR reactions (see Table 3.1 for details). Note that the HA tag was added to the C-terminal to avoid interference with the membrane targeting signal. PCR conditions for all reactions were exactly the same used to clone the rat wtPKBγ. The resulting PCR fragment was 1.4kb and was named myrPKBγ-HA (Figure 3.10).

**Table 3.1 PCR reactions to construct rat myrPKBγ-HA**

| PCR REACTION | TEMPLATE DNA | FORWARD PRIMER | REVERSE PRIMER |
|---|---|---|---|
| **1.** rat wtPKBγ with no stop codon | 2μl rat brain cDNA library | rat wtPKBγ forward primer | rat HA reverse primer 1 |
| **2.** rat myrPKBγ-1/2 HA | 1μl gel pure reaction **1.** PCR product | rat myrPKBγ forward primer | rat HA reverse primer 2 |
| **3.** rat myrPKBγ-HA | 1μl gel pure reaction **2.** PCR product | Spe/myr forward primer | rat HA reverse primer 3 |

### 3.3.2 Ligation of rat myrPKBγ-HA PCR product into the pGEM-T Easy vector.

The myrPKBγ-HA PCR product was gel-purified, 'A-tailed' then ligated into the pGEM-T

Easy vector by TA cloning as described previously (section 3.2.1). Following transformation

of JM109 calcium competent bacteria and blue-white screening, 6 ampicillin resistant white

bacterial colonies were picked for analysis. The colonies were grown up overnight and the

plasmid DNA mini-preped for restriction digest using EcoR1 (Figure 3.11). The rat

myrPKBγ-HA insert was found in all the plasmids. Colony 1 was used to make glycerol

stocks.

### 3.3.3 Sub-cloning the rat myrPKBγ-HA construct into the pcDNA3.1 mammalian expression vector and sequencing .

The rat myrPKBγ-HA was sub-cloned into the mammalian expression vector pcDNA3.1

(Invitrogen) as an EcoR1 fragment. Following ligations and transformation of JM109

bacteria, 6 ampicillin resistant colonies were picked for restriction analysis. Analysis by

BamH1 restriction digest revealed that the rat myrPKBγ-HA was inserted into plasmids from

colonies 1,3,4 and 6 (Figure 3.12). Glycerol stocks were prepared from Colony 1.

The pcDNA.ratmyrPKBγ-HA construct was sequenced by primer walking using the T7

forward primer as the initial primer (Figure 3.13). This sequence was confirmed as rat wild-

type PKBγ containing a N-terminal myristoylation/palmitoylation sequence and a C-terminal

HA tag as expected.
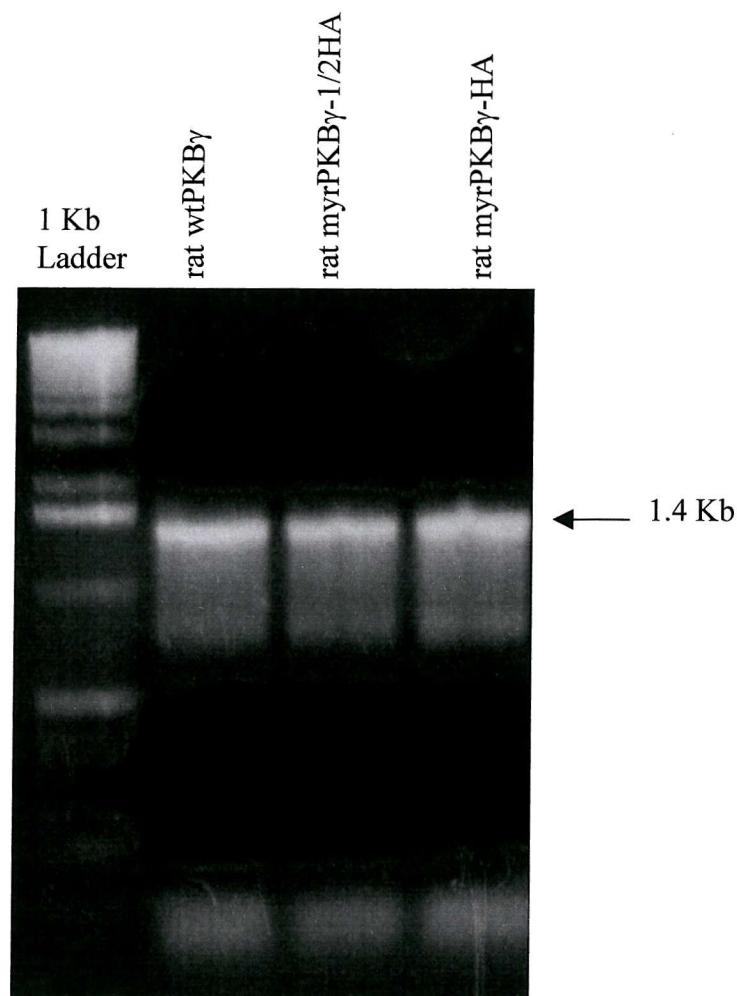
**Figure 3.10    PCR of rat myrPKBγ-HA.**

3 successive PCR reactions were set up to produce rat myrPKBγ-HA (see Table 3.1 for reaction details). For all 3 reactions, PCR conditions after initial denaturing at 95°C for 2mins were: 95°C for 30s; annealing at 60°C for 30s; then extension at 72°C for 4mins. This was repeated for 30 cycles with a final extension at 72°C for 5mins. 10μl of each PCR reaction was loaded onto a 1% agarose gel and subjected to electrophoresis for 1 hour at 100V.

**Figure 3.11    Restriction digest analysis of pGEM-T.ratmyrPKBγ-HA transformations.**

Following ligation of rat myrPKBγ-HA into the pGEM-T Easy vector by TA cloning, transformation of JM109 calcium competent *E. Coli* and blue-white screening, 6 ampicillin resistant, white bacterial colonies were picked and their plasmid DNA mini-preped (Promega). Restriction digests were set up using 5μl of plasmid DNA and 0.5μl EcoR1 restriction enzyme. Colonies 1,2,4,5 and 6 all contain the rat myrPKBγ-HA insert.

**Figure 3.12    Restriction digest analysis of rat pcDNA.myrPKBγ-HA transformations.**

Following ligation of rat myrPKBγ-HA into the pcDNA3.1 mammalian expression vector and transformation of JM109 calcium competent *E. Coli*, 6 ampicillin resistant bacterial colonies were picked and their plasmid DNA mini-preped (Promega). Restriction digests were set up using 5μl of plasmid DNA and 0.5μl of BamH1 restriction enzyme. Colonies 1,3,4 and 6 were found to contain the rat myrPKBγ-HA insert in the correct orientation. Glycerol stocks were prepared from Colony 1.
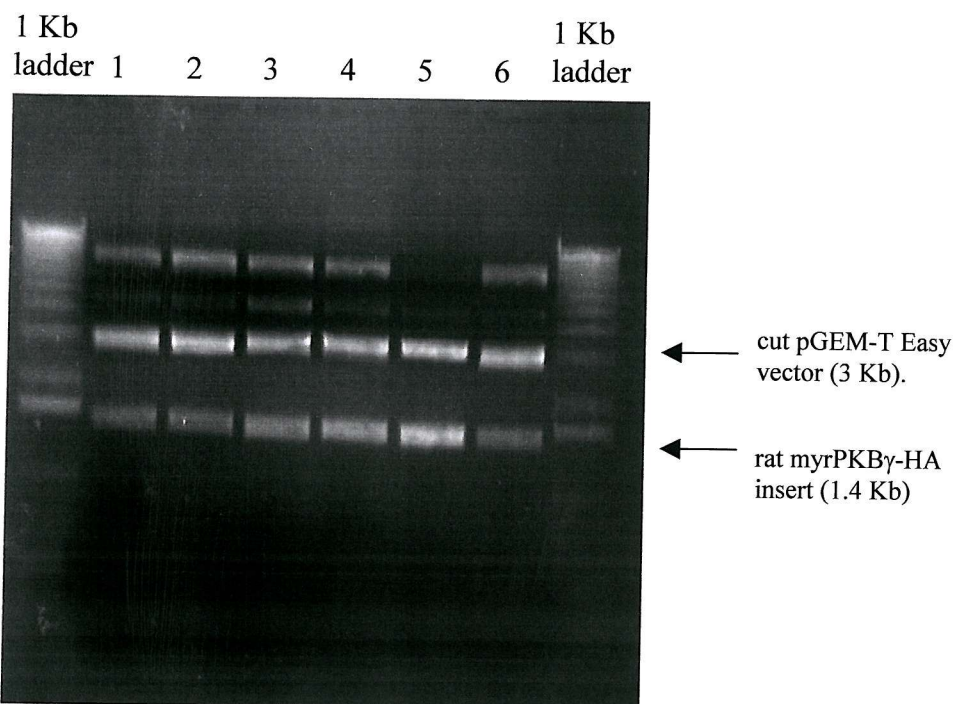
**Figure 3.11**     **Restriction digest analysis of pGEM-T.ratmyrPKBγ-HA transformations.**



**Figure 3.12**     **Restriction digest analysis of rat pcDNA.myrPKBγ-HA transformations.**

**ATG**GGCTGTGTCTGCAGCTCAAACCCTGAAGATGACAGCGATGTTACCATCGTTAAAGAAGGCTGGGTTCAGAAG

AGGGGAGAATATATAAAAAATTGGAGGCCAAGATACTTCCTTTTGAAGACAGACGGCTCATTCATAGGCTATAAA

GAGAAACCTCAAGATGTGGACTTACCTTATCCCCTCAACAACTTCTCAGTGGCAAAATGTCAATTAATGAAAACA

GAACGACCAAAGCCAAATACATTTATTATCAGATGTCTTCAGTGGACCACTGTTATAGAGAGAACATTTCATGTA

GATACTCCAGAGGAAAGAGAAGAATGGACAGAAGCTATCCAAGCTGTAGCCGATCGACTGCAGAGGCAAGAAGAG

GAGAGGATGAATTGTAGTCCAACGTCACAGATTGATAATATTGGAGAGGAAGAGATGGATGCATCTACAACCCAT

CATAAAAGAAAGACAATGAATGATTTTGACTATTTGAAACTGCTAGGTAAAGGCACTTTTGGGAAGGTTATTTTG

GTTCGAGAGAAGGCAAGTGGAAAATACTACGCTATGAAGATTCTGAAGAAAGAAGTCATTATCGCAAAGGATGAA

GTGGCACATACTCTGACTGAGAGCAGAGTTCTAAAGAACACCAGACATCCATTTCTAACATCCTTGAAATATTCC

TTCCAGACAAAAGACCGTTTGTGTTTTGTGATGGAATATGTTAATGGGGGAGAGCTGTTTTTCCATTTGTCGAGA

GAGCGGGTGTTCTCTGAGGACCGCACACGTTTCTATGGTGCAGAAATTGTCTCTGCTTTGGACTATCTACATTCT

GGAAAGATTGTGTACCGAGATCTCAAGTTGGAGAATTTGATGCTAGATAAGGATGGCCATATAAAAATTACGGAT

TTTGGACTTTGCAAAGAAGGGATCACAGATGCAGCTACCATGAAGACATTCTGTGGTACACCAGAGTATCTGGCA

CCAGAGGTATTAGAAGATAATGACTATGGCCGAGCTGTGGACTGGTGGGGCCTAGTGTTGTCATGTATGAAATGA

TGTGTGGGAGGTTGCCTTTCTACAACCAGGATCATGAGAAACTCTTTGAACTAATACTCATGGAAGACATCAAAT

TCCCCCGAACACTCTTCTTCAGATGCAAAGTCATTGCTTTCAGGGCTCTTGATAAAGGATCCAAATAAACGCCTT

GGTGGAGGGCCAGATGATGCAAAAGAGATAATGAGGCACAGTTTCTTTTCTGGAGTAAACTGGCAGGACGTATAT

GACAAAAAGCTTGTACCTCCTTTTAAGCCTCAAGTAACATCTGAGACAGACACCCGATATTTCGATGAAGAATTT

ACAGCTCAGACTATTCAATAACACCACCTGAAAAGTGGCACTATACCCATACGATGTGCCAGATTACGCC**TAA**

HA tag

**Figure 3.13    Full length sequencing of rat myrPKBγ-HA.**

Full length sequencing 5'-3'of rat myrPKBγ-HA (sequencing by Oswell). The translational initiation and stop codons ATG and TAA respectively are highlighted in bold. The N-terminal Lck myristoylation sequence is shown in blue and C-terminal HA tag sequence is shown in red.

## 3.4 Site-directed mutagenesis of rat PKBγ to produce constitutively active rat PKBγ(T305D)-HA.

### 3.4.1 Construction of rat PKBγ(T305D)-HA by site-directed mutagenesis using PCR.

The second constitutively active mutant of rat PKBγ was produced by site directed mutagenesis of the Thr305 phosphorylation site to Asp305. A series of four PCR reactions were used (Table 3.2). First, two separate PCR reactions were set up using rat brain cDNA as the template DNA and the mutating T305D primers to produce 940bp and 480bp products respectively. Next, these two fragments were combined as the template DNA in a third PCR reaction using the rat KpnI forward and rat HA reverse primer2 to produce rat PKBγ(T305D)-1/2HA. A fourth and final PCR reaction was used to construct full-length rat PKBγ(T305D)-HA with a 5' Kpn I site and 3' Xba I site (Figure 3.14).

**Table 3.2      PCR reactions to construct rat PKBγ(T305D)-HA.**

| PCR REACTION | TEMPLATE DNA | FORWARD PRIMER | REVERSE PRIMER |
|---|---|---|---|
| 1. Fragment 1 (940bp). | 2μl rat brain cDNA library | rat Kpn I forward primer | T305D reverse primer |
| 2. Fragment 2 (480bp) | 2μl rat brain cDNA library | T305D forward primer | rat HA reverse primer 1 |
| 3. PKBγ(T305D)-1/2HA | 1μl each of gel-purified fragments 1 and 2. | rat Kpn I forward primer | rat HA reverse primer 2 |
| 4. PKBγ(T305D)-HA | 1μl gel-purified reaction 3 PCR product. | rat Kpn I forward primer | Xba I HA reverse primer |

### 3.4.2 Direct ligation of rat PKBγ(T305D)-HA into the pcDNA3.1 mammalian expression vector and sequencing.

A change in my method of cloning and sub-cloning the PCR fragments was the use of the restriction sites Kpn I within the forward PCR primer and Xba I within the reverse primer. This results in PCR products containing these two different restriction sites at the 5'and 3'ends. The major advantage of doing this was that the PCR products could then be cloned directly into the pcDNA3.1 vector alleviating the need for ligation into the pGEM-T Easy vector first. In addition to this method being much faster, the PCR products are always inserted in the correct orientation.

The PKBγ(T305D)-HA PCR product was gel purified and digested with Kpn I and Xba I, then ligated directly into Kpn I/Xba I digested pcDNA3.1 expression vector. The ligation reaction was then used to transform calcium competent JM109 *E.coli* by heat shock. Six ampicillin resistant bacterial colonies were picked for mini-prep and restriction digest analysis using the restriction enzymes Kpn I and Xba I. Plasmids from colonies 2, 3 and 5 contain the rat PKBγ(T305D)-HA insert (Figure 3.15). Glycerol stocks were made from colony 2.

The pcDNA.ratPKBγ(T305D)-HA construct was sequenced by primer walking using the T7 forward primer as the starting primer. Figure 3.16 confirms that the construct is full length wild-type rat PKBγ with the Thr305 residue mutated to Asp305 and a C-terminal HA tag.

**Figure 3.14    Construction of rat PKBγ(T305D)-HA by site-directed mutagenesis using the polymerase chain reaction.**

**(A)** PCR of mutant fragments 1 and 2 of rat PKBγ changing Thr305 to Asp. Each PCR reaction was carried out using rat brain cDNA as the template DNA and the mutating T305D reverse and forward primers respectively. PCR conditions after initial denaturing at 95°C for 2mins were: 95°C for 30s; annealing at 60°C for 30s; then extension at 72°C for 2mins. This was repeated for 30 cycles with a final extension at 72°C for 5mins. 10μl of each PCR reaction was loaded onto a 1% agarose gel and subjected to electrophoresis for 1 hour at 100V.

**(B)** The two fragments were gel purified and used as template DNA in a third PCR to produce rat PKBγ(T305D)-1/2HA with half the HA tag. This PCR product was used as the template DNA in a fourth and final PCR reaction along with the rat Kpn I forward and Xba I HA reverse primers to produce full length rat PKBγ(T305D)-HA. PCR conditions for both reactions after initial denaturing at 95°C for 2mins were: 95°C for 30s; annealing at 60°C for 30s; then extension at 72°C for 4mins. This was repeated for 30 cycles with a final extension at 72°C for 5mins. 10μl of each PCR reaction from the reactions was loaded onto a 1% agarose gel and subjected to electrophoresis for 1 hour at 100V.
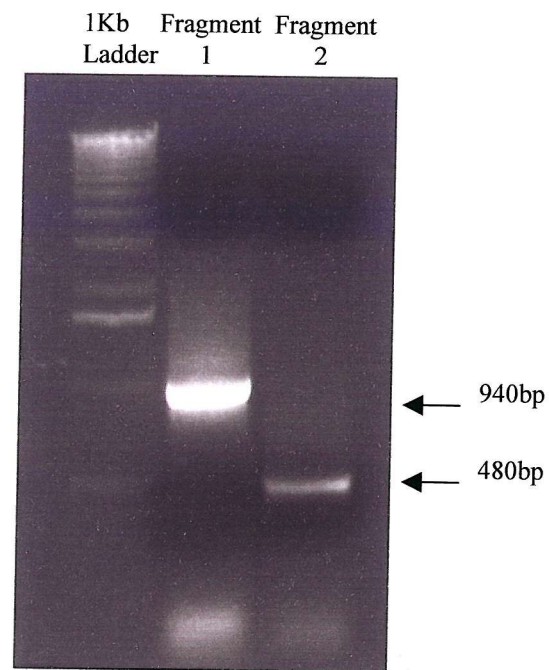
Figure 3.14(A). PCR of mutating fragments 1 and 2.
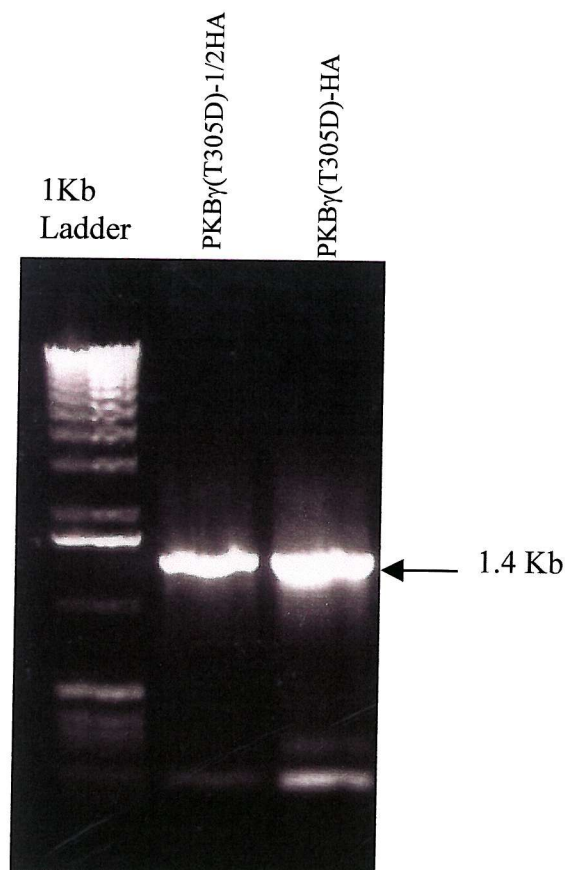


Figure 3.1(B). PCR of full length rat PKBγ(T305D)-HA.
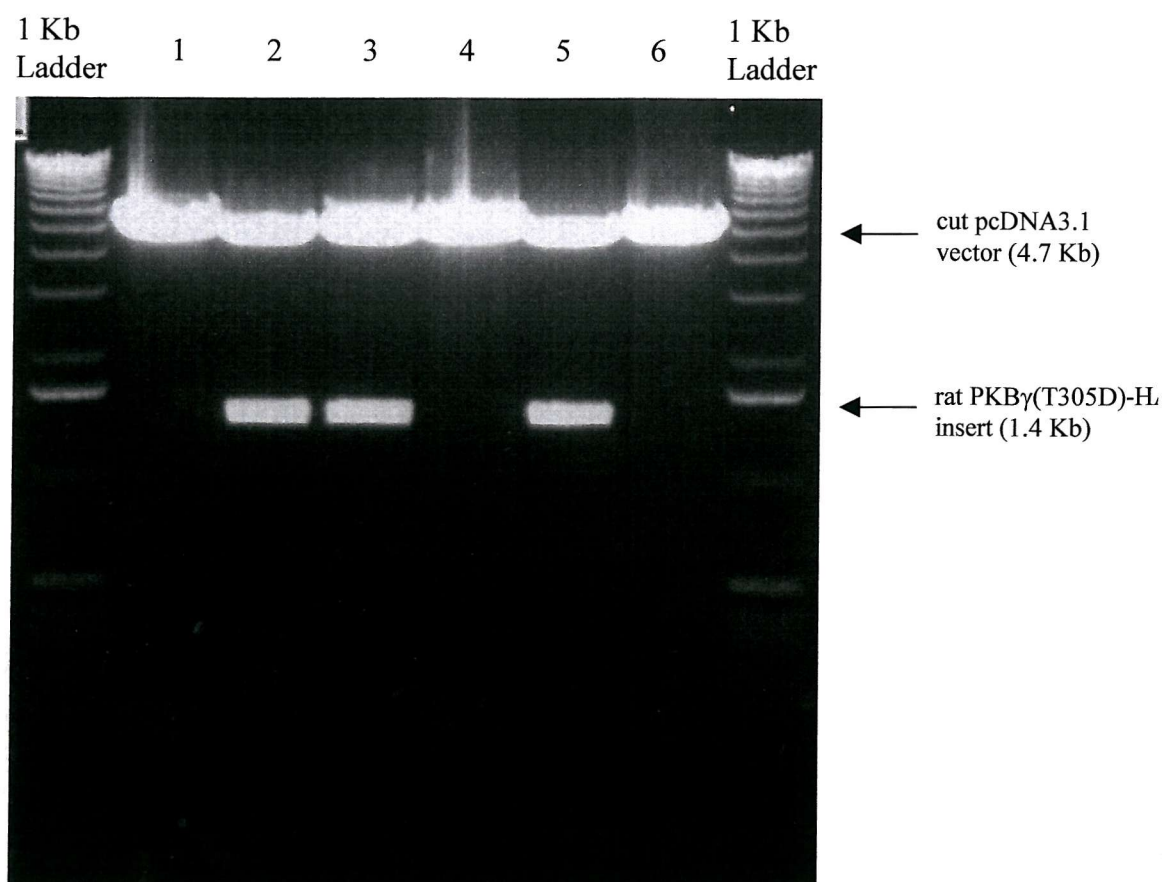
**Figure 3.15 Restriction digest analysis of rat pcDNA.PKBγ(T305D)-HA transformations.**

Following ligation of rat PKBγ(T305D)-HA into the pcDNA3.1 mammalian expression vector and transformation of JM109 calcium competent *E. coli*, 6 ampicillin resistant bacterial colonies were picked and their plasmid DNA mini-prepped (Promega). Restriction digests were set up using 5μl of plasmid DNA and 0.5μl each of Kpn I and Xba I restriction enzymes. Colonies 2,3 and 5 were found to contain the rat PKBγ(T305D)-HA insert in the correct orientation. Glycerol stocks were prepared from Colony 2.

