UNIVERSITY OF SOUTHAMPTON

BAG-1 expression and function in breast cancer

Presented by

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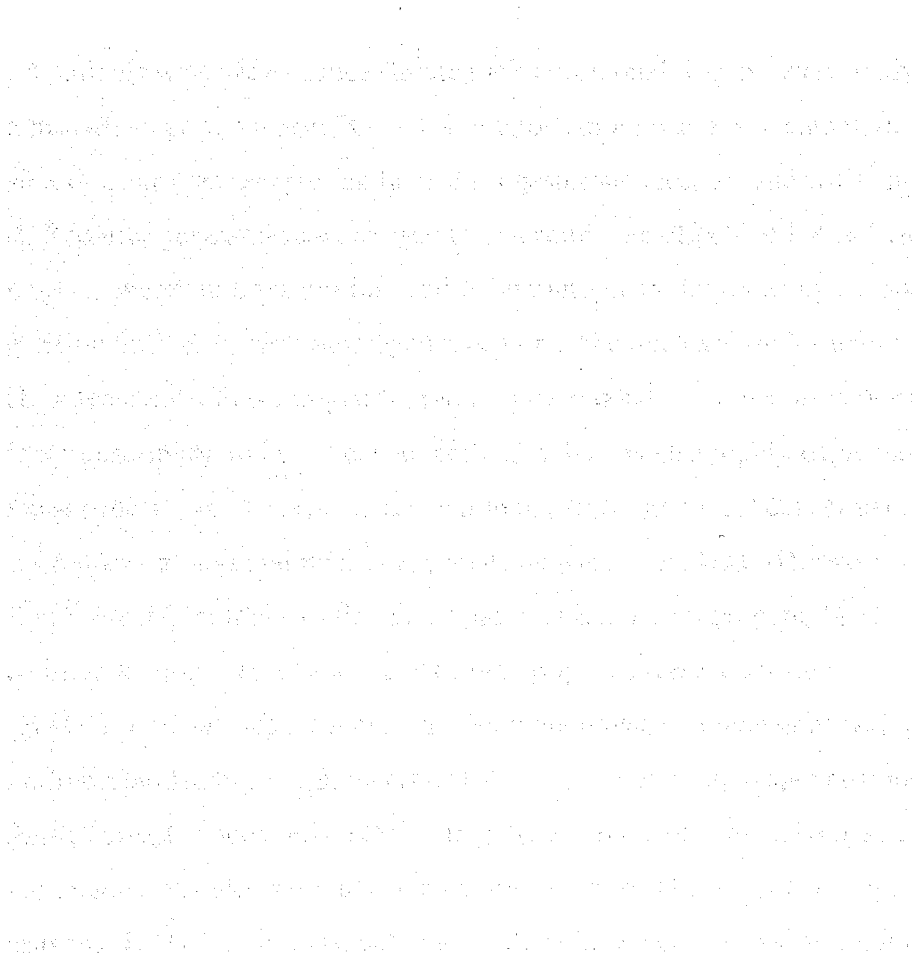
Declaration

This thesis is the result of original work performed whilst in registered postgraduate candidature under the supervision of Dr G Packham and Professor P Johnson within the Cancer Sciences Division of the University of Southampton. Unless otherwise stated I carried out this work entirely myself and it forms no part of any other degree or thesis.



UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES

CANCER SCIENCES DIVISION

Doctor of Philosophy

BAG-1 EXPRESSION AND FUNCTION IN BREAST CANCER

By Ramsey Ian Cutress

BAG-1 is a multifunctional protein that binds a wide range of cellular targets including heat shock proteins and some nuclear hormone receptors. BAG-1 exists as three isoforms, BAG-1L, BAG-1M and BAG-1S. BAG-1L contains a nuclear localisation signal, which is not present in the other isoforms and is predominantly localised in the cell nucleus.

To determine the significance of BAG-1 expression in breast cancer, tumours from 138 patients with breast cancer treated with hormonal therapy were analysed by immunohistochemistry. Nuclear BAG-1 immunostaining was associated with expression of oestrogen receptor alpha and progesterone receptor and with improved survival. Reporter gene assays were used to determine the effects of BAG-1 isoforms on oestrogen dependent transcription, and coimmunoprecipitation assays to analyse the interaction of BAG-1 with oestrogen receptors. The nuclear BAG-1 isoform, BAG-1L, interacted with oestrogen receptor alpha and beta and increased oestrogen dependent transcription in breast cancer cells. BAG-1S is also highly expressed in some breast cancers, and to investigate its role in protecting breast cancer cells from apoptosis reporter assays and microarray analysis were used. BAG-1S overexpression reduced p53 dependent transcription and candidate BAG-1 target genes that may be involved in protecting breast cancer cells from apoptosis were identified.

BAG-1 protects breast cancer cells from apoptosis and interferes with p53 function. Importantly, since high levels of BAG-1L can increase responsiveness to oestrogens in breast cancer cells, BAG-1 may be a marker of responsiveness to hormonal therapy, via direct effects on receptor function. These findings support the hypothesis that BAG-1 is an important molecule in breast cancer and suggest that BAG-1 may prove to be a novel target for cancer therapy.

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I have presented work described within this thesis at various scientific meetings. These include the British Association of Surgical Oncology Annual Scientific Meeting (November 2002), the British Cancer Research Meeting (July 2002) for which the presentation won the Hamilton-Fairley prize for best proffered paper and the 24th San Antonio Breast Cancer Symposium (December 2001). Work described within this thesis has formed a major part of, or contributed to, the following publications:

- Cutress, R. I., Townsend, P. A., Sharp, A., Maison, A., Wood, L., Brimmell, M., Brimmell, M., Mullee, M. A., Johnson, P. W., Royle, G. T., Bateman, A. C., & Packham, G. 2003, "The nuclear BAG-1 isoform, BAG-1L, enhances oestrogen-dependent transcription", *Oncogene*, vol. 22, no. 32, pp. 4973-4982.