**ATG**AGCGATGTTACCATCGTTAAAGAAGGCTGGGTTCAGAAGAGGGGAGAATATATAAAAAATTGGAGGCCAAGA

TACTTCCTTTTGAAGACAGACGGCTCATTCATAGGCTATAAAGAGAAACCTCAAGATGTGGACTTACCTTATCCC

CTCAACAACTTCTCAGTGGCAAAATGTCAATTAATGAAAACAGAACGACCAAAGCCAAATACATTTATTATCAGA

TGTCTTCAGTGGACCACTGTTATAGAGAGAACATTTCATGTAGATACTCCAGAGGAAAGAGAAGAATGGACAGAA

GCTATCCAAGCTGTAGCCGATCGACTGCAGAGGCAAGAAGAGGAGAGGATGAATTGTAGTCCAACGTCACAGATT

GATAATATTGGAGAGGAAGAGATGGATGCATCTACAACCCATCATAAAAGAAAGACAATGAATGATTTTGACTAT

TTGAAACTGCTAGGTAAAGGCACTTTTGGGAAGGTTATTTTGGTTCGAGAGAAGGCAAGTGGAAAATACTACGCT

ATGAAGATTCTGAAGAAGAAGTCATTATCGCAAAGGATGAAGTGGCACATACTCTGACTGAGAGCAGAGTTCTA

AAGAACACCAGACATCCATTTCTAACATCCTTGAAATATTCCTTCCAGACAAAAGACCGTTTGTGTTTTGTGATG

GAATATGTTAATGGGGGAGAGCTGTTTTTCCATTTGTCGAGAGAGCGGGTGTTCTCTGAGGACCGCACACGTTTC

TATGGTGCAGAAATTGTCTCTGCTTTGGACTATCTACATTCTGGAAAGATTGTGTACCGAGATCTCAAGTTGGAG

AATTTGATGCTAGATAAGGATGGCCATATAAAAATTACGGATTTTGGACTTTGCAAAGAAGGGATCACAGAT<span style="color:blue">GCA</span>

<span style="color:blue">GCTACCATGAAG</span><span style="color:blue">**<u>GAC</u>**</span><span style="color:blue">TTCTGTGGTACACCA</span>GAGTATCTGGCACCAGAGGTATTAGAAGATAATGACTATGGCCGA

GCTGTGGACTGGTGGGGCCTAGGTGTTGTCATGTATGAAATGATGTGTGGGAGGTTGCCTTTCTACAACCAGGAT

CATGAGAAACTCTTTGAACTAATACTCATGGAAGACATCAAATTTCCCCGAACACTCTCTTCAGATGCAAAGTCA

TTGCTTTCAGGGCTCTTGATAAAGGATCCAAATAAACGCCTTGGTGGAGGGCCAGATGATGCAAAAGAGATAATG

AGGCACAGTTTCTTTTCTGGAGTAAACTGGCAGGACGTATATGACAAAAAGCTTGTACCTCCTTTTAAGCCTCAA

GTAACATCTGAGACAGACACCAGATATTTCGATGAAGAATTTACAGCTCAGACTATTACAATAACACCACCTGAA

AAGTGTCCACTA<span style="color:red">TACCCATACGATGTGCCAGATTACGCC</span>**<span style="color:red">TAA</span>** |
<span style="color:red">HA tag</span>

**Figure 3.16    Full length sequencing of rat PKBγ(T305D)-HA.**

Full length sequencing 5'-3'of rat PKBγ(T305D)-HA (sequencing by Oswell). The translational initiation and stop codons ATG and TAA respectively are highlighted in bold. The mutated sequence is shown in blue with the GAC coding for Asp305 underlined. The C-terminal HA tag sequence is shown in red.

## 3.5 Cloning of mouse wild-type PKBγ by PCR.

As mentioned in the introduction to this chapter, early in 1999 two groups working independently published the sequences for mouse and human PKBγ (Nakatani et al., 1999; Brodbeck et al., 1999). Interestingly, in contrast to the rat PKBγ protein, both human and mouse PKBγ contain a carboxy-tail region homologous to that of PKBα and PKBβ. This region is 25 amino acids long and includes the regulatory serine phosphorylation site (Ser$^{472}$) corresponding to Ser$^{473}$ present in PKBα. With this in mind I decided to clone the mouse PKBγ in order to investigate any similarities or differences to the truncated rat PKBγ.

The method used to clone mouse PKBγ was essentially the same as used for the rat PKBγ. However there were two notable differences, firstly, in order to ligate the gene directly into the pcDNA vector the restriction sites Kpn I and Xba I were added to the 5'and 3'-ends of the gene respectively; and secondly, the HA tag was added onto the C-terminus.

### 3.5.1 PCR of mouse PKBγ-HA.

Total RNA was isolated from mouse brain tissue and then used to produce cDNA representing all of the genes expressed in the mouse brain. This cDNA was then used as template in the first of three successive PCR reactions as detailed in Table 3.3 below. The final 1.4Kb PCR product is full-length mouse wild-type PKBγ with a C-terminal HA tag (mouse PKBγ-HA) (Figure 3.17).

**Table 3.3 PCR reactions to construct mouse PKBγ-HA.**

| PCR REACTION | TEMPLATE DNA | FORWARD PRIMER | REVERSE PRIMER |
|---|---|---|---|
| 1. mouse PKBγ | 2µl mouse brain cDNA library | mouse wtPKBγ forward primer | mouse wtPKBγ reverse primer |
| 2. mouse PKBγ-1/2HA | 1µl gel pure reaction 1. PCR product | mouse wtPKBγ forward primer | mouse wtPKBγ-1/2HA reverse primer |
| 3. mouse PKBγ-HA | 1µl gel pure reaction 2. PCR product | mouse wtPKBγ forward primer | Xba I HA reverse primer |

## 3.5.2 Ligation of mouse PKBγ-HA into the pcDNA mammalian expression vector and sequencing by primer walking.

The mouse PKBγ-HA PCR product from reaction 3 was gel purified and digested using the restriction enzymes Kpn I and Xba I. The resulting digested product was then ligated directly into Kpn I/ Xba I digested pcDNA3.1 vector and used to transform JM109 *E.coli* by heat shock. six ampicillin resistant bacterial colonies were picked for restriction digest analysis. All of the colonies picked with the exception of colony 4 contained mouse pcDNA.PKBγ-HA (Figure 3.18). Glycerol stocks were prepared from colony 1.

The mouse pcDNA.PKBγ-HA plasmid was sequenced by primer walking using the T7 forward primer as the initiation primer (Figure 3.19). The sequencing confirmed that the gene was indeed mouse wild-type PKBγ with a C-terminal HA tag as expected.

**Figure 3.17    The cloning of mouse PKBγ-HA by PCR.**


Total RNA (2μg) extracted from a mouse brain using the RNazol reagent (Biogenesis) was used in a 25μl reaction to produce a cDNA library representing all the expressed genes in the rat brain tissue. Three PCR reactions were carried out to clone mouse PKBγ-HA (see table 3.3 for details). 2μl of cDNA was then used as template in the first PCR reaction along with the mouse PKBγ gene-specific forward and reverse primers to clone mouse PKBγ. Half of the HA tag was added onto the C-terminal of the mouse PKBγ gene using a second PCR reaction. The third and final PCR reaction yeilded full length mouse PKBγ-HA. For all three reactions, PCR conditions after initial denaturing at 95°C for 2mins were: 95°C for 30s; annealing at 60°C for 30s; then extension at 72°C for 4mins. This was repeated for 30 cycles with a final extension at 72°C for 5mins. 10μl of each PCR reaction was loaded onto a 1% agarose gel and subjected to electrophoresis for 1 hour at 100V.




**Figure 3.18    Restriction digest analysis of mouse pcDNA.PKBγ-HA transformations.**


Following ligation of mouse PKBγ-HA into the pcDNA3.1 mammalian expression vector and transformation of JM109 calcium competent *E. Coli*, 6 ampicillin resistant bacterial colonies were picked and their plasmid DNA mini-preped (Promega). Restriction digests were set up using 5μl of plasmid DNA and 0.5μl each of  Kpn I and Xba I restriction enzymes. Colonies 1,2,3,5 and 6 were found to contain the mouse pcDNA.PKBγ-HA construct in the correct orientation. Glycerol stocks were prepared from Colony 1.
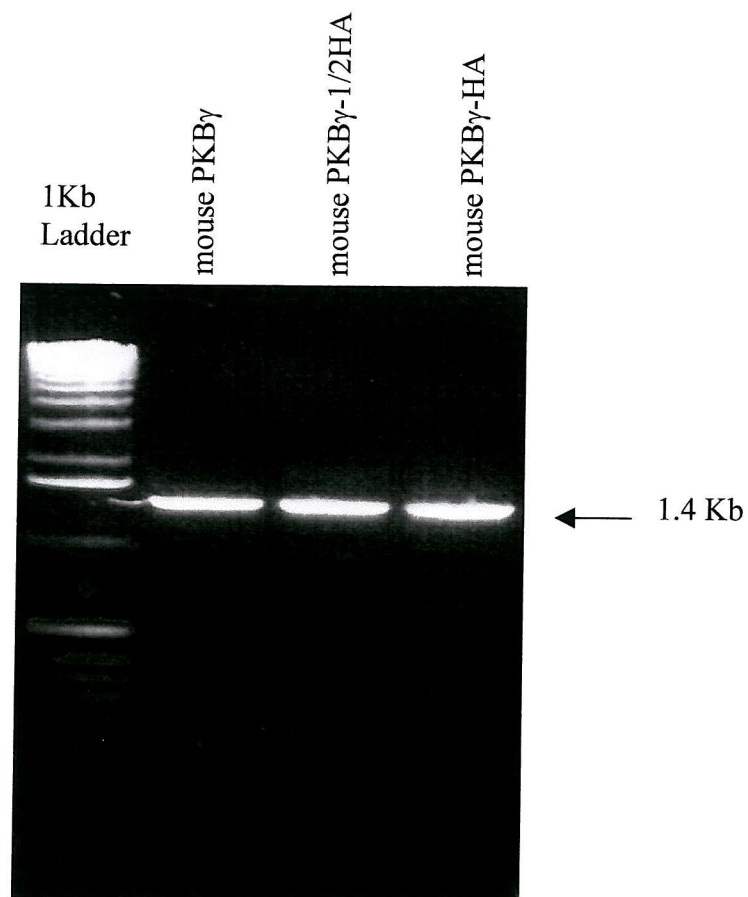
**Figure 3.17** **The cloning of mouse PKBγ-HA by PCR.**
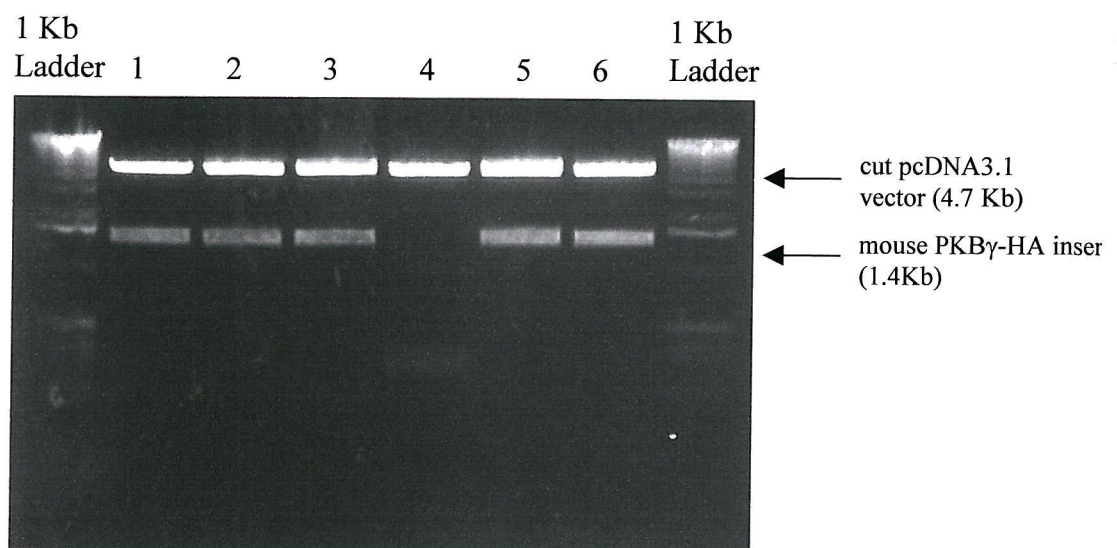


**Figure 3.18** **Restriction digest analysis of mouse pcDNA.PKBγ-HA transformations.**

**ATG**AGCGATGTTACCATTGTGAAAGAAGGTTGGGTTCAGAAGAGGGGAGAATATATAAAAAACTGGAGGCCAAGA

TACTTCCTTTTGAAGACAGATGGCTCATTCATAGGCTATAAGGAGAAACCTCAAGATGTGGACTTACCTTATCCC

CTCAACAACTTCTCAGTGGCAAAATGTCAGTTAATGAAAACAGAACGACCAAAGCCAAATACATTTATTATCAGA

TGTCTTCAGTGGACCACTGTTATAGAGAGAACATTTCATGTAGATACACCAGAGGAAAGAGAAGAGTGGACGGAA

GCTATCCAAGCCGTAGCCGACCGATTGCAGAGGCAAGAGGAGGAGAGGATGAATTGTAGCCCAACCTCACAGATT

GATAATATAGGAGAAGAAGAGATGGATGCGTCTACAACCCATCATAAAAGAAAGACGATGAATGATTTTGACTAT

TTGAAACTACTAGGTAAAGGCACTTTTGGGAAAGTTATTTTGGTTCGAGAGAAGGCAAGTGGAAAATACTATGCT

ATGAAGATTCTGAAGAAGAAGTCATTATTGCAAAGGATGAAGTGGCACACACTCTTACTGAAAGCAGAGTACTA

AAGAACACCAGACATCCATTTTTAACATCCTTGAAATATTCCTTCCAGACAAAAGACCGTTTGTGTTTTGTGATG

GAATATGTTAATGGCGGAGAGCTGTTTTTCCATTTGTCGAGAGAGCGAGTGTTCTCTGAGGACCGCACACGTTTC

TATGGTGCAGAAATTGTCTCTGCTTTGGACTATCTACATTCTGGAAAGATTGTGTACCGTGATCTCAAGTTGGAG

AATTTGATGCTAGATAAGGATGGCCATATAAAAATTACGGATTTTGGGCTTTGCAAAGAAGGGATCACAGATGCA

GCTACCATGAAGACATTCTGTGGCACACCAGAGTACCTGGCACCAGAGGTATTAGAAGATAATGACTATGGCCGA

GCCGTGGACTGGTGGGGCTTAGGTGTTGTCATGTATGAAATGATGTGTGGAAGGTTGCCTTTCTACAACCAGGAT

CATGAGAAACTCTTTGAATTAATACTAATGGAAGACATTAAATTCCCCCGAACACTCTCTTCAGATGCAAAATCA

TTGCTTTCAGGGCTCTTGATAAAGGATCCAAATAAACGCCTTGGTGGAGGGCCAGATGATGCAAAAGAAATCATG

AGGCATAGTTTTTTTTCTGGAGTAAACTGGCAAGATGTATATGACAAAAAGCTTGTACCTCCTTTTAAGCCTCAA

GTAACATCTGAAACAGACACCCGATATTTTGATGAAGAATTTACAGCTCAGACTATTACAATAACACCACCTGAA

AAGTATGACGACGACGGCATGGACGGCATGGACAACGAGCGGCGGCCACACTTCCCTCAGTTCTCCTACTCTGCA

AGCGGACGGGAA<span style="color:red">TACCCATACGATGTGCCAGATTACGCC</span>**TAA**

<span style="color:red">HA tag</span>

**Figure 3.19    Full length sequencing of mouse PKBγ-HA.**

Full length sequencing 5'-3'of mouse PKBγ-HA (sequencing by Oswell). The translational
initiation and stop codons ATG and TAA respectively are highlighted. The C-terminal HA
tag sequence (in red) encodes the amino acid sequence recognised by the anti-HA antibody
(U.B.I).

## 3.6 Construction of *N*-myristoylated constitutively active mouse PKBγ (mouse myrPKBγ-HA).

### 3.6.1 PCR cloning of mouse myrPKBγ-HA.

To create the membrane targeted, constitutively active, mouse PKBγ (mouse myrPKBγ-HA), the same mouse Lck myristoylation/palmitoylation signal (MGCVCSSNPEDD) as used to create rat myrPKBγ-HA was added to the N-terminus of mouse PKBγ. As before, the HA tag was at the C-terminus. This was achieved using 2 successive PCR reactions (see Table 3.4 for details). Note that the PCR conditions for these reactions were different than for previous reactions. The annealing temperature had to be increased to 72°C in order to produce a specific product. The resulting PCR fragment was 1.4Kb and named mouse myrPKBγ-HA (Figure 3.20). As for mouse PKBγ-HA, the mouse myrPKBγ-HA has a 5'Kpn I site and a 3'Xba I site to aid ligation into the pcDNA expression vector.

### Table 3.4 PCR reactions to construct mouse myrPKBγ-HA

| PCR REACTION | TEMPLATE DNA | FORWARD PRIMER | REVERSE PRIMER |
|---|---|---|---|
| **1.** mouse 1/2myrPKBγ-HA | 10ng mouse pcDNA.PKBγ-HA | mouse 1/2myrPKBγ forward primer | XbaI HA reverse primer |
| **2.** mouse myrPKBγ-HA | 1μl gel pure reaction 2. PCR product | mouse KpnI myr forward primer | XbaI HA reverse primer |

### 3.6.2 Ligation of mouse myrPKBγ-HA into the pcDNA3.1 mammalian expression vector and sequencing by primer walking.

The 1.4Kb PCR product mouse myrPKBγ-HA was gel purified and digested using the restriction enzymes Kpn I and Xba I. The digested product was then ligated directly into Kpn I/Xba I digested pcDNA3.1 vector. The ligations were then used to transform calcium competent JM109 *E. coli* by heat shock treatment. Six ampicillin resistant colonies were picked and grown up, their plasmid DNA minipreped and analysed by restriction digest (Figure 3.21). All 6 colonies were found to contain the mouse pcDNA.myrPKBγ-HA construct. Colony 1 was used to prepare glycerol stocks.

The mouse pcDNA.myrPKBγ-HA construct was sequenced by primer walking as before and found to be full length mouse PKBγ with a N-terminal myristoylation/palmitoylation sequence and the C-terminal HA tag as expected (Figure 3.22).