- Cutress, R. I., Townsend, P. A., Brimmell, M., Bateman, A. C., Hague, A., & Packham, G. 2002, "BAG-1 expression and function in human cancer", *British Journal of Cancer*, vol. 87, pp. 834-839.

- Cutress, R. I., Townsend, P. A., Bateman, A. C., Johnson, P. W., Ryder, K., Barnes, D. M., & Packham, G. 2001, "BAG-1 immunostaining and survival in early breast cancer", *Journal of Clinical Oncology*, vol. 19, no. 16, pp. 3706-3707.

- Townsend, P. A., Cutress, R. I., Sharp, A., Brimmell, M., & Packham, G. 2003, "BAG-1: a multifunctional regulator of cell growth and survival", *Biochimica et Biophysica Acta*, vol. 1603, no. 2, pp. 83-98.

- Townsend, P. A., Cutress, R. I., Sharp, A, Brimmell, M., & Packham, G. 2003, "BAG-1 Prevents Stress-Induced Long Term Growth Inhibition in Breast Cancer Cells via a Chaperone-Dependent Pathway", *Cancer Research*, vol. 63, no.14, pp. 4150-4157.

- Townsend, P. A., Dublin, E., Hart, I. R., Kao, R.-H., Hanby, A. M., Cutress, R. I., Poulsom, R., Ryder, K., Barnes, D. M., & Packham, G. 2002, "BAG-1 expression in human breast cancer: Inter-relationship between BAG-1 RNA, protein, hsc-70 expression and clinico-pathological data", *Journal of Pathology*, vol. 197, pp. 51-59.

- Arhel, N. J., Packham, G., Townsend, P. A., Collard, T. J., H-Zadeh, A. M., Sharp, A., Cutress, R. I., Malik, K., Hague, A., Paraskeva, C., & Williams, A. C. 2003, "The retinoblastoma protein interacts with Bag-1 in human colonic adenoma and carcinoma derived cell lines", *International Journal of Cancer*, vol. 106, no. 3, pp. 364-371.

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Abbreviations

AR	Androgen receptor
COX	Cytochrome c oxidase
DBD	DNA binding domain
DMEM	Dulbecco's modified Eagles medium
EMSA	Electrophoretic mobility shift assay
ER	Oestrogen receptor
ERE	Oestrogen response element
GR	Glucocorticoid receptor
GST	Glutathione-S-transferase
HB-EGF	Heparin-binding-epidermal growth factor
HBS	HEPES Buffered Saline
HGFR	Hepatocyte growth factor receptor
HRP	Horseradish peroxidase
IRES	Internal ribosome entry sequence
MR	Mineralocorticoid receptor
NHR	Nuclear hormone receptor
NLS	Nuclear localisation signal
PBS	Phosphate Buffered Saline
PgR	Progesterone receptor
PDGF	Platelet derived growth factor
RAR	Retinoic acid receptor
RXR	Retinoid X receptor
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
SEM	Standard error of the mean
TBE	Tris-Borate-EDTA Buffer
TE	Tris-EDTA buffer
TR	Thyroid hormone receptor
TS	Tris-Buffered Saline
ULD	Ubiquitin-like domain
VDR	Vitamin D receptor

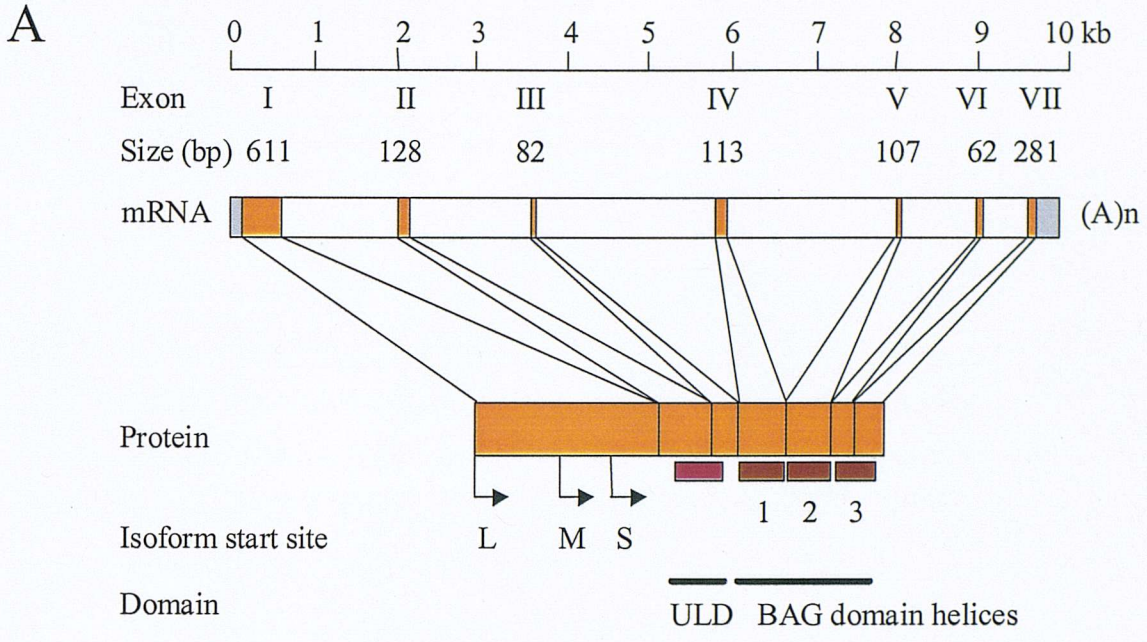
1. Introduction

1.1 Introduction

BAG-1 is a multifunctional protein that interacts with a diverse array of molecular targets. These include the 70 kDa heat shock proteins, HSC70 and HSP70, the Raf-1 kinase, components of the ubiquitylation/proteasome system, the BCL-2 protein, nuclear hormone receptors, and DNA. BAG-1 modulates many regulatory pathways important for both normal cells and which are deregulated in malignancy, including apoptosis, signalling, proliferation, transcription and cell motility. Alterations of BAG-1 expression occur at an early stage in malignant processes, and studies of BAG-1 expression and function may increase our understanding of normal and malignant processes and provide novel targets for cancer therapy.

1.2 BAG-1 Gene Structure

BAG-1 was initially identified in a screen for BCL-2 binding proteins (**B**CL-2 associated **A**thano**G**ene 1; athanos (Greek: anti-death)) (Takayama et al. 1995). It became apparent that this was the product of the same gene as a glucocorticoid receptor (GR) binding protein identified later in the same year and initially known as RAP46 (**R**eceptor **A**ssociated **P**rotein 46 kDa) (Zeiner & Gehring 1995). The BAG-1 gene comprises 7 exons and is located on chromosome 9 band 12 (Figure 1.1) (Takayama et al. 1996). This chromosomal region is not associated with frequent cytogenetic alterations in human cancer, although some single nucleotide polymorphisms of unknown significance occur. Some hereditary disorders, including Fraser syndrome, where neonates and stillborns are born with a failure of eye fissures to form (crypopthalmos), and webbed digits (syndactyly) may be associated with alterations of this part of chromososme 9 (Takayama et al. 1996). Although these conditions appear to involve defective apoptosis there is no direct evidence that BAG-1 is involved.



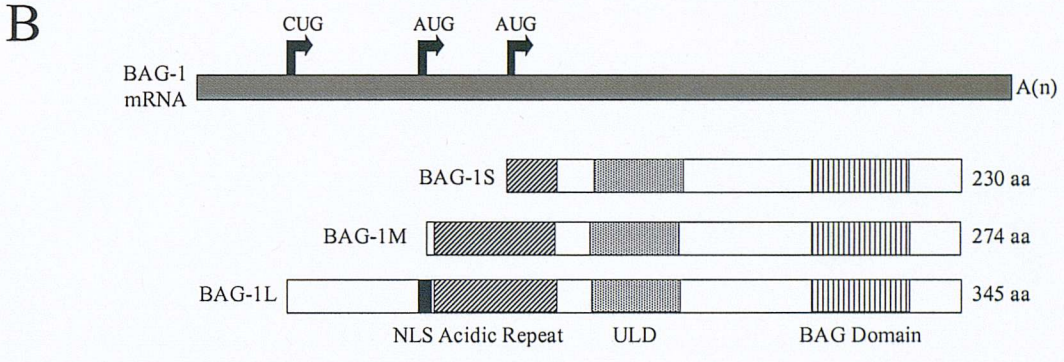


Figure 1.1: BAG-1 gene and protein structure

The human BAG-1 gene (A; adapted from Townsend et al. 2003b) comprises 7 exons and spans approximately 10 kb. The BAG-1 gene is transcribed to produce a single mRNA of approximately 1.5 kb. Alternate translation initiation from CUG and AUG codons generates the major BAG-1 isoforms (B) from different start sites. Each BAG domain helix is derived from a separate exon.

NLS, nuclear localisation sequence; ULD, ubiquitin-like domain

There are multiple BAG-1 isoforms of 36 kDa (BAG-1S), 46 kDa (BAG-1M/RAP46) and 50 kDa (BAG-1L) in human cells, and 32 kDa (BAG-1S) and 50 kDa (BAG-1L) in mouse cells. Mouse cells do not express an equivalent to the BAG-1M isoform. A further fourth BAG-1 isoform of 29 kDa has also been described (Yang et al. 1998), but is often present in much smaller quantities or not detected at all. Although there are multiple BAG-1 isoforms, multiple BAG-1 mRNAs have not been detected. The multiple BAG-1 proteins are generated from a single mRNA through the selection of different translation start sites (Packham et al. 1997; Takayama et al. 1998; Yang et al. 1998).

The majority of proteins are produced by cap-dependent translation. RNA transcripts are capped in the nucleus at their 5' end with 7-methylguanosine triphosphate. This cap is important for proper binding of the ribosome which then "scans" along the mRNA until it reaches an AUG (transcribed from ATG) which codes for methionine, within a good Kozak consensus sequence (Kozak 1989), and translation begins. Unusually, BAG-1L translation initiates at an upstream CUG codon whereas BAG-1M translation begins at the first in-frame AUG codon and BAG-1S at the second in-frame AUG. The first AUG is not conserved in mice explaining why mouse cells do not express BAG-1M. Translation initiation at CUG codons, although relatively rare, has been described for several other proteins including c-myc, Hck and Int2 (Hann et al. 1988; Lock et al. 1991). Interestingly similar to BAG-1, cells also express multiple isoforms of these proteins produced by translation from alternate start sites.

BAG-1S is generally the most abundant isoform expressed in cells, followed by BAG-1L and then BAG-1M (Brimmell et al. 1999; Packham et al. 1997; Takayama et al. 1998; Yang et al. 1998). Internal ribosome entry sequence (IRES)-dependent translation, provides an explanation for the surprising finding that BAG-1S is the most abundant isoform in cells, despite being downstream of multiple CUG and two AUG codons, some in frame and in good Kozak consensus. To produce BAG-1S solely by cap-dependent translation scanning ribosomes would have to disregard these, which would be unlikely to occur according to the scanning model of

translation initiation. Thus the smaller c-myc isoform and BAG-1S are produced by IRES-dependent translation where ribosomes are directed to the internal BAG-1S and c-myc AUG's. IRES-dependent translation is used by viruses and by proteins important for cell death or growth including, in addition to c-myc, XIAP, APAF-1 and PDGF. Cap dependant scanning is reduced during apoptosis, mitosis or under conditions of stress, and IRES-dependent translation enables continued production of certain important proteins despite the general shutdown of protein synthesis that occurs during these processes (Gray & Wickens 1998). IRES-dependent translation is dependent on complex structural elements within mRNA to direct ribosomes to internal start sites, and the sequence in the BAG-1 mRNA upstream of the AUG for BAG-1S is relatively long (410 nucleotides) and particularly GC-rich, and therefore has the potential to form extensive secondary structures. This region enhances translation of a downstream open reading frame in artificial bicistronic mRNAs 17 fold, binds directly to known IRES activating proteins (Pickering et al. 2003), and maintains BAG-1S production following heat shock (Coldwell et al. 2001). Together these findings demonstrate that IRES-dependent translation is responsible at least in part for expression of BAG-1S.