**Figure 3.20    PCR cloning of mouse myrPKBγ-HA.**

Using mouse pcDNA.PKBγ-HA as the template DNA, two successive PCR reactions were set up to clone mouse myrPKBγ-HA as described in table 3.4. PCR conditions after initial denaturing at 95°C for 2mins were: 95°C for 30s; annealing and extention at 72°C for 4mins. This was repeated for 30 cycles with a final extension at 72°C for 5mins. The final PCR product from reaction 2 is mouse myrPKBγ-HA. 10μl of each PCR reaction was loaded onto a 1% agarose gel and subjected to electrophoresis for 1 hour at 100V.

**Figure 3.21    Restriction digest analysis of mouse pcDNA.myrPKBγ-HA transformations.**

Following ligation of mouse myrPKBγ-HA into the pcDNA3.1 mammalian expression vector and transformation of JM109 calcium competent *E. Coli*, 6 ampicillin resistant bacterial colonies were picked and their plasmid DNA mini-preped (Promega). Restriction digests were set up using 5μl of plasmid DNA and 0.5μl each of  Kpn I and Xba I restriction enzymes. All 6 colonies were found to contain the mouse pcDNA.myrPKBγ-HA construct in the correct orientation. Glycerol stocks were prepared from Colony 1.

**Figure 3.20    PCR cloning of mouse myrPKBγ-HA.**



**Figure 3.21    Restriction    digest    analysis    of    mouse    pcDNA.myrPKBγ-HA transformations.**

**ATG**GGCTGTGTCTGCAGCTCAAACCCTGAAGATGACAGCGATGTTACCATTGTGAAAGAAGGTTGGGTTCAGAAG

AGGGGAGAATATATAAAAAACTGGAGGCCAAGATACTTCCTTTTGAAGACAGATGGCTCATTCATAGGCTATAAG

GAGAAACCTCAAGATGTGGACTTACCTTATCCCCTCAACAACTTCTCAGTGGCAAAATGTCAGTTAATGAAAACA

GAACGACCAAAGCCAAATACATTTATTATCAGATGTCTTCAGTGGACCACTGTTATAGAGAGAACATTTCATGTA

GATACACCAGAGGAAAGAGAAGAGTGGACGGAAGCTATCCAAGCCGTAGCCGACCGATTGCAGAGGCAAGAGGAG

GAGAGGATGAATTGTAGCCCAACCTCACAGATTGATAATATAGGAGAAGAAGAGATGGATGCGTCTACAACCCAT

CATAAAAGAAAGACGATGAATGATTTTGACTATTTGAAACTACTAGGTAAAGGCACTTTTGGGAAAGTTATTTTG

GTTCGAGAGAAGGCAAGTGGAAAATACTATGCTATGAAGATTCTGAAGAAAGAAGTCATTATTGCAAAGGATGAA

GTGGCACACACTCTTACTGAAAGCAGAGTACTAAAGAACACCAGACATCCATTTTTAACATCCTTGAAATATTCC

TTCCAGACAAAAGACCGTTTGTGTTTTGTGATGGAATATGTTAATGGCGGAGAGCTGTTTTTCCATTTGTCGAGA

GAGCGAGTGTTCTCTGAGGACCGCACACGTTTCTATGGTGCAGAAATTGTCTCTGCTTTGGACTATCTACATTCT

GGAAAGATTGTGTACCGTGATCTCAAGTTGGAGAATTTGATGCTAGATAAGGATGGCCATATAAAAATTACGGAT

TTTGGGCTTTGCAAAGAAGGGATCACAGATGCAGCTACCATGAAGACATTCTGTGGCACACCAGAGTACCTGGCA

CCAGAGGTATTAGAAGATAATGACTATGGCCGAGCCGTGGACTGGTGGGGCTTAGGTGTTGTCATGTATGAAATG

ATGTGTGGAAGGTTGCCTTTCTACAACCAGGATCATGAGAAACTCTTTGAATTAATACTAATGGAAGACATTAAA

TTCCCCCGAACACTCTCTTCAGATGCAAAATCATTGCTTTCAGGGCTCTTGATAAAGGATCCAAATAAACGCCTT

GGTGGAGGGCCAGATGATGCAAAAGAAATCATGAGGCATAGTTTTTTTTCTGGAGTAAACTGGCAAGATGTATAT

GACAAAAAGCTTGTACCTCCTTTTAAGCCTCAAGTAACATCTGAAACAGACACCCGATATTTTGATGAAGAATTT

ACAGCTCAGACTATTACAATAACACCACCTGAAAAGTATGACGACGACGGCATGGACGGCATGGACAACGAGCGG

CGGCCACACTTCCCTCAGTTCTCCTACTCTGCAAGCGGACGGGAA<span style="color:red">TACCCATACGATGTGCCAGATTACGCC**TAA**</span>

**Figure 3.22    Full length sequencing of mouse myrPKBγ-HA.**

Full length sequencing 5'-3'of mouse myrPKBγ-HA (sequencing by Oswell). The translational initiation and stop codons ATG and TAA respectively are highlighted in bold. The N-terminal Lck myristoylation sequence is shown in blue and C-terminal HA tag sequence is shown in red.

## 3.7 Cloning of mouse wild-type PKBα by PCR.

Wild-type mouse PKBα was cloned and the membrane targeted mutant (myrPKBα-HA) constructed as a positive control of the system. Work by Khwaja and co-workers has already shown that membrane targeted PKBα, when stably expressed in MDCK cells, protects the MDCK cells from a form of apoptosis known as aniokis (Khwaja et al., 1997). Therefore this would be a useful control to measure the level of protection against apoptosis provided by the constitutively active mutants of rat and mouse PKBγ when stably expressed in MDCK cells.

### 3.7.1 PCR of mouse PKBα-HA.

The mouse brain cDNA prepared for cloning mouse PKBγ was used as template in the first of three successive PCR reactions as detailed in Table 3.5 below. The final 1.4Kb PCR product is full-length mouse wild-type PKBα with a C-terminal HA tag (mouse PKBα-HA) (Figure 3.23).

**Table 3.5 PCR reactions to construct mouse PKBα-HA.**

| PCR REACTION | TEMPLATE DNA | FORWARD PRIMER | REVERSE PRIMER |
|---|---|---|---|
| **1.** mouse PKBα | 2μl mouse brain cDNA library | mouse wtPKBα forward primer | mouse wtPKBα reverse primer |
| **2.** mouse PKBα-1/2HA | 1μl gel pure reaction **1.** PCR product | mouse wtPKBα forward primer | mouse wtPKBα-1/2HA reverse primer |
| **3.** mouse PKBα-HA | 1μl gel pure reaction **2.** PCR product | mouse wtPKBα forward primer | Xba I HA reverse primer |

## 3.7.2 Ligation of mouse PKBα-HA into the pcDNA mammalian expression vector and sequencing by primer walking.

The mouse PKBα-HA PCR product from reaction 3 was gel purified and digested using the restriction enzymes Kpn I and Xba I. The resulting digested product was then ligated directly into Kpn I/ Xba I digested pcDNA3.1 vector and used to transform JM109 *E.coli* by heat shock. six ampicillin resistant bacterial colonies were picked for restriction digest analysis. All of the colonies picked contained mouse pcDNA.PKBα-HA (Figure 3.24). Glycerol stocks were prepared from colony 1.

The mouse pcDNA.PKBα-HA plasmid was sequenced by primer walking using the T7 forward primer as the initiation primer (Figure 3.25). The sequencing confirmed that the gene was indeed mouse wild-type PKBα with a C-terminal HA tag as expected.

**Figure 3.23    The cloning of mouse PKBα-HA by PCR.**

As for mouse PKBγ, three successive PCR reactions were used to construct mouse PKBα-HA. In the first reaction 2μl of mouse brain cDNA (prepared from mouse brain total RNA as described previously) was used as template DNA along with the mouse PKBα gene-specific forward and reverse primers. The product from this reaction was used as template DNA in a second reaction producing mouse PKBα-1/2HA. The final PCR product from reaction 3 was mouse PKBα-HA. PCR conditions after initial denaturing at 95°C for 2mins were: 95°C for 30s; annealing at 60°C for 30s; then extension at 72°C for 4mins. This was repeated for 30 cycles with a final extension at 72°C for 5mins. These conditions were used for all three PCR reactions. 10μl of each PCR reaction was loaded onto a 1% agarose gel and subjected to electrophoresis for 1 hour at 100V.

**Figure 3.24    Restriction digest analysis of mouse pcDNA.PKBα-HA transformations.**

Following ligation of mouse PKBα-HA into the pcDNA3.1 mammalian expression vector and transformation of JM109 calcium competent *E. Coli*, 6 ampicillin resistant bacterial colonies were picked and their plasmid DNA mini-preped (Promega). Restriction digests were set up using 5μl of plasmid DNA and 0.5μl each of  Kpn I and Xba I restriction enzymes. All 6 colonies were found to contain the mouse pcDNA.PKBα-HA construct in the correct orientation. Glycerol stocks were prepared from Colony 1.
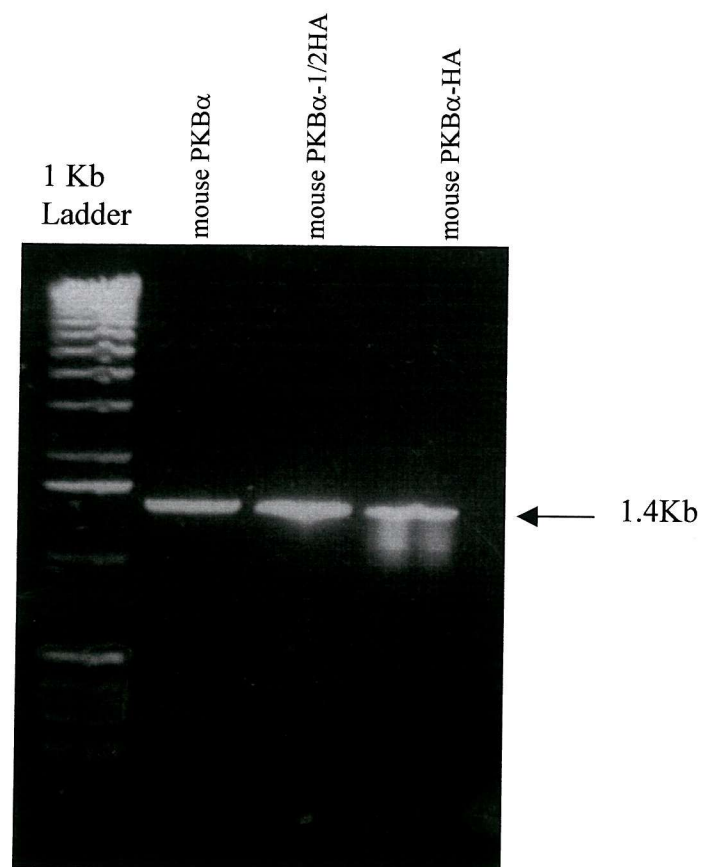
**Figure 3.23    The cloning of mouse PKBα-HA by PCR.**



**Figure 3.24    Restriction digest analysis of mouse pcDNA.PKBα-HA transformations.**

**ATG**AACGACGTAGCCATTGTGAAGGAGGGCTGGCTGCACAAACGAGGGGAATATATTAAAACCTGGCGGCCACGC

TACTTCCTCCTCAAGAACGATGGCACCTTTATTGGCTACAAGGAACGGCCTCAGGATGTGGATCAGCGAGAGTCC

CCACTCAACAACTTCTCAGTGGCACAATGCCAGCTGATGAAGACAGAGCGGCCAAGGCCCAACACCTTTATCATC

CGCTGCCTGCAGTGGACCACAGTCATTGAGCGCACCTTCCATGTGGAAACGCCTGAGGAGCGGGAAGAATGGGCC

ACCGCCATTCAGACTGTGGCCGATGGACTCAAGAGGCAGGAAGAAGAGACGATGGACTTCCGATCAGGCTCACCC

AGTGACAACTCAGGGGCTGAAGAGATGGAGGTGTCCCTGGCCAAGCCCAAGCACCGTGTGACCATGAACGAGTTT

GAGTACCTGAAACTACTGGGCAAGGGCACCTTTGGGAAAGTGATTCTGGTGAAAGAGAAGGCCACAGGCCGCTAC

TATGCCATGAAGATCCTCAAGAAGGAGGTCATCGTCGCCAAGGATGAGGTTGCCCACACGCTTACTGAGAACCGT

GTCCTGCAGAACTCTAGGCATCCCTTCCTTACGGCCCTCAAGTACTCATTCCAGACCCACGACCGCCTCTGCTTT

GTCATGGAGTATGCCAACGGGGGCGAGCTCTTCTTCCACCTGTCTCGAGAGCGCGTGTTCTCCGAGGACCGGGCC

CGCTTCTATGGTGCGGAGATTGTGTCTGCCCTGGACTACTTGCACTCCGAGAAGAACGTGGTGTACCGGGACCTG

AAGCTGGAGAACCTCATGCTGGACAAGGACGGGCACATCAAGATAACGGACTTCGGGCTGTGCAAGGAGGGGATC

AAGGATGGTGCCACTATGAAGACATTCTGCGGAACGCCGGAGTACCTGGCCCCTGAGGTGCTGGAGGACAACGAC

TACGGCCGTGCAGTGGACTGGTGGGGGCTGGGCGTGGTCATGTATGAGATGATGTGTGGCCGCCTGCCCTTCTAC

AACCAGGACCACGAGAAGCTGTTCGAGCTGATCCTCATGGAGGAGATCCGCTTCCCGCGCACACTCGGCCCTGAG

GCCAAGTCCCTGCTCTCCGGGCTGCTCAAGAAGGACCCTACACAGAGGCTCGGTGGGGGCTCTGAGGATGCCAAG

GAGATCATGCAGCACCGGTTCTTTGCCAACATCGTGTGGCAGGATGTGTATGAGAAGAAGCTGAGCCCACCTTTC

AAGCCCCAGGTCACCTCTGAGACTGACACCAGGTATTTCGATGAGGAGTTCACAGCTCAGATGATCACCATCACG

CCGCCTGATCAAGATGACAGCATGGAGTGTGTGGACAGTGAGCGGAGGCCGCACTTCCCCCAGTTCTCCTACTCA

GCCAGTGGCACAGCC<span style="color:red">TACCCATACGATGTGCCAGATTACGCC</span>**TAA**

<span style="color:red">HA tag</span>

**Figure 3.25     Full length sequencing of mouse PKBα-HA.**

Full length sequencing 5'-3'of mouse PKBα-HA (sequencing by Oswell). The translational

initiation and stop codons ATG and TAA respectively are highlighted. The C-terminal HA

tag sequence (in red) encodes the amino acid sequence recognised by the anti-HA antibody

(U.B.I).

## 3.8 Construction of _N_-myristoylated constitutively active mouse PKBα (mouse myrPKBα-HA).

### 3.8.1 PCR cloning of mouse myrPKBα-HA.

Two successive PCR reactions (Table 3.6) were used to create the membrane targeted, constitutively active, mouse PKBα (mouse myrPKBα-HA). Note that as for the mouse myrPKBγ-HA, the annealing temperature had to be increased to 72°C in order to produce a specific product. The resulting PCR fragment was 1.4Kb and named mouse myrPKBα-HA (Figure 3.26). As before, the mouse myrPKBα-HA has a 5'Kpn I site and a 3'Xba I site to aid ligation into the pcDNA expression vector.

**Table 3.6 PCR reactions to construct mouse myrPKBα-HA**

| PCR REACTION | TEMPLATE DNA | FORWARD PRIMER | REVERSE PRIMER |
|---|---|---|---|
| 1. mouse 1/2myrPKBα-HA | 10ng mouse pcDNA.PKBα-HA | mouse 1/2myrPKBα forward primer | Xba I HA reverse primer |
| 2. mouse myrPKBα-HA | 1μl gel pure reaction 2. PCR product | mouse KpnI myr forward primer | Xba I HA reverse primer |

### 3.8.2 Ligation of mouse myrPKBα-HA into the pcDNA3.1 mammalian expression vector and sequencing by primer walking.

The 1.4Kb PCR product mouse myrPKBα-HA was gel purified and digested using the restriction enzymes Kpn I and Xba I. The digested product was then ligated directly into

KpnI/XbaI digested pcDNA3.1 vector. The ligations were then used to transform calcium competent JM109 *E. coli* by heat shock treatment. Six ampicillin resistant colonies were picked and grown up, their plasmid DNA minipreped and analysed by restriction digest (Figure 3.27). All 6 colonies were found to contain the mouse pcDNA.myrPKBα-HA construct. Colony 1 was used to prepare glycerol stocks.

The mouse pcDNA.myrPKBα-HA construct was sequenced by primer walking as before and found to be full length mouse PKBα with a N-terminal myristoylation/palmitoylation sequence and the C-terminal HA tag as expected (Figure 3.28).

**Figure 3.26    PCR cloning of mouse myrPKBα-HA.**

Using mouse pcDNA.PKBα-HA as the template DNA, 2 successive PCR reactions were set up to clone mouse myrPKBα-HA as described in table 3.6. PCR conditions after initial denaturing at 95°C for 2mins were: 95°C for 30s; annealing and extention at 72°C for 4mins. This was repeated for 30 cycles with a final extension at 72°C for 5mins. The final PCR product from reaction 2 is mouse myrPKBα-HA. 10μl of each PCR reaction was loaded onto a 1% agarose gel and subjected to electrophoresis for 1 hour at 100V.
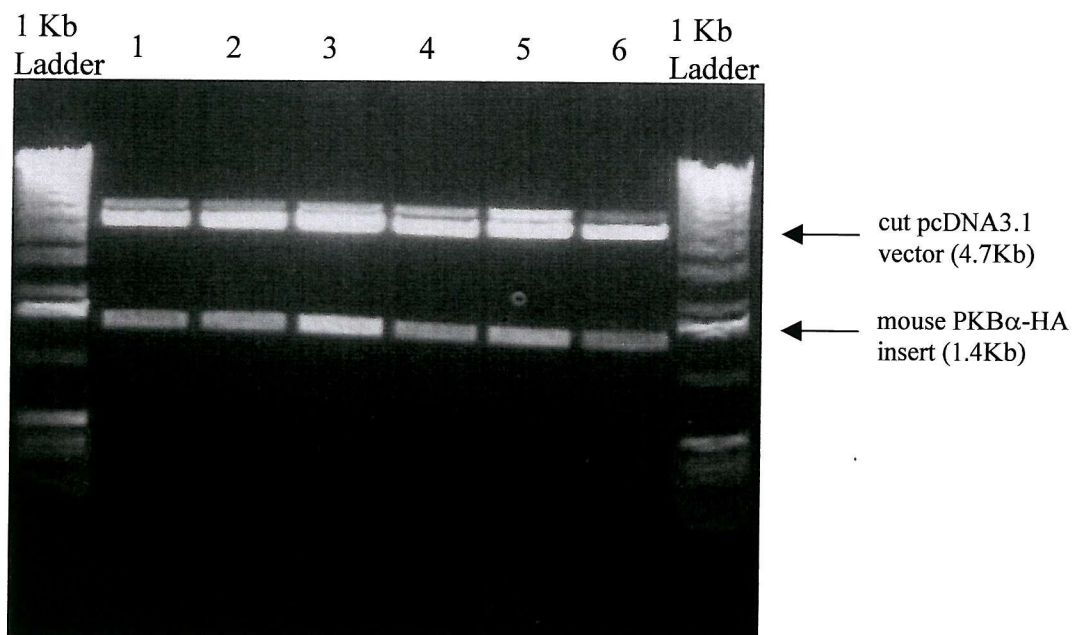
**Figure 3.27    Restriction digest analysis of mouse pcDNA.myrPKBα-HA transformations.**

Following ligation of mouse myrPKBα-HA into the pcDNA3.1 mammalian expression vector and transformation of JM109 calcium competent *E. Coli*, 6 ampicillin resistant bacterial colonies were picked and their plasmid DNA mini-preped (Promega). Restriction digests were set up using 5μl of plasmid DNA and 0.5μl each of Kpn I and Xba I restriction enzymes. All 6 colonies were found to contain the mouse pcDNA.myrPKBα-HA construct in the correct orientation. Glycerol stocks were prepared from Colony 1.