1.3 BAG-1 protein structure

1.3.1 BAG-1 nuclear localisation sequences

Various domains have been identified within BAG-1 proteins. A nuclear localisation signal (NLS) has been identified within the unique amino-terminal domain of BAG-1L, consistent with the predominantly nuclear localisation of this isoform (Brimmell et al. 1999; Packham et al. 1997; Takayama et al. 1998; Yang et al. 1998). This sequence is well conserved in human and mouse BAG-1 (human: PRMKKKT, mouse: PRVKKKV) and is very similar to the SV40 Tag NLS (PKKKRKV). By contrast, BAG-1S and BAG-1M lack this sequence and BAG-1S is largely located in the cytoplasm whilst BAG-1M partitions between the nucleus and cytoplasm (Packham et al. 1997; Takayama et al. 1998; Yang et al. 1998).

Five out of the seven of the amino acids of this NLS are present at the extreme amino terminus of BAG-1M, but this is unlikely to constitute a functional NLS since BAG-1M is often found in the cytoplasm and one of the missing amino-acids is a key charged residue (arginine). Indeed it has been suggested that this sequence may be part of a larger nucleoplasmin-like NLS (Takayama et al. 1998) and additional amino-terminal sequences outside of this core region are required for optimal nuclear localization (Knee et al. 2001). Although another potential NLS resides within BAG-1S the function of this has not been addressed directly (Zeiner & Gehring 1995). It is possible that this sequence may play a role in changes in BAG-1 localisation under certain conditions.

1.3.2 The BAG domain and BAG family proteins

All BAG-1 isoforms contain a carboxy-terminal "BAG domain" which plays a key role in mediating many BAG-1 functions (Figure 1.2). This domain of approximately 50 amino-acid residues defines a family of related BAG proteins which all contain a BAG domain close to their carboxy-terminus (Takayama & Reed 2001). This core of the BAG domain comprises two anti-parallel alpha-helices (helices two and three; Figure 1.2) that mediate interaction with the HSC70 and HSP70 heat shock proteins (Briknarova et al. 2001; Sondermann et al. 2001). A third helix (helix one; Figure 1.2) is not required for binding to the HSC70 and HSP70 heat shock proteins but may play a role in maintaining the overall structure of the region and is involved in binding to Raf-1 (Song et al. 2001). HSC70 and HSP70 molecular chaperones bind proteins in non-native states assisting them to reach functional confirmations, and comprise a peptide-binding domain that interacts with denatured polypeptides and a regulatory ATPase domain. BAG-1 interacts with the ATPase domain, leaving the peptide-binding domain available for further interactions with protein substrates. BAG-1 regulates the chaperone function of HSC70 and HSP70 (Hohfeld 1998) and mutation of specific amino-acid residues important for binding to chaperone proteins abrogates at least some BAG-1 functions (Briknarova et al. 2001).

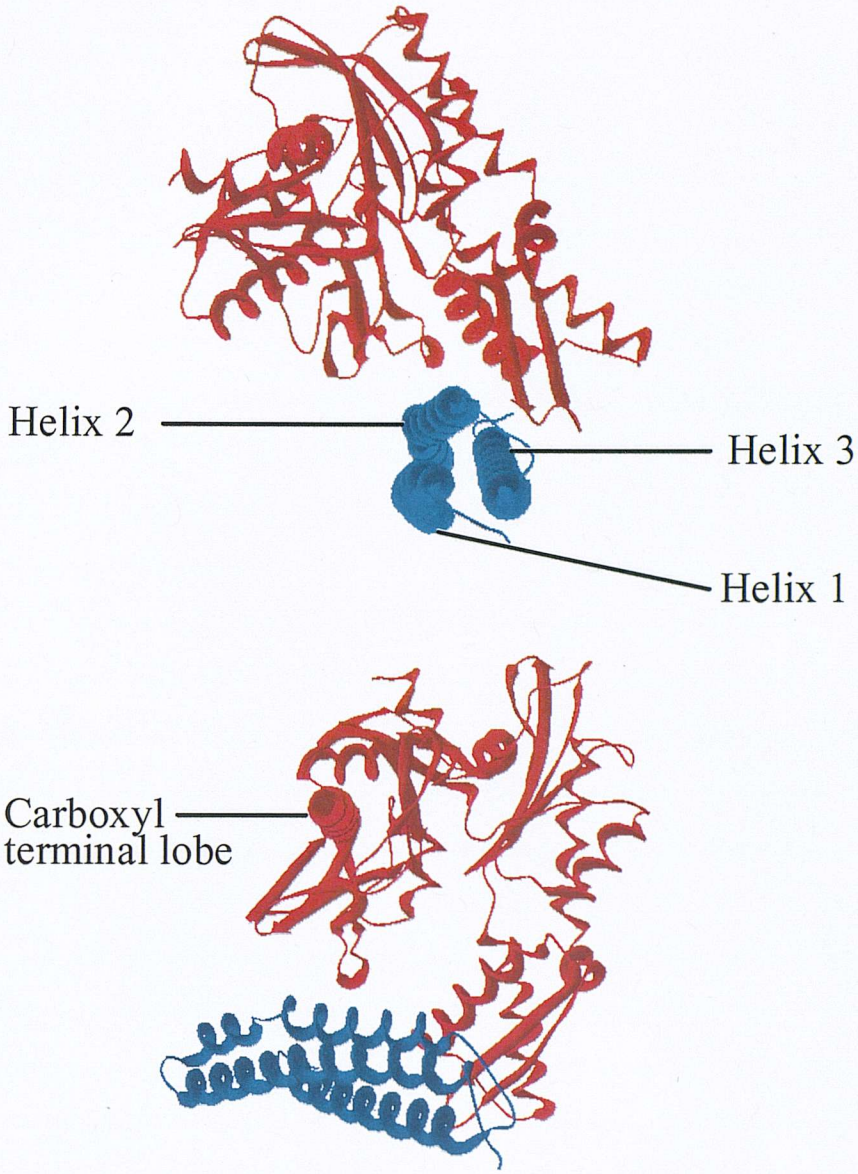


Figure 1.2: The BAG-1:HSC70 Interaction

Helices 2 and 3 of the BAG-1 BAG domain (blue) interact with the carboxyl-terminal lobe of the HSC70 ATPase domain (red). Figure drawn with "Swiss-pdb Viewer" using data downloaded from the "protein data bank" (www.rcsb.org/pdb) and described in Sondermann et al. 2002.

The BAG-1 carboxy-terminus also mediates interaction with the serine/threonine kinase Raf-1 through helices one and two of the BAG-1 domain. Raf-1 is a kinase normally activated by RAS to stimulate the mitogen-activated protein (MAP) kinase signalling cascade. This signalling pathway is important for proliferation and survival, and BAG-1 activates Raf-1 independent of RAS (Song et al. 2001). Thus, BAG-1 overexpression provides a potential mechanism by which tumours lacking oncogenic RAS mutations might activate MAP kinase pathway mediated proliferative and survival signals. Raf-1 and HSP70 interact at partially overlapping sites and therefore their binding to BAG-1 is competitive.

In addition to these direct binding partners, several other proteins have been reported to interact with BAG-1 (Figure 1.3). These include nuclear hormone receptors (NHR), the anti-apoptotic BCL-2 protein and some tyrosine kinase receptors such as the hepatocyte growth factor and platelet derived growth factor receptors (Townsend et al. 2003b; Cato & Mink 2001; Takayama & Reed 2001). Although definitive proof is often lacking, it is possible that much of this binding is indirect and mediated via the peptide binding activities of HSC70/HSP70 (Hohfeld 1998).

BAG-1 is the prototypical member of a family of BAG domain containing proteins, which are conserved throughout phylogeny and bind to and regulate chaperone molecules (Takayama et al. 1999). There are at least six BAG family proteins in the human and genes of the BAG family proteins are found in yeast (*Saccharromyces cerevisiae*), invertebrates (*Caenorhabditis elegans*), amphibians (*Xenopus laevis*), plants (*Oryza satia*) and mammals (humans and mice) (Takayama & Reed 2001). In addition to their BAG domains BAG family proteins generally contain other distinct domains that are often responsible for protein:protein interactions. BAG-6 (also known as Scythe or BAT3) for example, like BAG-1, contains a ULD but also interacts with the pro-apoptotic Reaper molecule (Thress et al. 2001). BAG-3 (also known as CAIR-1 or BIS) contains PXXP motifs which are responsible for it's interaction with phospholipase C-γ. BAG-4 (also known as SODD) interacts with the cytoplasmic death domain of certain death receptors