**Figure 3.26   PCR cloning of mouse myrPKBα-HA.**



**Figure 3.27   Restriction   digest   analysis   of   mouse   pcDNA.myrPKBα-HA transformations.**

ATGGGCTGTGTCTGCAGCTCAAACCCTGAAGATGACATGAACGACGTAGCCATTGTGAAGGAGGGCTGGCTGCAC

AAACGAGGGGAATATATTAAAACCTGGCGGCCACGCTACTTCCTCCTCAAGAACGATGGCACCTTTATTGGCTAC

AAGGAACGGCCTCAGGATGTGGATCAGCGAGAGTCCCCACTCAACAACTTCTCAGTGGCACAATGCCAGCTGATG

AAGACAGAGCGGCCAAGGCCCAACACCTTTATCATCCGCTGCCTGCAGTGGACCACAGTCATTGAGCGCACCTTC

CATGTGGAAACGCCTGAGGAGCGGGAAGAATGGGCCACCGCCATTCAGACTGTGGCCGATGGACTCAAGAGGCAG

GAAGAAGAGACGATGGACTTCCGATCAGGCTCACCCAGTGACAACTCAGGGGCTGAAGAGATGGAGGTGTCCCTG

GCCAAGCCCAAGCACCGTGTGACCATGAACGAGTTTGAGTACCTGAAACTACTGGGCAAGGGCACCTTTGGGAAA

GTGATTCTGGTGAAAGAGAAGGCCACAGGCCGCTACTATGCCATGAAGATCCTCAAGAAGGAGGTCATCGTCGCC

AAGGATGAGGTTGCCCACACGCTTACTGAGAACCGTGTCCTGCAGAACTCTAGGCATCCCTTCCTTACGGCCCTC

AAGTACTCATTCCAGACCCACGACCGCCTCTGCTTTGTCATGGAGTATGCCAACGGGGGCGAGCTCTTCTTCCAC

CTGTCTCGAGAGCGCGTGTTCTCCGAGGACCGGGCCCGCTTCTATGGTGCGGAGATTGTGTCTGCCCTGGACTAC

TTGCACTCCGAGAAGAACGTGGTGTACCGGGACCTGAAGCTGGAGAACCTCATGCTGGACAAGGACGGGCACATC

AAGATAACGGACTTCGGGCTGTGCAAGGAGGGGATCAAGGATGGTGCCACTATGAAGACATTCTGCGGAACGCCG

GAGTACCTGGCCCCTGAGGTGCTGGAGGACAACGACTACGGCCGTGCAGTGGACTGGTGGGGGCTGGGCGTGGTC

ATGTATGAGATGATGTGTGGCCGCCTGCCCTTCTACAACCAGGACCACGAGAAGCTGTTCGAGCTGATCCTCATG

GAGGAGATCCGCTTCCCGCGCACACTCGGCCCTGAGGCCAAGTCCCTGCTCTCCGGGCTGCTCAAGAAGGACCCT

ACACAGAGGCTCGGTGGGGGCTCTGAGGATGCCAAGGAGATCATGCAGCACCGGTTCTTTGCCAACATCGTGTGG

CAGGATGTGTATGAGAAGAAGCTGAGCCCACCTTTCAAGCCCCAGGTCACCTCTGAGACTGACACCAGGTATTTC

GATGAGGAGTTCACAGCTCAGATGATCACCATCACGCCGCCTGATCAAGATGACAGCATGGAGTGTGTGGACAGT

GAGCGGAGGCCGCACTTCCCCCAGTTCTCCTACTCAGCCAGTGGCACAGCCTACCCATACGATGTGCCAGATTAC

GCCTAA

**Figure 3.28    Full length sequencing of mouse myrPKBα-HA.**

Full length sequencing 5'-3'of mouse myrPKBα-HA (sequencing by Oswell). The translational initiation and stop codons ATG and TAA respectively are highlighted in bold. The N-terminal Lck myristoylation sequence is shown in blue and C-terminal HA tag sequence is shown in red.

## 3.9 Stable transfection of MDCK cells with rat pcDNA.myrPKBγ-HA, rat pcDNA.PKBγ(T305D)-HA, mouse pcDNA.myrPKBγ-HA and mouse pcDNA.PKBα-HA.

Wild-type MDCK cells were transfected with the constructs rat pcDNA.myrPKBγ-HA, rat pcDNA.PKBγ(T305D)-HA, mouse pcDNA.myrPKBγ-HA, mouse pcDNA.myrPKBα-HA and the pcDNA vector alone as a control using Lipofectamine reagent. After 48 hours, the cells were re-plated into media supplemented with geneticin, G418 (a neomycin analogue) to select for clones which had taken up the plasmid DNA. The neomycin resistence gene on the pcDNA vector encodes for a aminoglycoside phosphotransferase and expression of this gene in mammalian cells causes the inactivation of the G418. Neomycin is a bacterial antibiotic which interfers with prokaryotic ribosomes and its analogue, G418 blocks protein synthesis in mammalian cells in the same way by interfering with eukaryotic ribosomal function, therefore all non-transfected cells will die off after several weeks of selection (Southern and Berg, 1982).

Following 2 weeks of G418 selection 24 isolated colonies of cells were picked from each transfection by using cloning cylinders. From the rat pcDNA.myrPKBγ-HA transfection, 22 of the 24 colonies grew up. From the rat pcDNA.PKBγ(T305D)-HA transfection only 15 of the 24 colonies grew up. All 24 picked colonies from both the mouse pcDNA.myrPKBγ-HA and mouse pcDNA.myrPKBα-HA transfections grew up. These selected clones were expanded and analysed by western blots for expression of the recombinant protein (Figure 3.29).

## 3.10 Western blot analysis of picked clones

Whole cell lysates were prepared from all the clones and 20μg of each analysed by western blot for expression of HA-tagged recombinant proteins using the HA-tag antibody (UBI). Expressed HA-tagged rat PKBγ is identified as an immunoreactive band at about 55KDa, while expressed HA-tagged mouse PKBγ and PKBα are identified as a band at 57KDa.

From the rat pcDNA.myrPKBγ-HA transfection, clones 7, 22 and 24 all expressed myrPKBγ-HA with clone 7 found to be expressed at the highest level (Figure 3.29A). From the rat pcDNA.PKBγ(T305D)-HA transfection, clones 4, 5 and 7 expressed PKBγ(T305D)-HA and clone 7 had the highest level of expression(Figure 3.29B). Clones 3, 13 and 19 from the mouse pcDNA.myrPKBγ-HA expressed mouse myrPKBγ-HA with clone 19 expressing at the highest level(Figure 3.29C). The mouse pcDNA.myrPKBα-HA transfection was the most successful with clones 1, 4, 5, 9, 10, 12 and 13 all expressing the recombinant mouse myrPKBα-HA protein(Figure 3.29D). Clones 9, 12 and 13 were selected for experiments being the highest expressers. Clones 6 and 7 from the empty vector control transfection were picked and expanded.

Once the clones expressing the constitutively active mutants were identified they were frozen down in liquid nitrogen for long-term storage and used experimentally.

**Figure 3.29    Western blot analysis for recombinant protein expression in selected MDCK cell clones.**

(A) **Rat pcDNA.myrPKBγ-HA transfection.** 22 of the 24 isolated clones picked grew up successfully and of these clones 7, 22 and 24 were found to express the rat myrPKBγ-HA protein. These clones were expanded and stocks of the cell were frozen down in liquid nitrogen.

(B) **Rat pcDNA.PKBγ(T305D)-HA transfection.** Only 15 of the 24 isolated clones picked grew up and clones 4, 5 and 7 expressed the rat PKBγ(T305D)-HA protein. These clones were expanded and stocks made.

(C) **Mouse pcDNA.myrPKBγ-HA transfection.** All 24 picked clones grew up successfully with clones 3, 13 and 19 expressing the mouse myrPKBγ-HA protein. These clones were expanded and stocks made.

(D) **Mouse pcDNA.myrPKBα-HA transfection.** All 24 picked clones grew up and clones 1, 4, 5, 9, 10, 12 and 13 were found to express the mouse myrPKBα-HA protein. Clones 9, 12 and 13 were the highest expressers and were expanded and stocks made.
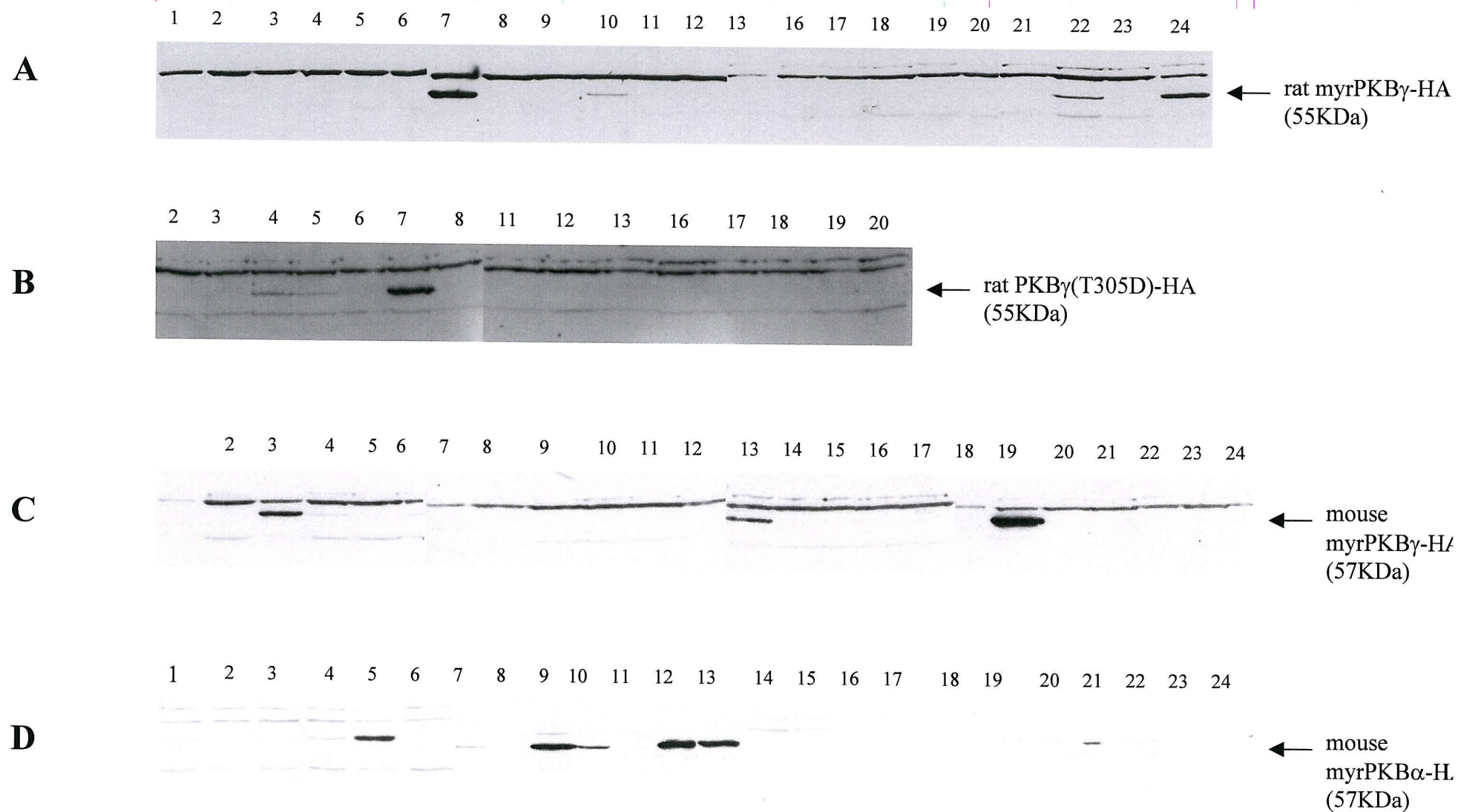
**A** → rat myrPKBγ-HA (55KDa)

**B** → rat PKBγ(T305D)-HA (55KDa)

**C** → mouse myrPKBγ-HA (57KDa)

**D** → mouse myrPKBα-HA (57KDa)

Figure 3.29    Western blot analysis for recombinant protein expression in selected MDCK cell clones.

## 3.11 Summary of results.

In this chapter I have reported the successful cloning and sequencing of rat PKBγ with a haemagglutinin tag. The sequence obtained differed at two positions to the original reported sequence (Konishi et al., 1995) changing Asn10 to Gly, and Pro396 to Ala which was in accordance with the findings of Walker et al., 1998. In addition to rat PKBγ, the recently discovered mouse PKBγ was also cloned, again adding a haemagglutinin tag. In this case there were no differences from the reported sequence. Two different, constitutively active mutants of ratPKBγ were then constructed in order to study the downstream signalling of rat PKBγ. The first, rat myrPKBγ-HA, has a N-terminal myristoylation signal to target the protein to the plasma membrane where it will be phosphorylated on Thr305 by the constitutively active PDK-1, even in the absence of growth factor stimulation. The second mutant, rat PKBγ(T305D)-HA, has a key mutation of the Thr305 phosphorylation site to the amino acid Asp in order to mimic phosphorylation by introducing a negative charge. A membrane-targeted mutant of mouse PKBγ (mouse myrPKBγ-HA) was also constructed to identify whether or not there are any significant differences in signalling between the rat and mouse forms of PKBγ. Finally, a haemagglutinin-tagged mouse PKBα was cloned and a membrane targeted mutant constructed (mouse myrPKBα-HA) as a positive control for our PKBγ stable cell lines.

The constructs were all sub-cloned into the pcDNA3.1 mammalian expression vector and then used to produce stable cell lines expressing the constitutively active mutant PKB proteins in MDCK cells. Three different clones expressing each constitutively active mutant were obtained and expanded for use in subsequent experiments.

# CHAPTER 4

# PKBγ AND GSK-3.

## 4.1 Introduction.

### 4.1.1 GSK-3 and its substrates.

Early work on glycogen metabolism established that the enzyme involved in glycogen synthesis, glycogen synthase (GS), was regulated by phosphorylation at multiple sites. Indeed, it was discovered that inactive GS was phosphorylated on at least 9 sites by multiple protein kinases and became rapidly dephosphorylated in response to insulin and growth factors (Cohen, 1985). The protein kinases responsible for the phosphorylation of GS were soon purified. In the terminology of Cohen, Glycogen synthase kinase 1 (GSK-1) equates to cyclic-AMP-dependent protein kinase, GSK-2 is phosphorylase kinase and GSK-5 is casein kinase II. GSK-3 and GSK-4 were, at the time, novel enzymes of unknown function. It was soon found that GSK-3 phosphorylated three clustered serine residues in GS, which resulted in dramatic inactivation of GS (Cohen, 1985; Poulter et al., 1988). Following on from the discovery that GSK-3 phosphorylated and inactivated GS, a number of other substrate proteins for GSK-3 were identified. These include the transcription factors c-jun (de Groot et al., 1992; Boyle et al., 1991) and the cAMP-response element binding protein (CREB) (Fiol et al., 1994); a translation factor (eIF2B) (Welsh and Proud, 1993; Welsh et al., 1998); and other cytosolic proteins, ATP-citrate lyase (Hughes et al., 1992) inhibitor-2 (Hemmings et al., 1996) and more recently, Tau (Hong and Lee, 1997).

The sequence motif which is preferentially phosphorylated by GSK-3 is Ser-Xaa-Xaa-Xaa-Ser(P) (Fiol et al., 1990). This same motif is found not only in GS but also in other GSK-3

substrates such as ATP-citrate lyase (Hughes et al., 1992). The phosphorylation of substrates by GSK-3 requires 'priming'via prior phosphorylation of a proximal residue by a second protein kinase. Indeed, the phosphorylation of GS by GSK-3 only occurs if the protein has already been phosphorylated by casein kinase II (Fiol et al., 1990).

GSK-3 is a serine/threonine protein kinase which is ubiquitously expressed in mammalian cells. It exists as at least two distinct isoforms, GSK-3α and GSK-3β (Woodgett, 1990). It must be noted that GSK-3 also has a close homologue in *Drosophila*, the product of the zeste-white 3 or shaggy homeotic gene (Seigfreid et al., 1990; Bourouis et al., 1990).

## 4.1.2 The regulation of GSK-3 by PKB.

Until relatively recently, little was known about the regulation of GSK-3 by growth factors and hormones such as insulin. Initally it was shown that insulin rapidly decreased the activity of GSK-3 in rat adipocytes (Hughes et al., 1992; Welsh and Proud, 1993). In addition, the inactivation of GSK-3 in response to insulin and growth factors was reversed by treatment with serine/threonine phosphatases, indicating that it was due to increased phosphorylation of Ser/Thr residues in GSK-3. This phosphorylation was found to occur on a single serine residue near to the N-terminus, Ser21 on GSK-3α and Ser9 on GSK-3β (Saito et al., 1994).

In 1995 Cross and co-workers found that GSK-3 activity is inhibited by PKBα (Cross et al., 1995). It was discovered that the 40%-50% inhibition of GSK-3 activity by insulin was unaffected by agents which prevented the activation of MAPKAP-K1 (8-bromo-cyclicAMP or PD98059) and/or p70S6 kinase (rapamycin) *in vivo*. This result suggested that neither MAPKAP-K1 nor p70S6 kinase where responsible for the insulin or growth factor stimulated

inhibition of GSK-3. PKB was eventually identified as the serine/threonine protein kinase

probably responsible for phosphorylating and inactivating GSK-3 *in vivo* (Cross et al., 1995).

These results also explained why adding wortmannin increased the GSK-3 activity since

wortmannin inhibits PI-3 kinase, which lies upstream of PKB.


So the main signalling pathway to GSK-3 is now tentatively accepted as the PI-3 kinase/PKB

pathway although GSK-3β may also be regulated by other PI-3 kinase-dependent, but PKB-

independent pathways (Delcommenne et al., 1998; Kobayashi and Cohen, 1999)


## 4.1.3 GSK-3 and apoptosis.


As I have mentioned, GSK-3 is involved in a wide range of cellular processes including

glucose metabolism, protein synthesis (c-jun and eIF2B), poliferation and differentiation

(Wnt signalling pathway in *Drosophila* and *Xenopus*). In addition to these processes, there is

ever mounting evidence implying a role for GSK-3 in the regulation of apoptosis. GSK-3 was

identified as a putative physiological target of PKBα (Cross et al., 1995) and since the PI3-

kinase/PKB signalling pathway is a key pathway in cell survival signalling, it was

hypothesised that GSK-3 may also be an important component in this signalling pathway

(Yao and Cooper, 1995; Dudek et al., 1997; Kauffmann-Zeh et al., 1997; Kulik et al., 1997).

Pap and Cooper, working on Rat-1 and PC12 cells, found that overexpressing an active

mutant of GSK-3 was sufficient to induce apoptosis in these cells demonstrating that GSK-3

may be involved in the regulation of apoptosis (Pap and Cooper, 1998). In contrast,

overexpressing a dominant negative form of GSK-3 inhibited apoptosis induced by the

inhibition of PI3-kinase. These results indicated that GSK-3 is a key target of PI3-

kinase/PKB cell survival signalling. The findings by Pap and Cooper were backed up by

similar work on primary cultured CNS neurons (Hetman et al., 2000). These cells undergo apoptosis after serum withdrawal, or treatment with PI3-kinase inhibitors. It was found that serum starvation in these cells inhibits PI3-kinase and activates GSK-3$\beta$. In addition, activation of GSK-3$\beta$ was sufficent to induce apoptosis in these cells, even in the presence of serum (Hetman et al., 2000).

Following the identification of GSK-3$\beta$ as potentially a key mediator of apoptosis in cells, the next step now is to discover the mechanism(s) by which activated GSK-3$\beta$ could induce apoptosis. Several theories have been put forward concerning this. Initially $\beta$-catenin degradation was thought to be the GSK-3 substrate responsible for inducing apoptosis in cells. GSK-3$\beta$ phosphorylates four serine residues at the N-terminus of $\beta$-catenin and causes $\beta$-catenin degradation (Miller and Moon, 1996; Yost et al., 1996). It has been proposed that destabilisation of $\beta$-catenin potentiates neuronal apoptosis induced by $\beta$-amyloid peptide (Z. Zang et al., 1998). However, this hypothesis was not supported by the findings of Hetman and co-workers. They found that overexpressing a stable mutant of $\beta$-catenin did not rescue cortical neurons from serum-starvation-induced apoptosis (Hetman et al., 2000). Recent work on GSK-3$\beta^{-/-}$ embryonic mice cells has yielded evidence suggesting that the transcription factor NF-$\kappa$B is regulated by GSK-3$\beta$ at the level of the transcriptional complex, thus facilitating NF-$\kappa$B function (Hoeflich et al., 2000; Karin and Ben-Neriah, 2000; Khawaja, 1999). These findings also back up the observations that NF-$\kappa$B is activated following activation of the PI3-kinase/PKB signalling pathway (Ozes et al., 1999). In addition to $\beta$-catenin and glycogen synthase, several other possible substrates for GSK-3 have been identified, some of which may be mediators of GSK-3-induced apoptosis. One such substrate is mitochondrial pyruvate dehydrogenase that is phosphorylated and inhibited by GSK-3$\beta$.

Therefore, it is conceivable that the resulting metabolic failure results in apoptotic cell death (Hoshi et al., 1996). GSK-3β phosphorylates the microtubule-associated protein tau converting it into Alzheimers's disease-like forms (PH-tau) found in paired helical filaments (Hanger et al., 1992; Mandelkow et al., 1992; Ishiguro et al., 1993; Mulot et al., 1994; Hong and Lee, 1997). The appearance of PH-tau is associated with early alterations in neurites associated with Alzheimer's disease (Goedert et al., 1995). Tau phosphorylation by GSK-3β may cause axonal dysfunction and trigger neuronal apoptosis (Hong and Lee, 1997). Finally, GSK-3β has also been found to phosphorylate the insulin receptor substrate-1 (IRS-1) converting it into an inhibitor of insulin receptor tyrosine kinase activity (Eldar-Finkelmann and Krebs, 1997). IRS-1 is crucial for insulin and IGF-1 cell survival signalling therefore disruption of the receptor may result in apoptosis.

All these findings strongly implicate GSK-3β as a crucial mediator of apoptosis. In addition the inhibition of GSK-3β by PKB could be one of the main pathways responsible for cell survival signalling.