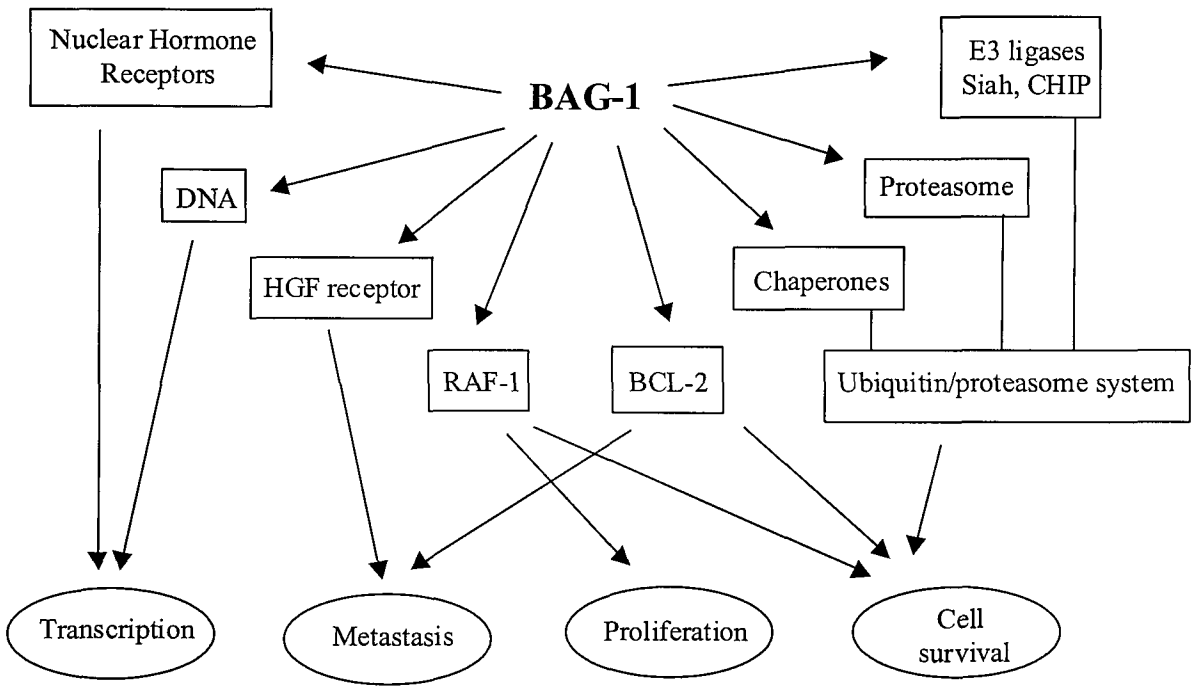


Figure 1.3: BAG-1 binding partners and functions

BAG-1 interaction partners are indicated. Some of these interactions are direct, whereas others are probably mediated via binding to chaperone molecules, e.g., NHR. Interactions of BAG-1 with chaperones, E3 ligases and the proteasome suggest a key role in regulating the ubiquitin/proteasomal degradation system. Biological activities ascribed to BAG-1 are indicated below along with some potential molecular targets that might contribute to these effects. However, it is important to note that definitive evidence linking specific BAG-1 target molecules to biological responses is often lacking.

(Jiang et al. 1999; Miki & Eddy 2002), and BAG-3 and BAG-4, like BAG-1 interact with BCL-2 (Antoku et al. 2001). The BAG-5 protein is unusual in that it contains four BAG domains. It has been suggested that these BAG family proteins act as "adapter" proteins linking chaperone molecules via their BAG domains to other molecular targets through distinct amino-terminal domains (Takayama & Reed 2001).

1.3.3 Ubiquitin, the BAG-1 ubiquitin-like domain and ubiquitin like proteins

All BAG-1 isoforms contain a ubiquitin-like domain (ULD), similar to ubiquitin and ubiquitin-like proteins that appears to be essential for at least some of BAG-1's biological effects (Hohfeld et al. 2001; Luders et al. 2000a; Takayama & Reed 2001). Ubiquitin is a 76 amino-acid residue protein present in all eukaryotic cells. When ubiquitin is covalently attached as a multiple chain to a lysine residue of target proteins it serves to target these proteins for ATP-dependent degradation via the proteasome, the major non-lysosomal proteolytic complex. Ubiquitin attachment occurs via the action of a series of enzymes including an E1 activating enzyme that activates ubiquitin through an ATP dependent process, an E2 ubiquitin carrier enzyme that accepts ubiquitin from the E1 and ubiquitin E3 ligases that transfer ubiquitin to specific substrate lysine residues. The substrate specificity increases down the chain so whilst there are few E1 enzymes there are many more highly substrate specific E3 enzymes. Ubiquitin also plays other, less well characterized roles and so whilst multiubiquitylation generally targets proteins for degradation, monoubiquitylation does not (Hershko & Ciechanover 1998; Pickart 2001). In addition there are different ways of building multiubiquitin side chains by using different lysine residues of ubiquitin and these have different functional consequences. As a result ubiquitin and ubiquitylation, similar to postranslational modification by phosphorylation, has been implicated in the regulation of a myriad of cellular processes. In addition to protein degradation these processes include cell cycle progression and cellular differentiation, apoptosis and stress responses, protein transport, antigen processing and DNA repair (Weissman 2001).

In addition to ubiquitin, eukaryotic cells express a whole host of proteins related to ubiquitin in sequence or function. These proteins can be grouped into two distinct classes; the ubiquitin like modifiers (UBL) and ubiquitin domain proteins (Jentsch & Pyrowolakis 2000). Ubiquitin like modifiers function in a manner analogous to ubiquitin and can be catalysed by specific enzmes to covalently attach to other proteins. The best known of these UBL's are perhaps the SUMO (for **S**mall **U**biquitin-related **MO**difier) proteins of which there are at least three distinct proteins. Conjugation of SUMO proteins to substrates (sumoylation) occurs through E1 and E2 enzymes and may play a role in protein targeting or association such as targeting RanGAP1, a GTPase activating protein, to associate with RanBP2 at the nuclear pore (Jentsch & Pyrowolakis 2000). Ubiquitin domain proteins, of which BAG-1 is a member, contain a protein domain with sequence homology to ubiquitin, but otherwise are structurally and functionally heterogenous to each other. At least two of these ubiquitin like proteins, RAD23 and BAG-1 associate with the proteasome (Jentsch & Pyrowolakis 2000; Luders et al. 2000a), and RAD23 appears to be required for nucleotide excision repair. Other ULD-containing proteins such as BAT3 and UBP6 are also important for regulating ubiquitylation/proteolysis or associate with chaperones.

1.3.4 The BAG-1 repeat sequence

BAG-1 proteins contain different numbers of copies of six amino-acid repeats rich in acidic residues (TR/QSEEX consensus sequence). Human BAG-1L and BAG-1M contain nine copies whereas BAG-1S contains three copies (Packham et al. 1997). Fewer copies are present in the mouse homologues. This region is predicted to form an alpha-helix with charged residues lining up along the axis of the helix (Hohfeld 1998). The function of the repeats remains unclear since this part of the molecule is not essential for suppression of apoptosis, but is required for regulating glucocorticoid receptor (GR)-dependent transcription (Schneikert et al. 1999). In addition these repeat elements create a consensus recognition site for phosphorylation by creatine kinase-2 (Takayama et al. 1998) and BAG-1M is phosphorylated at its amino terminus in vivo (Schneikert et al. 2000). The significance of BAG-1

phosphorylation is not known but it has been suggested that a larger molecular weight form of BAG-1S, detected in CLL cells, normal human tonsil and peripheral blood mononuclear cells may represent phosphorylated BAG-1 (Hayashi et al. 2000; Kitada et al. 1998; Takayama et al. 1998).