## 4.1.4 GSK-3 and PKBγ.

In this chapter a new GSK-3 kinase assay set-up by Neil Jones in our lab will be employed to test whether or not activation of PKBγ affects GSK-3, a putative target of PKBγ (Shaw and Cohen, 1999). This assay, detailed below, was used to measure the levels of endogenous GSK-3 activity from control MDCK cells and the stable MDCK cell lines expressing constitutively active rat and mouse PKBγ (set up in Chapter 3). The results obtained should show two things: (1) if the recombinant PKBγ mutants are indeed constitutively active, even

in the absence of growth factor stimulus; and (2) if GSK-3 is indeed a downstream target of PKBγ.

### 4.1.5 A kinase assay suitable for measuring GSK-3 activity in crude cell extracts.

In the past a variety of assay procedures have been used to measure GSK-3 activity. Most employ peptide substrates whose sequences are based on those around the GSK-3 phosphorylation sites known to be phosphorylated by GSK-3 in vitro (Fiol et al., 1990; Woodgett, 1989; Wang et al., 1994; Van Lint et al., 1993; Roach, 1991). However these peptides pose two major problems for the user: (1) they require prior phosphorylation by a 'priming' kinase (e.g. casein kinase II in the case of GS-based peptides); and (2) the GS-based peptides are phosphorylated by a wide range of cellular kinases and therefore cannot be used with a crude cell extract. Rather, the GSK-3 isoform must be immunoprecipitated prior to assay (Van Lint et al., 1993).

These problems were overcome by Welsh and co-workers who designed novel phosphpeptides based on the sequence which includes the single GSK-3 phosphorylation site in eIF2B, for the assay of GSK-3 (Welsh et al., 1997). The sequence of this phospho-peptide is RRAAEELDSRAGS(P)PQL and it not only alleviates the requirement of priming, but may also be used with crude cell extracts without the need for immunoprecipitation.

This phospho-peptide and a non-phosphorylated control peptide, were synthesised in our lab by Neil Jones who set up the kinase assay conditions required. This was the kinase assay I used for measuring the GSK-3 activity from the control and stable MDCK cells.

## 4.2 Serum starved MDCK cell lines expressing constitutively active PKBγ and PKBα show reduced endogenous GSK-3 activity.

Cell lysates from 24 hour serum starved control and stable MDCK cells were assayed for endogenous GSK-3 activity using the phospho-peptide described in the introduction (Figure 4.1). All the stable cell lines expressing constitutively active PKBγ and PKBα showed ~40-50% reduction in GSK-3 activity compared to control MDCK cells. As a positive control, control MDCK cells were stimulated with serum for 10mins and showed 40-50% reduction in GSK-3 activity.

More specifically, the slight differences in GSK-3 activity among the different constitutively active mutants may be accounted for as differences in the levels of protein expression. From the western blot accompanying the graph it can be seen that the levels of expression of both rat PKBγ(T305D)-HA and mouse myrPKBγ-HA are slightly less that those of rat myrPKBγ-HA and mouse myrPKBα-HA. Therefore the GSK-3 activities from both the rat PKBγ(T305D)-HA and mouse myrPKBγ-HA MDCK cells are slightly higher than those for the rat myrPKBγ-HA and mouse myrPKBγ-HA MDCK cells.

**Figure 4.1 GSK-3 activity in stable MDCK cell lines expressing constitutively active PKBγ and PKBα.**

Endogenous GSK-3 activity was measured in stable MDCK cell lines following 24 hrs serum starvation. The value for 100% activity is GSK-3 activity from control MDCK cells following 24hrs serum starvation. As a positive control, control MDCK cells were serum stimulated by incubating for 10 mins with 25% FBS. This resulted in a reduction of GSK-3 activity to 43%. Even following 24hrs serum starvation there is reduced GSK-3 activity in all the stable cell lines expressing constitutively active mutants of rat and mouse PKBγ and mouse PKBα. This indicates that the mutants of PKBγ and PKBα are indeed constitutively active since even in the absence of growth factor stimulus since they have the ability to inhibit the endogenous GSK-3 activity. The data (mean $\pm$ SEM) are from 4 independent experiments. Also shown is a western blot for a comparison of HA-tagged constitutively active mutant PKBγ and PKBα protein expression.
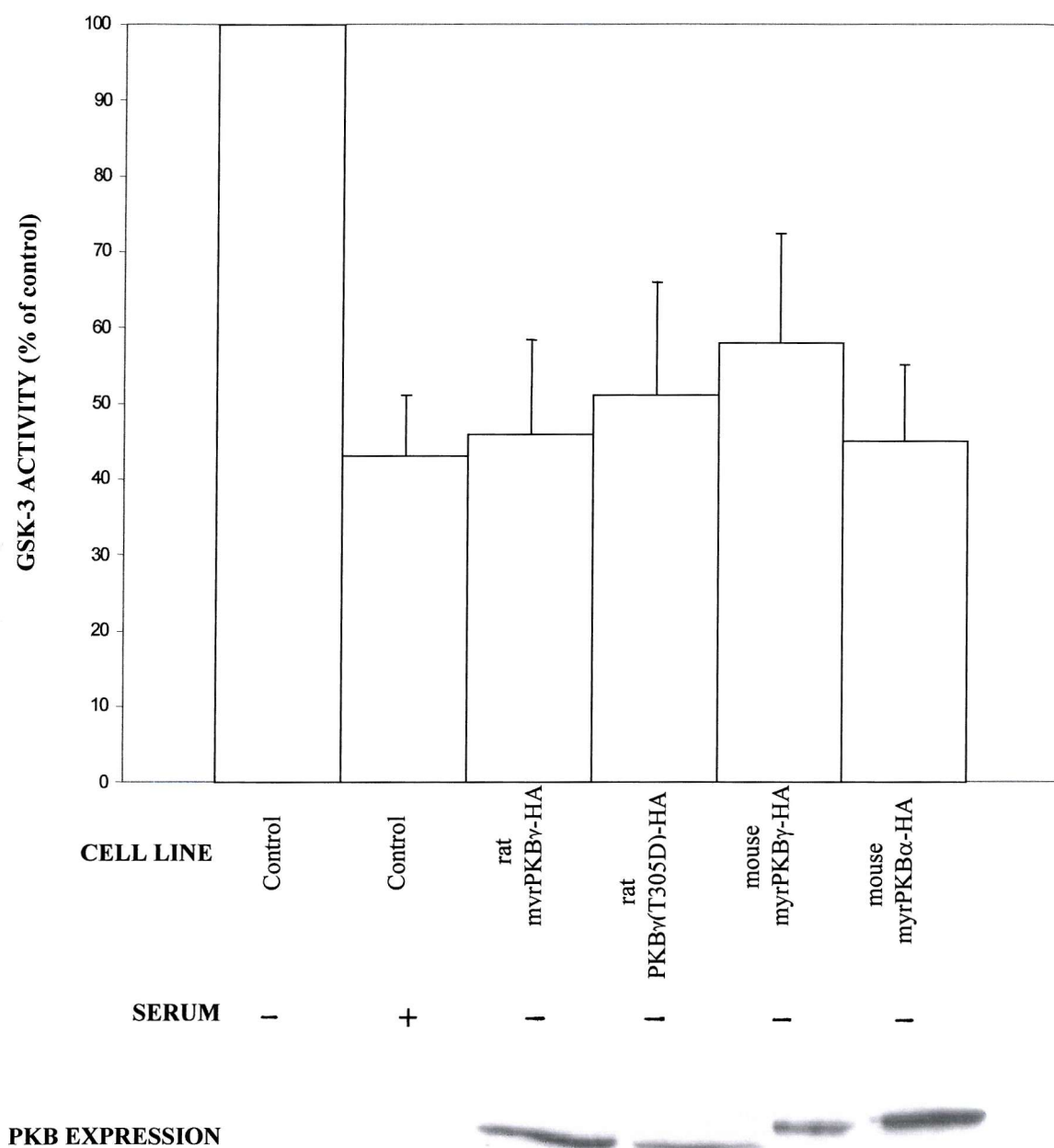
**Figure 4.1    GSK-3 activity in stable MDCK cell lines expressing constitutively active PKBγ and PKBα.**

## 4.3 Summary.

In this chapter an assay for measuring endogenous GSK-3 activity in crude cell extracts was used. This method employs the use of a phospho-peptide based on the GSK-3 phosphorylation site of eIF2B and alleviates the need for either peptide 'priming' or prior immunoprecipitation of the GSK-3. The results obtained demonstrate that, even when serum starved for 24 hours, the stable MDCK cell lines expressing constitutively active mutants of PKBγ and PKBα show a ~40-50% reduction in GSK-3 activity as compared to serum starved control MDCK cells (100% GSK-3 activity). This reduction in activity is similar to that achieved by serum stimulating control MDCK cells for 10mins to activate the PI-3 kinase/PKB pathway. Therefore, this result proves that GSK-3 is an *in vivo* target for PKBγ as well as PKBα. This work backs up the findings of Shaw and Cohen working with Swiss 3T3 cells. They discovered that endogenous PKBγ was activated in response to both IGF-1 and EFG stimulation and following activation of PKBγ, GSK-3α became phosphorylated on Ser21 causing ~50% inactivation of GSK-3 (Shaw and Cohen, 1999).

Therefore, these results demonstrate that the mutants of PKBγ are indeed constitutively active, even in the absence of serum and would be ideal for using to investigate the antiapoptotic signalling of PKBγ in future experiments.

# CHAPTER 5

# THE ROLE OF PKBγ IN CELL SURVIVAL SIGNALLING AND APOPTOSIS.

# CHAPTER 5: The role of PKBγ in cell survival signalling and Apoptosis.

## 5.1 Introduction.

### 5.1.1 Morphological and biochemical hallmarks of apoptosis.

Apoptosis, or programmed cell death, is one of the most important biochemical mechanisms present in cells. It ensures that potentially harmful cells such as cancerous cells are removed and therefore maintains appropriate cell numbers (Jacobson et al., 1997). However, inappropriate apoptosis can be a liability as shown by the onset of neurodegenerative diseases such as Alzheimer's (Thompson, 1995). Apoptosis is characterised by a series of typical morphological events such as shrinkage and fragmentation of the cell, condensation of the nucleus, membrane blebbing, loss of microvilli and rapid phagocytosis by neighbouring cells (Saraste and Pulkki, 2000). Internucleosomal fragmentation of the cell's genomic DNA has been the main biochemical hallmark of apoptosis for many years and it is this effect which I have used as the primary method for analysing apoptosis in this study (Nagata, 2000; Saraste and Pulkki, 2000).

As discussed in Chapter 1, apoptosis can be induced in cells by a wide variety of factors including growth factor withdrawal, UV irradiation, death factors such as the FasL (Fas Ligand), TNF (tumour necrosis factor) and oxidative stress (Ashkenazi and Dixit, 1998; Nagata, 1997; Raff, 1998; Green and Reed, 1998). The ultimate effect is the activation of the caspases, a family of cysteine proteases present in growing cells as inactive precursors called pro-caspases (for review see Bratton et al., 2000). Once activated, initiator caspases such as caspase 9 go on to activate the effector caspases such as caspase 3 and 6. These effector

caspases ultimately cleave a wide variety of essential cellular proteins resulting in the morphological and biochemical changes that characterise apoptotic cell death. The degradation of genomic DNA into nucleosomal fragments is one of the best-characterised biochemical features of apoptotic cell death (Nagata, 2000; Saraste and Pulkki, 2000).

## 5.1.2 DNA Fragmentation as a method for studying apoptosis.

The factor which causes apoptotic DNA fragmentation is a 40kDa protein called caspase-activated DNase (CAD). It is present in cells as a complex with a 45kDa protein ICAD (Inhibitor of CAD) (Enari et al., 1998). Figure 5.1 shows the current model for CAD activation and degradation of DNA during apoptosis. In normal growing cells when CAD is synthesised on ribosomes ICAD is required for correct folding of the protein. Therefore the ICAD:CAD complex is automatically formed. After CAD synthesis is complete, ICAD remains complexed to CAD inhibiting the CAD activity. Upon induction of apoptosis, the ICAD protein is rapidly cleaved in two places by caspase 3 and dissociates from the CAD protein. This results in active CAD which is then free to degrade the cell's genomic DNA into 180-200bp fragments which can be seen as a distinct DNA 'ladder' when run out on an agarose gel.

**Figure 5.1     CAD-mediated apoptotic DNA fragmentation.**

Whenever CAD is synthesized, its inhibitor, ICAD, binds to it in order to ensure correct and productive folding. Therefore, the CAD:ICAD complex exists in normal growing cells as an inactive complex. Apoptotic signals such as growth factor removal result in the release of Cytochrome C from the mitochondria. The release of Cytochome C causes the activation of Caspase 9 and concurrent activation of downstream effector caspases such as Caspase 3. Caspase 3 cleaves a wide range of cellular proteins including ICAD resulting in the inactivation of its CAD-inhibitory activity. Active CAD is therefore released from the ICAD and degrades the chromosomal DNA.
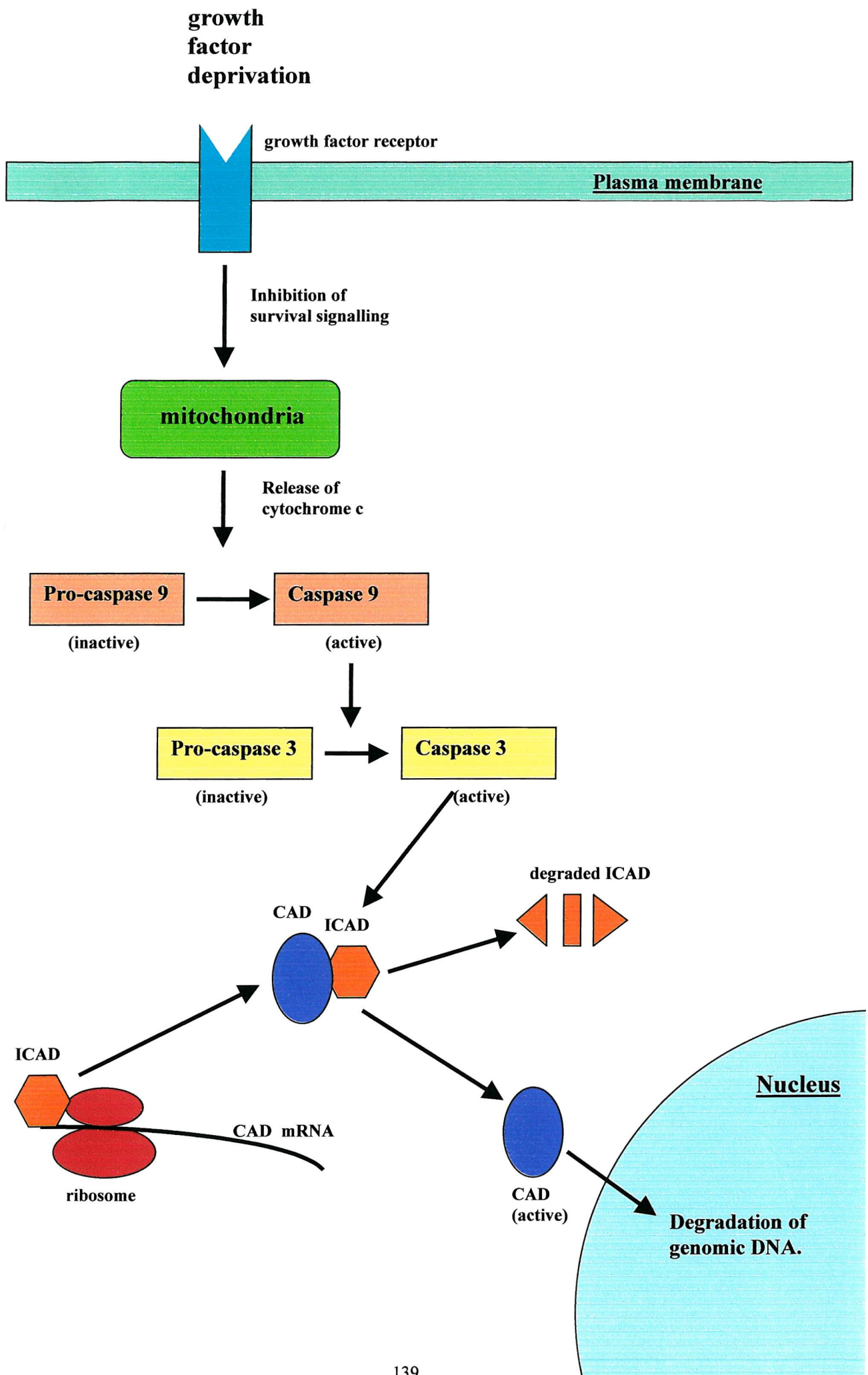
### 5.1.3 Changes in membrane morphology as a marker of apoptosis.

One of the initial morphological changes in apoptosis is the change in the position of phosphatidylserine (PS) within the cell membrane. In normal growing cells, the majority of the PS molecules are localised at the inner layer of the plasma membrane, however, soon after the induction of apoptosis the PS relocates to the outer layer of the membrane, and becomes exposed to the extracellular environment (Martin et al., 1995). Once again it is cleavage by the caspases which has been implicated in the externalisation of PS and this process may mediate the recognition of apoptotic bodies for phagocytosis by neighbouring cells (Vanags et al., 1996; Fadok et al., 1992; Martin et al., 1996). In the second part of this chapter the Apoalert Annexin V kit (Clontech) was employed to detect apoptotic cells. Exposed PS can be detected with annexin V, a 35kDa protein that has a strong affinity for PS. The specificity of the annexin V-PS interaction is reflected by competitive binding assays in which annexin V binding to apoptotic cells was specifically inhibited by PS liposomes, but was unaffected by liposomes containing other phospholipids (Martin et al., 1995). The annexin V protein can be labelled with a fluorescent marker in order to identify the apoptotic cells under a laser microscope.

### 5.1.4 Measuring cell survival: The MTS method.

An additional method available for use to investigate the extent of apoptosis within a population of cells is the MTS method. This is a useful assay sold as the CellTitre 96 AQueous One Solution Cell Poliferation Assay (Promega). It is a colorimetric method for determining the number of viable cells in poliferation or cytotoxicity assays. The solution consists of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-

carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES). The MTS tetrazolium compound (Owen's reagent) is bioreduced by cells into a coloured formazan product that is soluble in tissue culture media. This conversion is accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. The quantity of formazan product as measured by the absorbance at 490nm is directly proportional to the number of living cells in culture. This method will be employed in this study to back up the results obtained by the DNA fragmentation described above and provide a quantitative analysis of the extent of apoptosis occurring in the cells examined.

## 5.1.5 PKBγ and apoptosis.

A substantial number of studies have already shown that transfection of constitutively active PKBα into cells blocks apoptosis induced by a variety of ways including growth factor withdrawal, UV irradiation, matrix detachment, DNA damage, and treatment of cells with anti-Fas antibody (Dudek et al., 1997; Kauffmann-Zeh et al., 1997; Kennedy et al., 1997; Kulik et al., 1997; Philpott et al., 1997; Songyang et al., 1997; Xiong and Parsons, 1997; Eves et al., 1998; Gerber et al., 1998; Kulik and Weber, 1998; Hausler et al., 1998).

So far no studies have been reported concerning PKBγ and its possible role in cell survival signalling and apoptosis. In this chapter the techniques of DNA laddering, MTS cell survival measurement and the Apoalert Annexin V kit were employed to investigate the role of PKBγ in growth factor withdrawal induced apoptosis. This was achieved initially using the stablily transfected Madin-Darby Canine Kidney (MDCK) cell lines expressing two different constitutively active rat PKBγ mutants, constitutively active mouse PKBγ and constitutively

active mouse PKBα (see Chapter 3). In addition, a dominant negative rat PKBγ was cloned. Dominant negative mutants of PKBα have been used successfully to inhibit endogenous PKB in a variety of cell types (Wang et al., 1999; Hajduch et al., 1998; Pascale et al., 1998). Although their mode of operation is not exactly known, many people suspect that the overexpression of the dominant negative mutant acts as a competitive inhibitor to the endogenous PKB isoforms present in the cell. The rat PKBγ dominant negative mutant was used to transfect wild-type MDCK cells in order to attempt to induce apoptosis by inhibiting endogenous PKB. MDCK cells are canine embryonic epithelial cells and were used in this study since they are a particularly good model for observing growth factor induced apoptosis (Frisch and Francis., 1994; Khawaja et al., 1997).

## 5.2 Growth factor withdrawal consistently induces apoptosis in wild-type MDCK cells.

Before setting out to investigate the involvement of PKBγ in cell survival and apoptosis it was first important to establish a reliable system for inducing apoptosis in the wild-type MDCK cells. The three methods commonly used to induce apoptosis in MDCK cells are growth factor withdrawal, UV irradiation and matrix detachment (Khwaja et al., 1997). Of these three methods growth factor withdrawal and matrix detachment were tested in our lab since I did not have access to the correct UV transilluminator required for γ-irradiation.

Of the two methods tested, growth factor withdrawal (serum starvation) was found to reproducibly induce apoptosis in the wild-type MDCK cells. This method is relatively simple to carry out and very cheap in comparison to other ways of inducing apoptosis. 1 x10$^6$ wild-type MDCK cells were plated out onto 10cm tissue culture plates, then the following day the media was removed from the cells and the cells washed 3-5 times in serum-free DMEM media in order to remove all traces of FBS. The cells were then serum starved in serum-free DMEM media for set timepoints and then analysed for DNA laddering (Figure 5.2). These results show that the onset of apoptosis occurs following 48 hours of serum starvation with a clearly distinguishable DNA ladder after 72 hours. The DNA laddering was as expected and showed a smear of degraded chromosomal DNA fragments comprising all sizes with clearly distinguishable 180-200bp bands further down the gel (Nagata, 2000).

Over many repeats of this experiment the results obtained were consistent. Therefore I decided that this method of inducing apoptosis in the MDCK cells would be the most reliable and reproducible rather than the matrix detachment method (data not shown).

## 5.3 Growth factor withdrawal induces apoptosis in control MDCK cells but not in cells expressing constitutively active PKBγ as measured by DNA laddering.

With the conditions for inducing apoptosis in the MDCK cells now set the next step was to use this system to investigate the role PKBγ has to play in cell survival signalling. The stable MDCK cell lines I had produced expressing the constitutively active mutants of rat PKBγ, mouse PKBγ and mouse PKBα were tested for their ability to survive after growth factor withdrawal. This was achieved initally by DNA laddering as before. $1 \times 10^6$ control and mutant MDCK cells were plated out onto 10cm tissue culture plates and the following day serum starved by washing 3-5 times in serum free DMEM media. The cells where then incubated in serum-free media for 48 hours to allow for the induction of apoptosis. Following this time the cells were harvested (including floating apoptotic bodies) and the genomic DNA extracted and run on a 1.5% agarose gel in order to visualise the extent of DNA laddering (Figure 5.3). The results obtained showed that all the stable cells expressing constitutively active PKBγ and PKBα exhibited considerably less DNA laddering than the control MDCK cells. This result therefore indicates that rat and mouse constitutively active PKBγ mutants have the ability to protect MDCK cells against growth factor withdrawal induced apoptosis to the same extent as constitutively active PKBα.
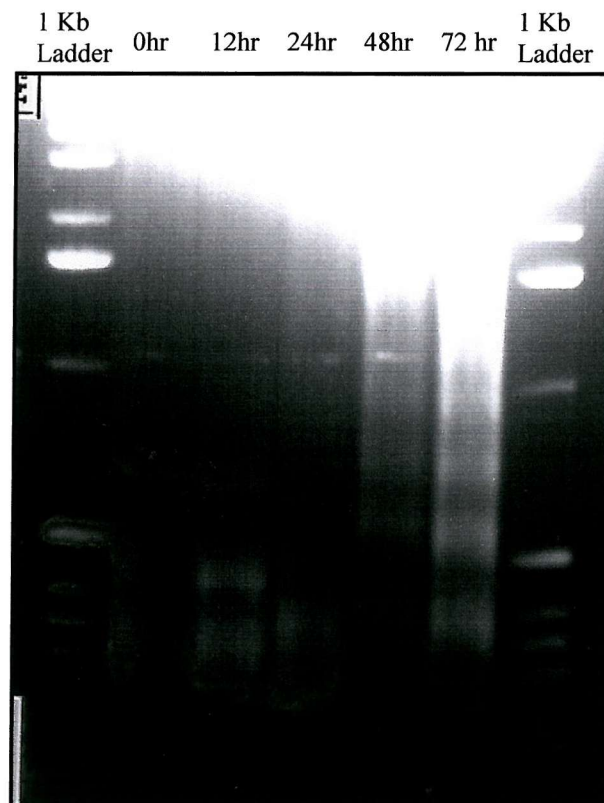
**Figure 5.2    Timecourse of serum starvation induced DNA laddering in wild-type MDCK cells.**

$1 \times 10^6$ wild-type MDCK cells were plated out onto 10cm plates in DMEM media containing 10% FBS. The following day, the cells were washed 3-5 times with serum-free DMEM media to remove all traces of FBS. The cells were then incubated in the same serum-free media for the times indicated in order to assess the extent of apoptosis by DNA laddering. For DNA laddering, cells were harvested by scraping (including floating cells) and total genomic DNA extracted. This genomic DNA was then loaded onto a 1.5% agarose gel containing ethidium bromide and ran for 1 hour at 100V. DNA laddering was visualised under UV light.
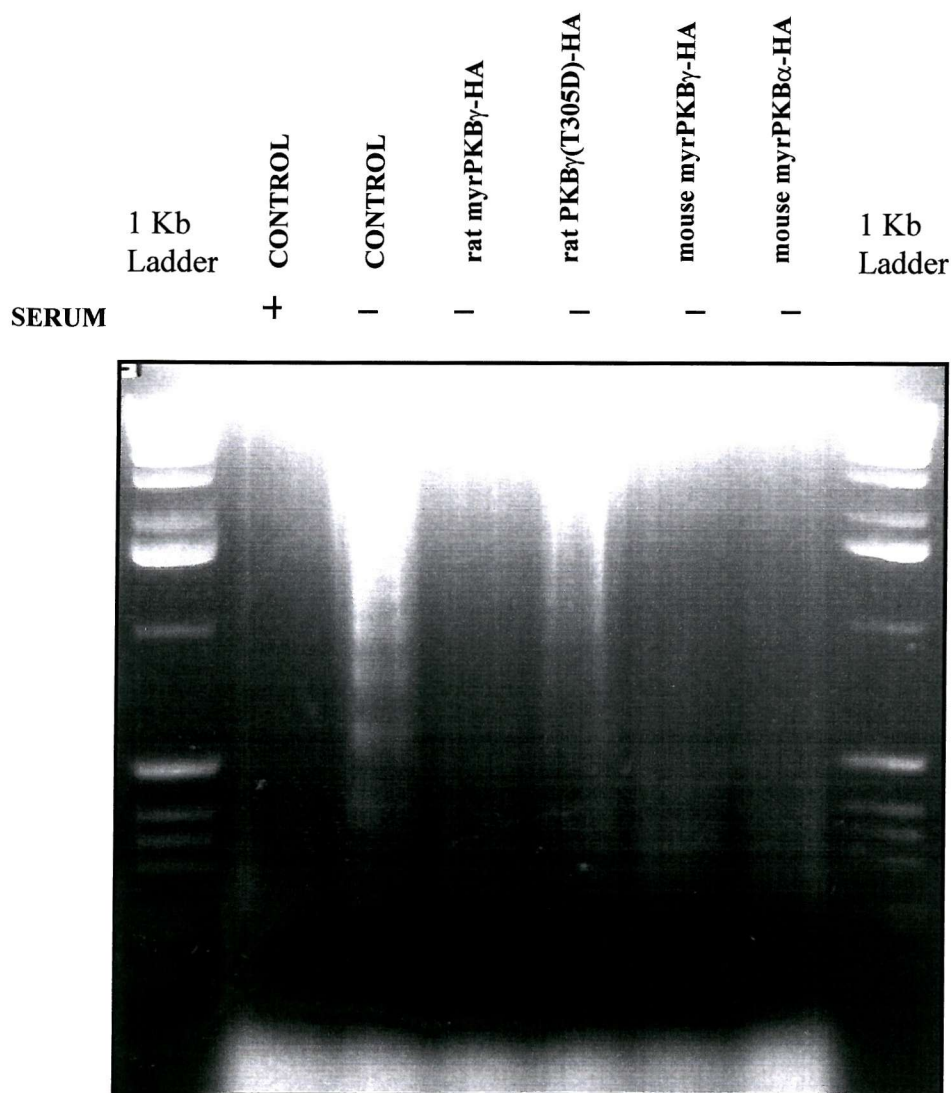
**Figure 5.3     Effect of serum starvation on apoptosis of control and stabily transfected MDCK cells.**

$1 \times 10^6$ MDCK cells were plated out on 10cm plates in DMEM media containing 10% FBS. The following day the cells were serum starved as described before and incubated in serum-free DMEM media for 48 hours to induce apoptosis. Genomic DNA was then extracted, run out on a 1.5% agaorose gel containing ethidium bromide and visualised under UV light. The no laddering control (+serum) is MDCK cells grown in the presence of 10% FBS for 48 hours. Similar results were obtained for all the constructs tested in at least two distinct clones.

## 5.4 Growth factor withdrawal induces apoptosis in control MDCK cells but not in cells expressing constitutively active PKBγ as measured by the MTS method.

Following on from the DNA laddering experiment, I decided to gain a more quantitative result by using the MTS method to measure cell survival after the induction of apoptosis (Figure 5.5). The results obtained were consistent with those obtained for the DNA laddering experiments. Control MDCK cells serum starved for 48 hours are highly susceptible to apoptosis with only 46% cell survival rates observed. In contrast, the stable cell lines expressing constitutively active rat PKBγ, mouse PKBγ and mouse PKBα are all highly protected against apoptosis. In fact the mouse myrPKBγ mutant showed >90% survival rates, almost as much as cells grown in the presence of serum for 48 hours. This experiment was repeated several times and proved an excellent way of backing up the results obtained from the earlier DNA laddering experiments.
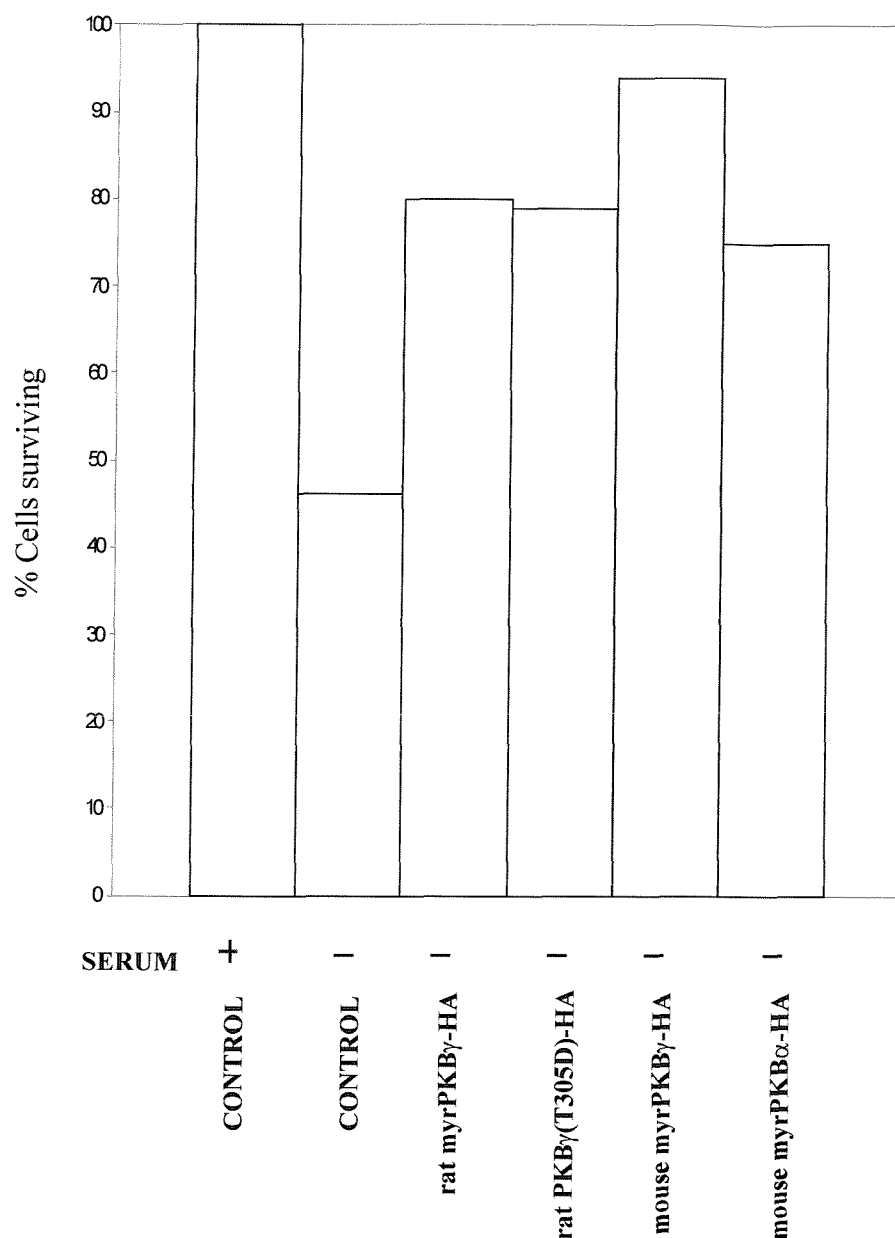
**Figure 5.4    Cell survival as measured by the MTS method after serum starvation induced apoptosis.**

Control and transfected MDCK cells were serum starved for 48 hours to induce apoptosis. Points were assayed in quadruplicate in each experiment. The results shown are from a typical experiment and similar results were obtained for all the constructs tested in four separate experiments using two distinct clones.

## 5.5 PCR cloning and expression of rat PKBγ dominant negative mutant (rat PKBγ(K179A,T305A)-HA).

### 5.5.1 Using site-directed mutagenesis to clone dominant negative rat PKBγ(K177A,T305A)-HA by PCR.

In order to construct a dominant negative HA-tagged rat PKBγ (rat PKBγ(K177A,T305A)-HA) alanine residues were substituted at the regulatory phosphorylation site (Thr305) and the phosphate transfer residue in the catalytic site (Lys177). This was achieved by two rounds of site directed mutagenesis similar to the method used to construct the constitutively active rat PKBγ(T305D)-HA construct. In the first round of mutagesesis the phosphorylation site (Thr305) was mutated to Ala305 in a series of 4 PCR reactions (Table 5.1). Reaction 1 was carried out using rat brain cDNA as the template, the rat KpnI forward primer and mutating T305A reverse primer. The PCR product was a 940bp fragment. The second PCR reaction used the same template DNA, the T305A forward primer and the rat HA reverse primer 1. The PCR product was a 480bp fragment. The two PCR products from reactions 1 and 2 were combined as template DNA in a third PCR reaction along with the rat Kpn I forward primer and rat HA reverse primer 2. The 1.4Kb PCR product from this reaction was rat PKBγ(T305A)-1/2HA with the Thr305 phosphorylation site mutated to Ala305 and half of the HA tag added. In order to produce the full length rat PKBγ(T305A)-HA the PCR product from reaction 3 was gel-purified and used as template with the rat Kpn I forward and Xba I HA reverse primers in a fourth PCR reaction. So the final PCR product from this first round of PCR was rat PKBγ(T305A)-HA with a 5'Kpn I site and a 3'Xba I site (Figure 5.5).

**Table 5.1**     **First round of PCR reactions to construct rat PKBγ(T305A)-HA.**

| PCR REACTION | TEMPLATE DNA | FORWARD PRIMER | REVERSE PRIMER |
|---|---|---|---|
| 1. Fragment 1 (940bp). | 2μl rat brain cDNA library | rat Kpn I forward primer | T305A reverse primer |
| 2. Fragment 2 (480bp) | 2μl rat brain cDNA library | T305A forward primer | rat HA reverse primer 1 |
| 3. PKBγ(T305A)-1/2HA | 1μl each of gel-purified fragments 1 and 2. | rat Kpn I forward primer | rat HA reverse primer 2 |
| 4. PKBγ(T305A)-HA | 1μl gel-purified reaction 3 PCR product. | rat Kpn I forward primer | Xba I HA reverse primer |

The second round of PCR to construct full length rat PKBγ(K177A,T305A)-HA involved a series of 3 PCR reactions (Table 5.2). The first PCR reaction used the gel-purified rat PKBγ(T305A)-HA PCR product as the template DNA with the rat KpnI forward primer and K177A reverse primer. The PCR product was a 530bp fragment. The second PCR reaction used the same template DNA as reaction 1 with the K177A forward primer and XbaI HA reverse primer. The PCR product from this reaction was a 870bp fragment. The two PCR products from reactions 1 and 2 were combined and used as the template DNA in the third and final PCR reaction along with the rat KpnI forward and the XbaI reverse primers. The 1.4Kb PCR product from this reaction was full length rat PKBγ(K177A,T305A)-HA with a 5'Kpn I site and a 3'Xba I site for cloning (Figure 5.6).

**Table 5.2** **Second round of PCR reactions to construct rat PKBγ(K177A,T305A)-HA.**

| PCR REACTION | TEMPLATE DNA | FORWARD PRIMER | REVERSE PRIMER |
|---|---|---|---|
| 1. Fragment 1 (530bp) | 1μl gel-purified PKBγ(T305A)-HA | rat Kpn I forward primer | K177A reverse primer |
| 2. Fragment 2 (870bp) | 1μl gel-purified PKBγ(T305A)-HA | K177A forward primer | Xba I HA reverse primer |
| 3.PKBγ(K177A,T305A)-HA | 1μl each of gel-purified fragments 1 and 2. | rat Kpn I forward primer | Xba I HA reverse primer |

## 5.5.2 Direct ligation of rat PKBγ(K177A,T305A)-HA into the pcDNA3.1 mammalian expression vector and sequencing.

The PKBγ(K177A,T305A)-HA PCR product was gel purified and digested with Kpn I and Xba I, then ligated directly into KpnI/XbaI digested pcDNA3.1 expression vector. The ligation reaction was then used to transform calcium competent JM109 *E.coli* by heat shock.. six ampicillin resistant bacterial colonies were picked for mini-prep and restriction digest analysis using the restriction enzymes Kpn I and Xba I. Plasmids in all 6 colonies contain the rat PKBγ(T305D)-HA insert (Figure 5.7). Glycerol stocks were made from colony 1.

The pcDNA.ratPKBγ(K177A,T305A)-HA construct was sequenced by primer walking using the T7 forward primer as the starting primer. Figure 5.8 confirms that the construct is full length wild-type rat PKBγ with the Lys177 and Thr305 residues mutated to Ala and a C-terminal HA tag.

### 5.5.3 Transient transfection of dominant negative rat PKBγ in wild-type MDCK cells.

The rat pcDNA.PKBγ(K177A,T305A)-HA construct was transiently expressed in wild-type MDCK cells to ensure good expression for later experiments (Figure 5.9). The levels of expression were compared to the expression of wild-type rat HA-PKBγ and it was found that there were slightly lower levels of dominant negative protein expression. One possible reason for this may that the expression of the dominant negative PKBγ mutant in the MDCK cells is inducing apoptosis and therefore less cells are surviving when harvested. Note that the Effectene reagent (Qiagen) was used in these and all future transfections since it ensured high levels of transfected cells (~ 40%) and consequently, higher levels of protein expression.
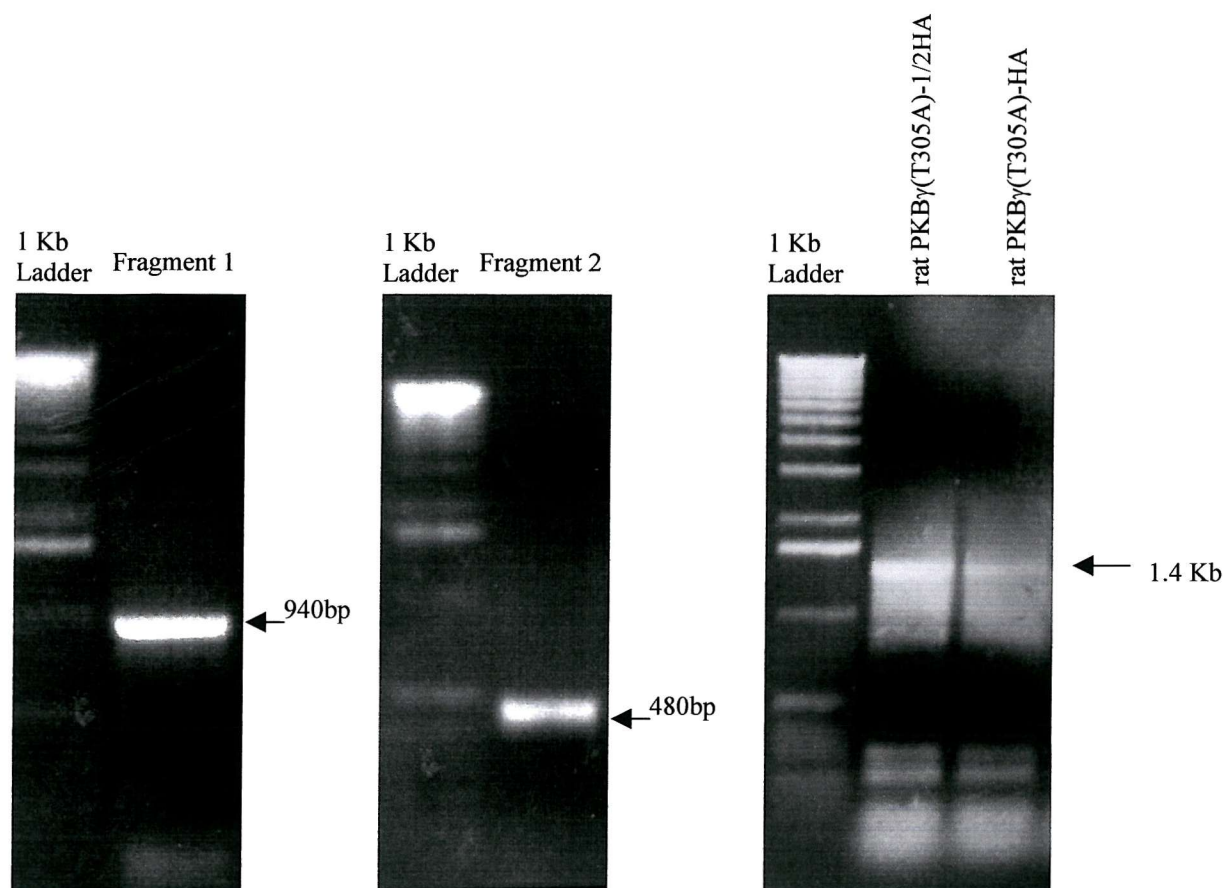
**Figure 5.5    First round of PCR reactions to construct rat PKBγ(T305A)-HA.**