1.4 BAG-1 expression and cellular localisation

1.4.1 Control of BAG-1 cellular expression

The 5' upstream flanking region of the BAG-1 gene has been cloned from human genomic DNA. Subsequent sequence analysis of the BAG-1 promoter region identified sequence motifs that are involved in transcriptional control including a CCAAT box and several GC boxes but no TATA box. A 272 base pair CpG island was also identified 200 base pairs upstream of the start site. CpG islands are short stretches of DNA where the frequency of the CG sequence is higher than other regions. The "p" simply indicates that a normal phosphodiester bond links C and G. CpG islands are normally located around the promoters of important genes or other genes frequently expressed in cells, and here the CG sequences are not methylated. CpG islands on the promoters of inactive genes are methylated at the 5' position of cytosine to form 5-methylcytosine and this suppresses gene expression. Promoter methylation and demethylation often plays a role in the derepression of oncogenes and supression of tumour suppresser gene expression, and the presence of a CpG island in the promoter of BAG-1 suggests that such processes may play a role in BAG-1 expression in normal and tumour tissue. Despite these possibilities the role of the CpG island and methylation in the regulation of BAG-1 expression has not been specifically addressed.

The BAG-1 promoter also contains some transcription factor binding sites (GATA-1, Ets and WT1) also found in the promoter regions of BCL-2 and BCL-X (Yang et al. 1999b). It has been suggested that this may lead to the co-expression of these apoptosis regulators (Yang et al. 1999b). Deletion analysis of reporter constructs suggested that repressive elements were present upstream of the CCAAT box, and the highest activity was obtained from regions just downstream of the

CCAAT box. The role of specific transcription factors on BAG-1 promoter activity has not been addressed however.

The BAG-1 promoter is moderately active in various transfected tumour cell lines and is also transactivated by some "gain-of-function" p53 mutants, although the BAG-1 promoter lacks any putative p53 binding sites (Yang et al. 1999b). A wide range of survival signals induces the expression of BAG-1 including interleukin (IL)-3 which is necessary for proliferation and survival of murine pro-B cells. In these cells IL-3 withdrawal results in reduced BAG-1 expression and cell death and this can be delayed by BAG-1S overexpression (Clevenger et al. 1997; Jeay et al. 2000; Sekiya et al. 1997).

Induction of BAG-1 by survival factors often involves increases in BAG-1 RNA expression and is consistent with a role for BAG-1 in the suppression of apoptosis. In addition BAG-1 RNA and protein levels appear to correlate directly in some cell lines suggesting that transcriptional control is an important mechanism in the regulation of BAG-1 expression in some instances (Yang et al. 1998; Yang et al. 1999a). There is however increasing evidence that post-transcriptional control also plays an important role in the control of BAG-1 expression (Coldwell et al. 2001; Townsend et al. 2002). For example no association was found between BAG-1 RNA and protein expression in a group of primary breast cancers (Townsend et al. 2002). Additionally, although ubiquitylation does not generally appear to lead to BAG-1 degradation, ubiquitin mediated degradation of BAG-1 does appear to play a role in the wave of olfactory neuronal apoptosis that follows surgical ablation of the mouse olfactory bulb (Sourisseau et al. 2001).

1.4.2 Control of BAG-1 subcellular localisation

The regulation of subcellular localisation is an important mechanism that controls the function and activity of many proteins, and BAG-1 isoforms are differentially localised in cells. Subcellular fractionation studies of human breast cancer MCF7 cells demonstrated that BAG-1L is predominantly nuclear, whereas BAG-1S is predominantly a cytoplasmic protein and BAG-1M partitions between the two compartments (Brimmell et al. 1999). Similar distribution of BAG-1 proteins

have been detected in 267 prostate and HeLa cells (Takayama et al. 1998). The amino-terminus of BAG-1L, which as described contains an NLS, is sufficient to confer nuclear localization to heterologous proteins (Brimmell et al. 1999; Hague et al. 2002; Knee et al. 2001). Additionally BAG-1L localises to nucleoli within the nucleus of oral carcinoma cells, and the amino terminus of BAG-1L is also sufficient for this (Hague et al. 2002). Loss of residues 1-16 of BAG-1L by deletion, does not significantly alter BAG-1L nuclear localisation whilst loss of residues 1-50 leads to reduced retention in the nucleus and diffuse cytoplasmic staining. It has therefore been suggested that sequences between residues 17 to 50 are required in addition to the BAG-1 SV-40 like NLS (residues 70 to 76), for optimal nuclear localisation (Knee et al. 2001).

The localisation of BAG-1 isoforms is regulated under certain conditions. This might represent a mechanism controlling the activity of BAG-1 proteins. For example, a BAG-1M-GFP fusion protein relocalises from the cytoplasm to the nucleus following heat shock and this is thought to be important for its effects on enhancing transcription in this system (Zeiner et al. 1999). BAG-1M moves into the nucleus with liganded glucocorticoid receptor and this may play a role in downregulating the receptor (Schneikert et al. 1999). BAG-1 localisation also changes during differentiation and BAG-1 has been reported to move from the nucleus to the cytoplasm during epidermal and neuronal differentiation, and breast epithelial involution (Schorr et al. 1999; Takayama et al. 1998; Kermer et al. 2002). It is therefore important to recognise that nuclear BAG-1 immunostaining in cancer cells may not be a reliable measure of BAG-1L expression. Thus, nuclear BAG-1 expression may indicate either high levels of BAG-1L or relocalisation of BAG-1S or BAG-1M to the nucleus in response to specific signals in the tumour microenvironment.