PCR of mutant fragments 1 and 2 of rat PKBγ to alter Thr305 to Ala. Each PCR reaction was carried out using rat brain cDNA as the template DNA and the mutating T305A reverse and forward primers respectively. The 940bp and 480bp products from these reactions were combined as the template DNA in a third reaction to construct rat PKBγ(T305A) with half of the HA tag. Finally, full length rat PKBγ(T305A)-HA was produced in a fourth reaction using the previous reactions product as template DNA with the rat Kpn I forward primer and the Xba I HA reverse primer. PCR conditions for all 4 reactions after initial denaturing at 95°C for 2mins were: 95°C for 30s; annealing at 55°C for 30s; then extension at 72°C for 2mins. This was repeated for 30 cycles with a final extension at 72°C for 5mins. 10μl of each PCR reaction was loaded onto a 1% agarose gel and subjected to electrophoresis for 1 hour at 100V.
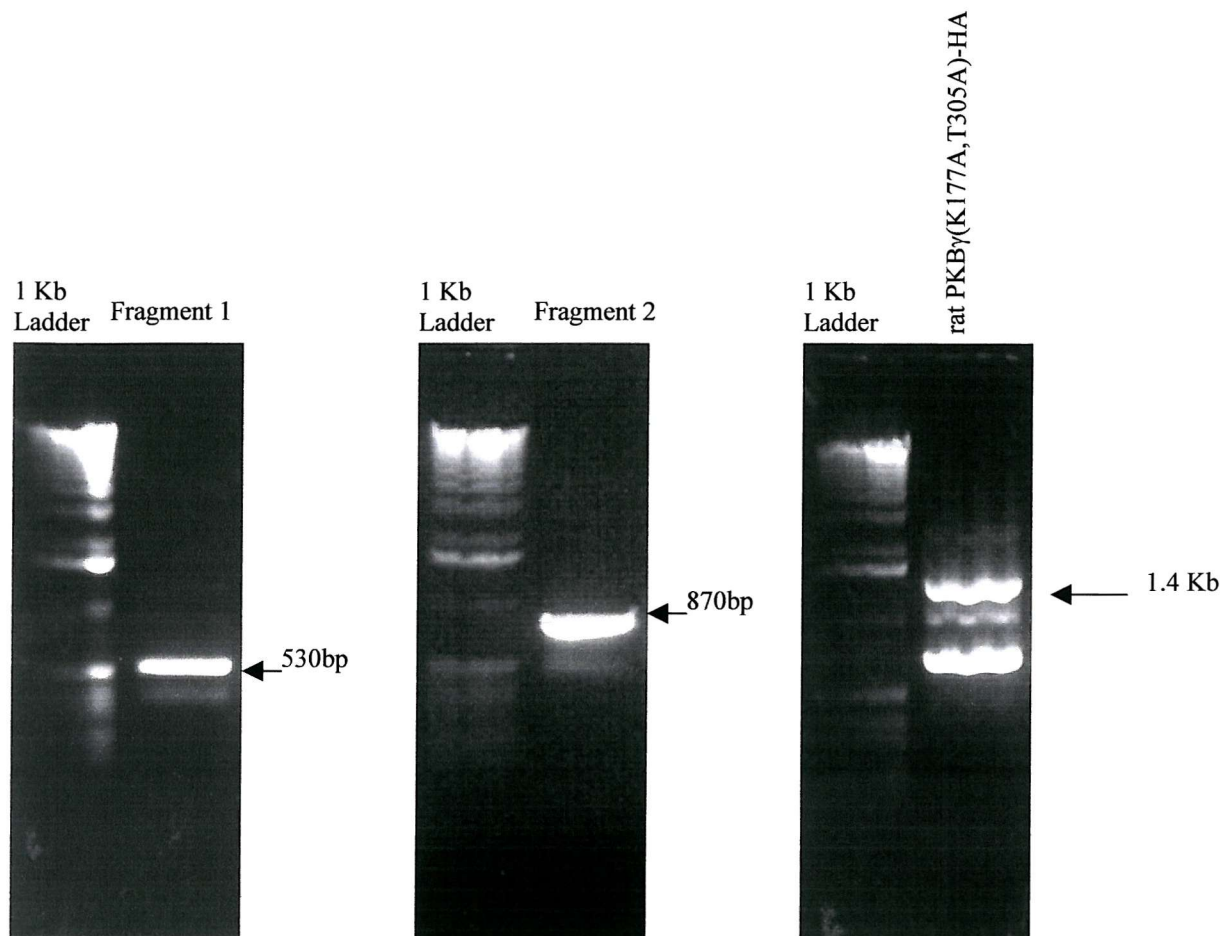
**Figure 5.6     Second round of PCR to construct rat PKBγ(K177A,T305A)-HA.**

PCR of mutant fragments 1 and 2 of rat PKBγ(T305A)-HA to alter Lys177 to Ala. Each PCR reaction was carried out using rat PKBγ(T305A)-HA from the first round of PCR reactions as the template DNA and the mutating K177A reverse and forward primers respectively. The 530bp and 870bp products from these reactions were combined as the template DNA in a third and final reaction to construct rat PKBγ(K177A,T305A)-HA. PCR conditions for all 4 reactions after initial denaturing at 95°C for 2mins were: 95°C for 30s; annealing at 55°C for 30s; then extension at 72°C for 2mins. This was repeated for 30 cycles with a final extension at 72°C for 5mins. 10μl of each PCR reaction was loaded onto a 1% agarose gel and subjected to electrophoresis for 1 hour at 100V.
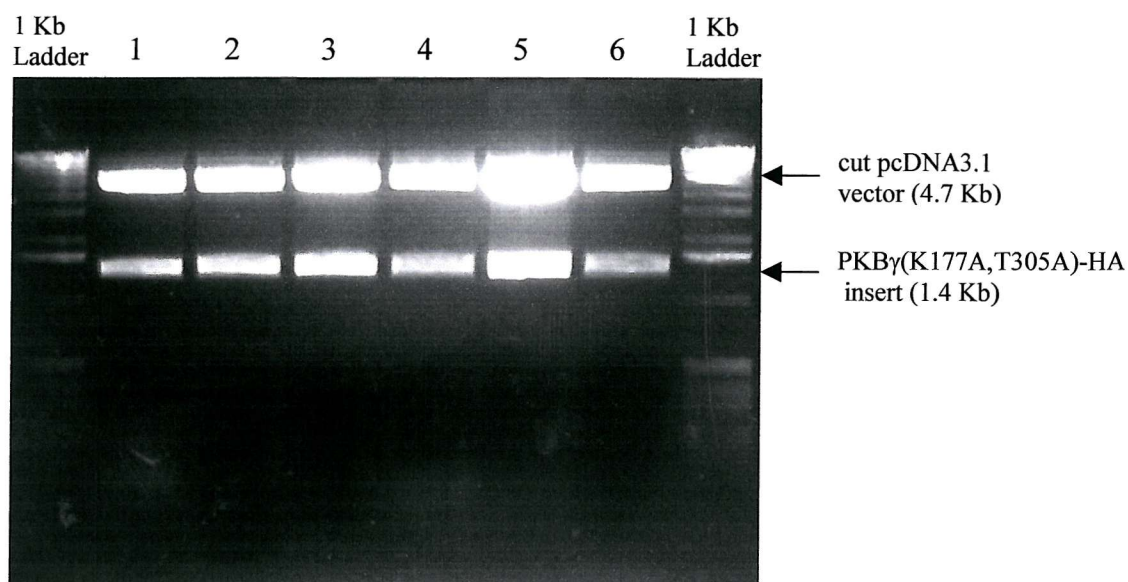
**Figure 5.7** **Restriction digest analysis of rat pcDNA.PKBγ(K177A,T305A)-HA transformations.**

Following ligation of rat PKBγ(K177A,T305A)-HA into the pcDNA3.1 mammalian expression vector and transformation of JM109 calcium competent *E. coli*, 6 ampicillin resistant bacterial colonies were picked and their plasmid DNA mini-preped (Promega). Restriction digests were set up using 5μl of plasmid DNA and 0.5μl each of Kpn I and Xba I restriction enzymes. All 6 colonies tested were found to contain the rat PKBγ(K177A,T305A)-HA insert in the correct orientation. Glycerol stocks were prepared from Colony 1.

**ATG**AGCGATGTTACCATCGTTAAAGAAGGCTGGGTTCAGAAGAGGGGAGAATATATAAAAAATTGGAGGCCAAGA

TACTTCCTTTTGAAGACAGACGGCTCATTCATAGGCTATAAAGAGAAACCTCAAGATGTGGACTTACCTTATCCC

CTCAACAACTTCTCAGTGGCAAAATGTCAATTAATGAAAACAGAACGACCAAAGCCAAATACATTTATTATCAGA

TGTCTTCAGTGGACCACTGTTATAGAGAGAACATTTCATGTAGATACTCCAGAGGAAAGAGAAGAATGGACAGAA

GCTATCCAAGCTGTAGCCGATCGACTGCAGAGGCAAGAAGAGGAGAGGATGAATTGTAGTCCAACGTCACAGATT

GATAATATTGGAGAGGAAGAGATGGATGCATCTACAACCCATCATAAAAGAAAGACAATGAATGATTTTGACTAT

TTGAAACTGCTAGGTAAAGGCACTTTTGGGAAGGTTATTTTGGTTCGAGAGAAGGCAAGTGGA<span style="color:blue">AAATACTACGCT</span>

<span style="color:blue">ATG**GCT**ATTCTGAAGAAAGAA</span>GTCATTATCGCAAAGGATGAAGTGGCACATACTCTGACTGAGAGCAGAGTTCTA

AAGAACACCAGACATCCATTTCTAACATCCTTGAAATATTCCTTCCAGACAAAAGACCGTTTGTGTTTTGTGATG

GAATATGTTAATGGGGGAGAGCTGTTTTTCCATTTGTCGAGAGAGCGGGTGTTCTCTGAGGACCGCACACGTTTC

TATGGTGCAGAAATTGTCTCTGCTTTGGACTATCTACATTCTGGAAAGATTGTGTACCCGAGATCTCAAGTTGGA

GAATTTGATGCTAGATAAGGATGGCCATATAAAAATTACGGATTTTGGACTTTGCAAAGAAGGGATCACAGAT<span style="color:blue">GC</span>

<span style="color:blue">AGCTACCATGAAG**GCT**TTCTGTGGTACACCA</span>GAGTATCTGGCACCAGAGGTATTAGAAGATAATGACTATGGCCG

AGCTGTGGACTGGTGGGGCCTAGTGTTGTCATGTATGAAATGATGTGTGGGAGGTTGCCTTTCTACAACCAGGAT

CATGAGAAACTCTTTGAACTAATACTCATGGAAGACATCAAATTCCCCCGAACACTCTCTTCAGATGCAAAGTCA

TTGCTTTCAGGGCTCTTGATAAAGGATCCAAATAAACGCCTTGGTGGAGGGCCAGATGATCCAAAAGAGATAATG

AGGCACAGTTTCTTTTCTGGAGTAAACTGGCAGGACGTATATGACAAAAAGCTTGTACCTCCTTTTAAGCCTCAA

GTAACATCTGAGACAGACACCAGATATTTCGATGAATTTACAGCTCAGACTATTACAATAACACCACCTGAAAAG

TGTCCACTA<span style="color:red">TACCCATACGATGTGCCAGATTACGCC</span>**TAA**

<span style="color:red">        └─────────────────────────────┘</span>

<span style="color:red">HA tag</span>

**Figure 5.8** **Full length sequencing of dominant negative rat PKBγ(K177A,T305A)-HA.**

Full length sequencing 5'-3'of rat PKBγ(K177A,T305A)-HA (sequencing by Oswell). The translational initiation and stop codons ATG and TAA respectively are highlighted in bold. The mutating regions around the Lysine (K177) residue and the Threonine (T305) residues mutated to Alanine are shown in blue with the mutated codons underlined. The C-terminal HA tag sequence (in red) encodes the amino acid sequence recognised by the anti-HA antibody (U.B.I).

rat
HA-PKBγ          rat dom. negative

← 55 kDA
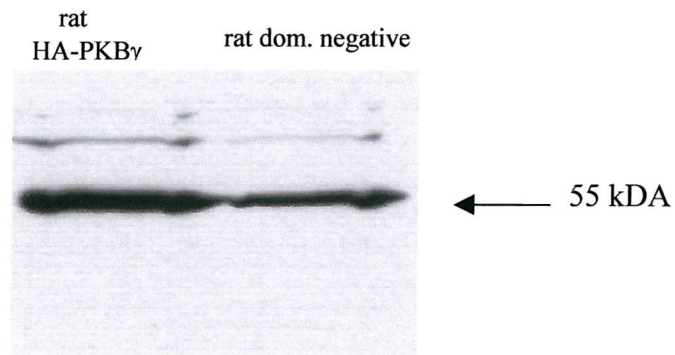
**Figure 5.9      Transient transfection of wild-type MDCK cells with dominant negative rat PKBγ - comparison to wild type rat HA-PKBγ expression.**

Wild-type MDCK cells were transiently transfected with the rat pcDNA.wild-type HA-PKBγ and the dominant negative rat pcDNA.PKBγ(K177A,T305A)-HA construct using the Effectene reagent. Cells were harvested 48 hours later and whole cell protein extracts prepared. 20µg protein was loaded per well and recombinant protein detected using the anti-HA tag antibody.

## 5.6 Growth factor withdrawal induced apoptosis in wild-type MDCK cells can be detected using Annexin V staining.

Using the Apoalert Annexin V kit (Clontech) it is possible to detect early signs of apoptosis in serum starved wild-type MDCK cells (Figure 5.10). Wild-type MDCK cells were seeded onto glass coverslips and the following day either left in 10% FBS (A) or serum starved for 48 hours to induce apoptosis (B). Following this 48 hour incubation period, the cells were stained with fluorescent-labelled Annexin V protein (green), which binds to any externalised PS at the plasma membrane of apoptotic cells. The cells were also counter-stained with Propidium iodide (red) which stains the nuclei of all the cells present. The vast majority of cells growing in the presence of 10% FBS only stained up red, whereas >50% of the serum starved cells stained up with both green and red. This result therefore shows that the serum starvation causes externalisation of PS at the plasma membrane, an early indicator of apoptosis. In addition, the numbers of cells left on the coverslip from the serum starved samples was significantly lower than from the sample growing in the presence of 10% FBS suggesting that many cells had already undergone apoptosis and detached from the surface of the coverslip. An interesting feature of the annexin V staining present in the serum starved sample is that it shows quite clearly membrane blebbing another morphological feature of apoptosis.

**(A)**
**Control**

**(B)**
**Serum starved**
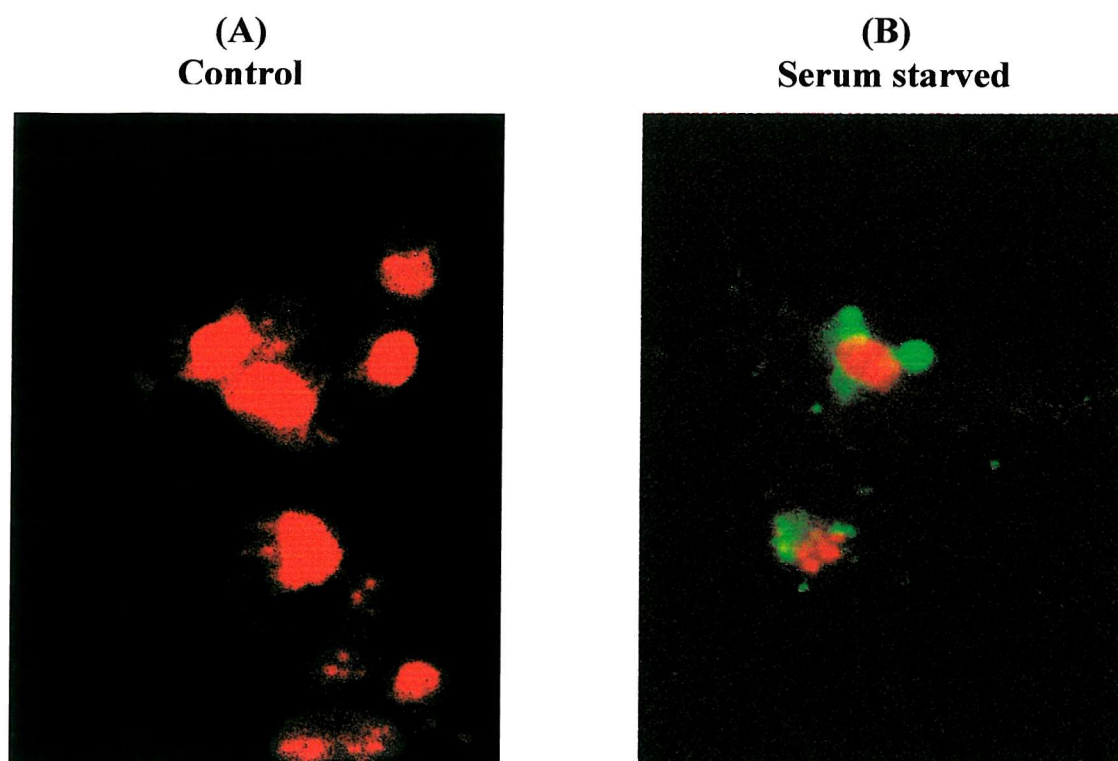
**Figure 5.10   Confocal microscopy of serum starvation induced apoptosis in MDCK cells after annexin V staining.**

**(A)** Wild-type MDCK cells were seeded onto 13mm glass coverslips and grown in 10% FBS for 48 hours. The cells were washed once with PBS, once with 1x binding buffer then incubated in the presence of 5µl of Annexin V (green) and 10µl of propidium iodide (red) stain for 15mins at room temperature (in the dark). Following incubation the cells were fixed in 2% formaldehyde for 10mins then mounted in Citiflour/glycerol/PBS solution. Laser scanning confocal microscopy was performed, magnification X400. A merged image of both green and red is shown.

**(B)** Wild-type MDCK cells were seeded onto 13mm coverslips and serum starved for 48 hours to induce apoptosis. Annexin V staining (green) and propidium iodide staining (red) was as described in (A) as was confocal microscopy.

## 5.7 Transfecting wild-type MDCK cells with dominant negative rat PKBγ induces apoptosis as detected by Annexin V staining.

Expression of the dominant negative rat PKBγ(K177A,T305A)-HA protein in wild-type MDCK cells results in the induction of apoptosis after 48 hours of transfection as detected by Annexin V staining (Figure 5.11D). As a comparison, wild-type untransfected MDCK cells were subjected to serum starvation for 48 hours to show the onset of apoptosis (Figure 5.11C). In contrast, control cells transfected with the empty pcDNA3.1 vector alone showed no signs of apoptosis (Figure 5.11B) and this was comparable to cells growing in 10% FBS (Figure 5.11A). This result proves that overexpression of the dominant negative rat PKBγ mutant inhibits the activity of endogenous PKB in the transfected cells and in doing so causes apoptosis in these cells.

**Figure 5.11  Annexin V staining of MDCK cells following transfection with empty vector or dominant negative rat PKBγ(K177A,T305A)-HA.**

1 x10$^5$ wild-type MDCK cells were seeded onto each 13mm glass coverslip per well of a 12 well plate. The following day cells were treated as follows: **(A)** Untransfected and growing in 10% FBS (negative control); **(B)** Transfected with empty pcDNA3.1 vector only and growing in 10% FBS (vector only control); **(C)** Untransfected and serum starved for 48 hours (positive control); and **(D)** Transfected with rat pcDNA.PKBγ(K177A,T305A)-HA and growing in 10% FBS (dominant negative). Following 48 hours of transfection, the media was removed from the cells and the cells washed, stained and fixed as in Figure 5.9. Magnification X400. These results are representative of two separate experiments.
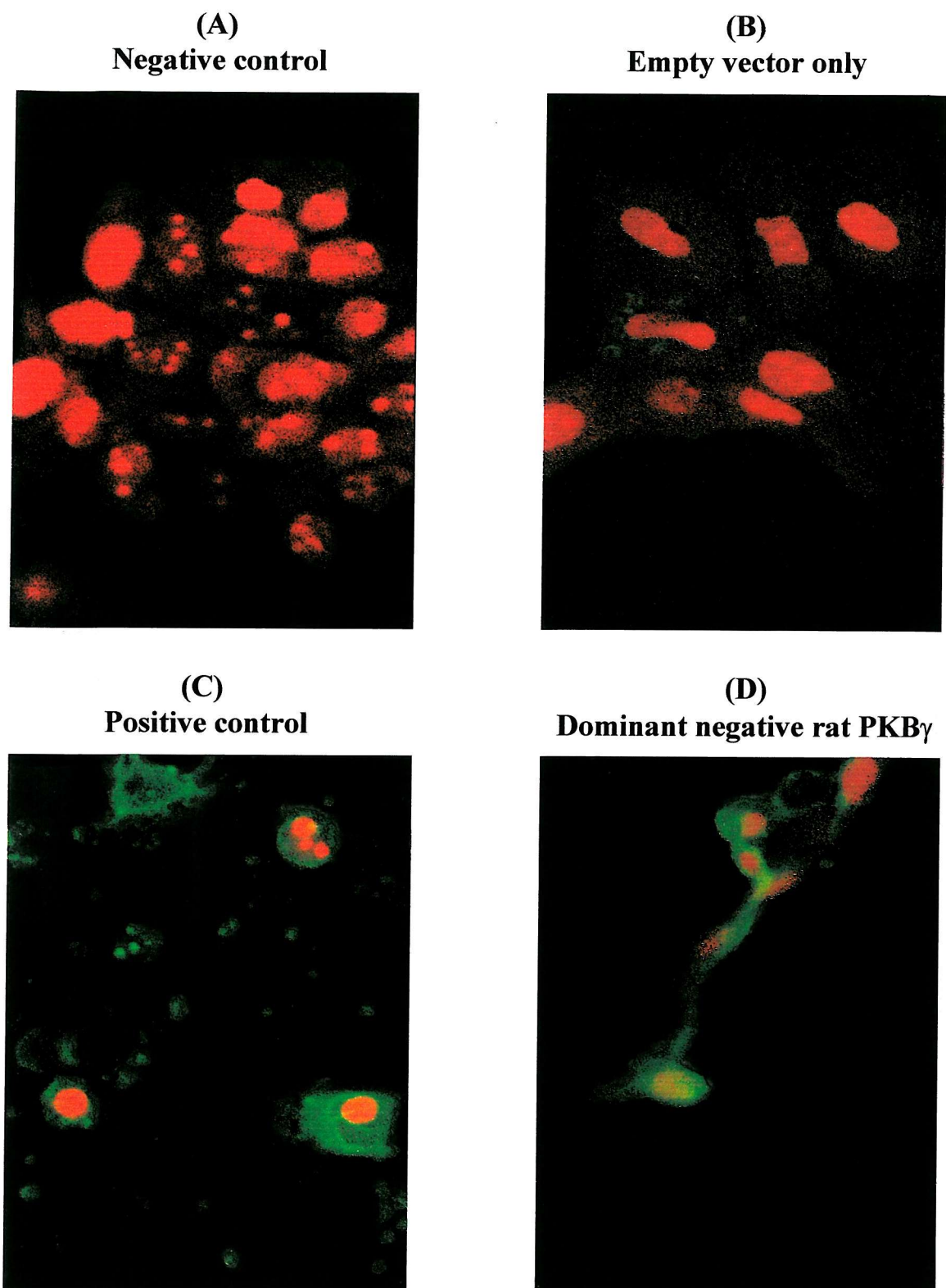
**(A)**
**Negative control**

**(B)**
**Empty vector only**

**(C)**
**Positive control**

**(D)**
**Dominant negative rat PKBγ**

**Figure 5.11** Annexin V staining of MDCK cells following transfection with empty vector or dominant negative rat PKBγ(K177A,T305A)-HA.

## 5.8 Summary.

From the results presented in this chapter it is clear that PKBγ has an important role to play in cellular survival signalling pathways and apoptosis. To ensure good reliable and reproducible results, the technique of growth factor removal (serum starvation) was used as the method of choice to induce apoptosis in our MDCK cells. These cells show characteristic signs of apoptosis following 48 hours serum starvation. This was measured initially by DNA laddering which is one of the biochemical hallmarks of apoptosis. In addition, cells undergoing apoptosis were identified morphologically using flourescently-labelled Annexin V protein. The basis of this assay relies on the binding of the Annexin V protein to externalised phosphatidylserine molecules on the plasma membrane of early apoptotic cells. This technique ensured specific identification of apoptotic cells following serum starvation. Having set up a reliable system for inducing apoptosis in the wild-type MDCK cells, the stable cell lines produced in Chapter 3 were then tested. These cell lines are MDCK cells stably expressing contitutively active mutants of rat PKBγ (rat myrPKBγ-HA and rat PKBγ(T305D)-HA), mouse PKBγ (mouse myrPKBγ-HA) and mouse PKBα (mouse myrPKBα-HA). Following 48 hours of serum starvation, all of the stable MDCK cell lines expressing constitutively active PKBγ and PKBα showed high levels of protection against apoptosis, in contrast to control MDCK cells that show extensive DNA laddering. This result was also confirmed by using the MTS method to measure the percentage of cells that survive following the induction of apoptosis.

Using a dominant negative mutant of rat PKBγ provided additional evidence demonstrating the essential role PKB plays in cell survival. This mutant, when overexpressed in cells, has the ability to inhibit the activity of endogenous PKB, possibly by competing for the upstream

kinases and /or phospholipids. From these results it is clear that overexpressing this dominant negative mutant in wild-type MDCK cells causes apoptosis, even when the cells are growing in the presence of 10% FBS. Taken together, these results demonstrate the potential role PKBγ plays in the growth and survival of cells.

# CHAPTER 6

# GENERAL DISCUSSION AND FUTURE WORK.

# CHAPTER 6: General discussion and future work.

## 6.1 General discussion.

The main findings of this study are: (1) that constitutively active rat and mouse PKBγ both protect cells against growth factor withdrawal induced apoptosis; and (2) GSK-3 is a downstream target of PKBγ. These results prove that PKBγ is a crucial protein serine/threonine kinase mediating the survival signalling of growth factors and that this cell survival signalling may well involve GSK-3, a downstream substrate of PKBγ.

Rat PKBγ was first cloned from rat brain tissue cDNA in 1995 by Konishi and co-workers and found to be slightly different from the previously cloned PKBα and PKBβ isoforms in that it was missing 23 amino acids from the C-terminus (Konishi et al., 1995). This difference in sequence was significant because the regulatory $Ser^{473}$ residue was missing. At the time of initiating this Ph.D project the general belief was that this rat PKBγ was simply a truncated isoform of PKB (Konishi et al., 1995; Walker et al., 1998; Andjelkovic et al., 1997). For this reason PKBγ was cloned from rat brain tissue cDNA using the published sequence to design PCR primers. Two differences were observed between the sequence obtained and that published by Konishi and co-workers. $Asn^{10}$ was changed to Gly and $Pro^{396}$ was changed to Ala. The equivalent residues in PKBα and PKBβ are also Gly and Ala. These changes were also found by Walker and co-workers (Walker et al., 1998).

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In 1999, two groups working independently cloned mouse and human PKBγ (Nakatani et al., 1999; Brodbeck et al., 1999). The importance of this was that these PKBγ clones were full-length PKBγ isoforms and contained the Ser$^{472}$ regulatory residue as found in PKBα and PKBβ. These results have brought into question the original cloning of rat PKBγ and one must address the possibility that the truncated rat PKBγ is a product of alternative splicing of the rat PKBγ mRNA. This now seems the case following the very recent cloning of a full-length rat PKBγ cDNA containing the regulatory Ser$^{472}$ residue (Vanhasenbroeck and Alessi, 2000). Following the publication of the full-length mouse PKBγ cDNA sequence I decided that it was important to clone this PKBγ isoform in order to compare it to the truncated rat PKBγ. In addition, I also cloned mouse PKBα to use as a positive control. Sequencing of both my cloned mouse PKBγ and PKBα isoforms revealed no differences to the published sequences.

To produce constitutively active mutants of my cloned PKBγ and PKBα isoforms I added the mouse Lck myristoylation sequence MGCVCSSNPEDD onto the N-terminals of wild-type ratPKBγ, mouse PKBγ and mouse PKBα. During recombinant protein expression myristic acid is covalently bonded to the N-terminal Gly$^2$ residue of this Lck sequence which targets the recombinant protein to the plasma membrane, even in the absence of growth factor stimulus. At the plasma membrane the mutant PKB proteins associate with the upstream kinases PDK1 and the putative Ser472 kinase, PDK2. Since PDK1 and PDK2 are constitutively active, they phosphorylate and activate the membrane targeted PKB mutants (Bellacosa *et al.*, 1991; Andjelkovic et al., 1997). In addition to the membrane-targeted mutant of rat PKBγ, a second constitutively active mutant was

constructed by site-directed mutagenesis of the Thr$^{305}$ regulatory residue to Asp. Changing the Thr$^{305}$ residue to the negatively charged amino acid Asp mimics the effect of phosphorylation and so the mutant rat PKBγ is active even in the absence of growth factor stimulus (Alessi et al., 1996). Stabily transfected MDCK cell lines were set up expressing these constitutively active mutants of rat PKBγ, mouse PKBγ and mouse PKBα and three individual clones from each cell line were picked for subsequent experiments.

The stable cell lines were used to first determine if the expressed mutant PKB proteins were indeed constitutively active. For this purpose a kinase assay was employed to measure the activity of GSK-3, a putative *in vivo* target of PKB, found in most cells (Cross et al., 1995). This kinase assay was an excellent way of measuring the activity of the PKB mutants for several reasons: (1) it could be carried out quickly using whole cell extracts, without the requirement for any prior purification of GSK-3; (2) it measures the activity of an endogenous substrate of PKB already in the cells, unlike the usual PKB kinase assays which involve first immunoprecipitating the PKB then using an exogenous substrate (e.g. Histone H2B or the peptide Crosstide) to measure activity; and (3) it would confirm that GSK-3 is a downstream target of PKBγ *in vivo*. The results obtained show that, even following 24 hours of serum starvation, the activity of GSK-3 in the stable cell lines expressing contitutively active rat PKBγ, mouse PKBγ and mouse PKBα is reduced by 40-50% as compared to the activity of GSK-3 from serum-starved wild-type MDCK cells. This demonstrates that active PKBγ phosphorylates and inactivates GSK-3 *in vivo*, backing up the findings of Shaw and co-workers (Shaw et al., 1997; Shaw and Cohen, 1999). See figure 6.1 for details of the PI 3-kinase/PKBγ/GSK-3 signaling pathway.

**Figure 6.1    Apoptosis mediated by GSK-3 activation.**

Apoptotic stimuli, such as the withdrawal of growth factors, cause the inactivation of the PI 3-kinase/PKBγ pathway. GSK-3 is inactivated by phosphorylation and has been identified as a downstream target of PKB. Therefore, down-regulation of PKB results in dephosphorylation and activation of GSK-3. Several substrates of GSK-3 have been tentatively linked to the induction of apoptosis. Glycogen synthase is phosphorylated and inactivated by GSK-3 resulting in a general decrease in glucose metabolism (Cohen, 1985). GSK-3 phosphorylates β-catenin causing β-catenin degradation (Millar and Moon, 1996). β-catenin is important both as a component of cytoskeletal structure and as a transcription factor of Wnt genes. Therefore, degradation and loss of β-catenin disrupts cytoskeletal structure and down-regulates gene transcription possibly resulting in apoptosis. Mitochondrial pyruvate dehydrogenase may be phosphorylated and inactivated by GSK-3 causing metabolic failure (Hoshi et al., 1996). GSK-3 phosphorylates IRS-1 converting it into an inhibitor of insulin receptor tyrosine kinase activity (Eldar-Finkelman and Krebs, 1997). Since insulin and IGF-1 both promote cell survival, then inhibition of the receptor for these growth factors may result in apoptosis. Finally, GSK-3 phosphorylates Tau converting it into PH-Tau. Hyperphosphorylated Tau is a major component of paired helical filaments (PHF's), the building block of neurofibrillary lesions in Alzheimers disease, typified by neuronal apoptosis (Hanger et al., 1992).
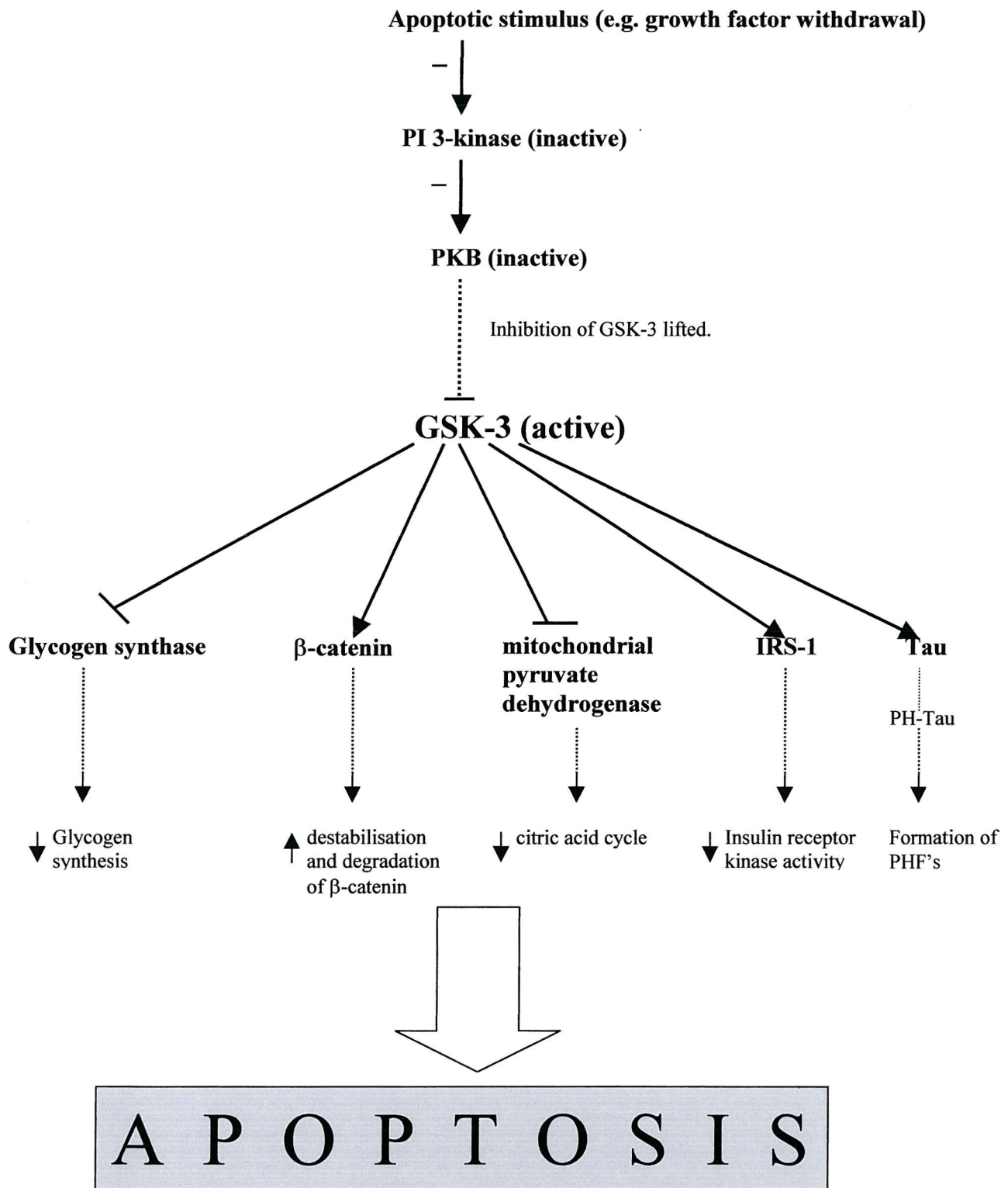
**Figure 6.1    Apoptosis mediated by GSK-3 activation.**

The second part of this study involved the potential role PKBγ has to play in promoting cell survival signalling by blocking apoptosis. Over the past few years work on several types of neuronal cells, fibroblasts and epithelial cells have implicated PKBα in just such a role (Dudek et al., 1997; Kauffmann-Zeh et al., 1997; Kennedy et al., 1997; Khwaja et al., 1997; Kulik et al., 1997; Philpott et al., 1997; Songyang et al., 1997; Xiong and Parsons, 1997; Eves et al., 1998; Gerber et al., 1998; Kulik and Weber, 1998; Hausler et al., 1998). For the purpose of this study, stable MDCK cell lines were set up expressing constitutively active mutants of rat PKBγ, mouse PKBγ and mouse PKBα. MDCK cells were chosen as a model system in this study since they undergo apoptosis in response to several different apoptotic stimuli. Matrix detachment, UV-irradiation and serum starvation have all been used in previous studies (Frisch and Francis, 1994; Khwaja et al., 1997; Chen et al., 1999). In the present study matrix detachment and serum starvation were both tested in wild-type MDCK cells to establish a reproducible and reliable system for inducing apoptosis in these cells. Serum starvation was found to be the most consistent method for inducing apoptosis and was used to test the stable MDCK cell lines. I found that constitutively active rat PKBγ, mouse PKBγ and mouse PKBα protected MDCK cells against serum starvation induced apoptosis. This result was confirmed both by DNA fragmentation gels (DNA laddering) and by cell survival measurements (MTS method).

In addition, a dominant negative mutant of rat PKBγ (rat PKBγ(K177A,T305A)-HA) was cloned by site-directed mutagenesis, changing the $Lys^{177}$ and $Thr^{305}$ residues to the neutral amino acid Ala. This mutant rat PKBγ was transiently expressed in wild-type

MDCK cells and found to induce apoptosis, even in the presence of serum. This result was shown by Annexin V staining of the transfected cells where a fluorescent-labeled Annexin V protein binds to externalized PS molecules at the plasma membrane. Dominant negative mutants are thought to work by competiting with endogenous PKB for the PI(3,4,5)P$_3$ and PDK1(2) and in doing so inhibit endogenous PKB signalling. Therefore, by blocking PKB the cell losses a major cell survival signalling pathway and pro-apoptotic Bcl-2 family members prevail over pro-survival Bcl-2 family members. The end result is the activation of the caspase cascades and the onset of apoptosis.

Taken together, these results demonstrate that PKB$\gamma$ can mediate the cell survival signalling pathways activated by growth factors such as insulin, IGF-1, EGF and PDGF. Upon withdrawal of these growth factors the PKB$\gamma$ pathway is down regulated resulting in the onset of apoptosis. The downstream targets of PKB$\gamma$ involved in apoptosis pathways have yet to be identified although this study has for the first time identified GSK-3 as a probable *in vivo* target of PKB$\gamma$. Even though GSK-3 has been traditionally implicated in glucose metabolism by regulating the activity of glycogen synthase, recently a new role is emerging for GSK-3 in mediating apoptosis signalling (Pap and Cooper, 1998; Hetman et al., 2000; Hoeflich et al., 2000). Figure 6.1 demonstrates the possible substrates of GSK-3 implicated in apoptosis.

## 6.2 Future work.

This study provides compelling evidence that PKBγ mediates cell survival signalling in MDCK cells. However, to gain a greater understanding of PKBγ's involvement in cell survival signalling, the same work must be carried out in other cell types to see if similar results are obtained. Specifically, neuronal cells will be an interesting model to use since PKBγ is the highest expressed of the three PKB isoforms in brain tissue (Walker et al., 1998; Nakatani et al., 1999).

Although this study has identified GSK-3 as a putative downstream target of PKBγ *in vivo*, it is important that the other downstream targets of PKBγ be identified. This information will be crucial in further resolving the signalling pathways involved in cell survival and apoptosis. In particular, focus must be turned on putative apoptotic PKB substrates such as BAD, Caspase-9, the forkhead family members and NF-κB.

Much work has already been carried out to identify the mechanism of PKB activation, however the identity of the $Ser^{473}$-phosphorylating protein kinase, tentatively named PDK2, remains to be discovered. Recently it has been suggested that PDK-1 can actually interact with other proteins in order to modify its function. PDK-1 may complex with a fragment of another kinase, PRK-2 (Balendran et al., 1999). This PDK-1/PRK-2 complex is then capable of phosphorylating PKBα at Ser473. In addition, this complex is up regulated by PI-3 kinase –generated 3'-phosphorylated phospholipids. Other evidence suggests that Integrin-linked kinase (ILK) has the ability to phosphorylate Ser473 of

PKBα *in vitro* (Delcommenne et al., 1998) and since this enzyme is activated by PI 3'-kinase it may well be PDK2.

## 6.3 Summary

In summary, this study has demonstrated that PKBγ is an essential component and mediator of growth factor-stimulated cell survival pathways. In the presence of growth factors, PKBγ is active and protects cells from apoptosis, promoting cell survival. These results have important implications for a number of common diseases involving disruption of normal intracellular signalling. Under normal physiological conditions a balance exists between cell proliferation and apoptosis (Thompson, 1995). However, biochemical or genetic changes, which either constitutively activate PKBγ or over-express PKBγ, could potentially result in increased protection against apoptosis and as a consequence, cell proliferation. This could then conceivably lead to the onset of cancer. Conversely, down-regulation of PKBγ results in the onset of apoptosis, as demonstrated by my work using the dominant negative rat PKBγ mutant. If this occurred in the brain, the result would be the loss of neuronal cells leading to neurodegenerative disease such as Alzheimer's disease, Parkinson's disease or ALS. Finally, down-regulation or inactivation of PKBγ in skeletal muscle and/or adipose tissue could possibly result in the insulin resistance typically observed in patients suffering Type II diabetes. Therefore it is of the utmost importance that we do everything to identify and develop specific activators and inhibitors of PKBγ in order to treat these diseases therapeutically.

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