1.4.3 BAG-1 expression pattern in normal tissue and development

An extensive study of BAG-1 expression in normal adult tissue demonstrated widespread immunoreactivity localised to the nucleus, cytoplasm or both depending on tissue or cell type (Takayama et al. 1998). Combinations of diffuse cytoplasmic

and nuclear labelling for BAG-1 occur in chondrocytes, cardiac myocytes, colonic enterocytes, bladder urotherlium, spermatagonia and breast epithelial cells. Predominantly nuclear labelling is found in cells of the gastric glands, corpus luteum, bone magakaryocytes, cortical and spinal cord neurons and adrenal chromaffin cells, whilst predominantly coarse cytoplasmic with or without nuclear labelling is observed in bronchial epithelial cells, alveolar macrophages, exocrine pancreas, renal collecting duct epithelium and bladder epithelium. Such widespread, but tissue specific patterns of distribution indicate that whilst BAG-1 is likely to be involved in widespread generic cellular processes, these processes and BAG-1 functions may be different in different tissue types. Patterns of BAG-1 expression were also examined by Western blotting in a panel of 67 tumour cell lines. BAG-1 was found at relatively high levels in several cell lines and in particular in leukaemia, breast, prostate and colon cancer cell lines. Levels of the BAG-1L protein were particularly high in leukaemia, breast and prostate cell lines, raising the possibility that it may be involved in glucocorticoid, oestrogen and androgen receptor signalling in these cell lines. Others have observed Takayama et al.'s findings in breast cancer cell lines by Western blotting of breast cancer tissue (Yang et al. 1999a). The absence of controls for tumour cell lines and the presence of contaminating stromal and other cells in tumour samples compromise studies involving Western blotting however since the proportion of malignant tumour cells in breast cancer samples can be relatively low (10 – 20%). Expression analysis in tumours by immunohistochemistry is discussed in detail in section 1.10.

BAG-1 expression may be involved in developmental regulation and BAG-1 is downregulated upon initiation of interdigital apoptosis in development of mouse limbs. In retinoic acid receptor null mice BAG-1 expression remains unaltered, interdigital apoptosis does not occur and severe interdigital webbing results (Crocoll et al. 2002a). It is likely therefore that BAG-1 downregulation is required for interdigital apoptosis, and possible therefore that disruption of these processes could be one possible cause of syndactyly, which occurs in Fraser syndrome, associated with alterations within chromosome 9 band 12 to which the BAG-1 gene maps. There are suggestions that BAG-1 and BCL-2 homologues may be involved in the

development of human intestinal villi. BAG-1 expression decreases by thirty percent between ten to twenty weeks and villus tip apoptosis emerges in the foetus at the twenty week stage (Vachon et al. 2000; Vachon et al. 2001). There is also evidence that BAG-1 may regulate sensitivity of the developing kidney to glucocorticoids as its expression correlates closely with that of the glucocorticoid but not the mineralocorticoid receptor in the developing kidney (Crocoll et al. 2000a; Crocoll et al. 2000b).

1.5 BAG-1 and HSP70 and HSC70 function

1.5.1 The 70 kDa heat shock protein family

Heat shock proteins are molecular chaperones and one of their key functions is to interact with other proteins and minimise inappropriate protein:protein interactions or aggregation. These ubiquitous proteins recognise, bind to, and renature proteins or peptides in non-native conformations. This process is generally energy dependent and so is usually regulated by, or occurs in association with nucleotide hydrolysis. Newly synthesised and assembled proteins, and those recently re-localised to different cellular compartments commonly require folding by chaperones. Proteins or peptides may additionally be in non-native conformations following cellular stress including heat shock, and thus also require chaperone mediated refolding. Chaperone function is therefore required for maintaining proteins in correct conformations for organellar localisation or import, for minimising aggregation of non-native proteins and for targeting non-native or aggregated proteins for degradation (Feder & Hofmann 1999). Although heat shock proteins were initially recognised as gene products whose expression was induced by heat and other stresses not all heat shock proteins are stress inducible.

There exists a plethora of different heat shock proteins commonly assigned to families according to molecular weight and sequence homology. For example families include those with molecular weights of 110, 100, 90, 70, 60, 40 and 10 kDa. Each family comprises multiple members that differ in inducibility, intracellular localisation and function. The 70 kDa heat shock proteins are one of the best

characterised of these families of molecular chaperones (Mayer et al. 2001; Schlesinger 1990). Even this family alone consists of at least eleven distinct genes producing highly related proteins, but the best known and most highly expressed of these genes are HSP70 (gene locus 6p21.3) and HSC70 (gene locus 11q24) (Tavaria et al. 1996). HSC70 is abundantly expressed under normal conditions and is not significantly heat inducible whilst HSP70 is highly inducible. BAG-1 interacts with both these proteins but not all proteins of the 70 kDa family. BAG-1 does not for example interact with BiP/GRP78 localised in the endoplasmic reticulum and with mHsp70 localised in mitochondria (Zeiner et al. 1997).

HSC70 and HSP70 comprises an amino terminal ATPase domain, a central peptide binding domain, and a carboxy terminal region that can form a "lid" over the peptide binding domain (Figure 1.4). The peptide binding domain forms a pocket that interacts with short hydrophobic segments in substrate polypeptides. Substrate refolding is energy dependent and requires the hydrolysis of ATP, which drives the conformational changes. In the ATP bound state, the substrate binding pocket is open and peptide binding affinity is low and exchange rates fast whereas in the ADP-bound state the "lid" is effectively "closed" over the peptide binding pocket and substrates bind with high affinity. Cochaperone molecules regulate ATP hydrolysis and nucleotide exchange, and in eukaryotes, ATPase activity is stimulated by Hsp40 and nucleotide exchange activity is inhibited by Hip (Bukau & Horwich 1998).

1.5.2 BAG-1 structure and the structural basis of the BAG-1 HSC70/HSP70 interaction

An understanding of the structure and physical characteristics of a protein, or of the structural basis and physical properties of the interaction between two proteins may enable prediction of key interaction points and may help with the rational design of drugs or inhibitors of protein function. Structural studies are therefore an important approach, not only for their contribution to the biological understanding of proteins, but also as an informed starting point for the design of potential protein inhibitors or drugs. The physical characteristics of the interaction between BAG-1 and HSC70 have now been clearly defined. In intact cells such as MCF-7 cells the levels of

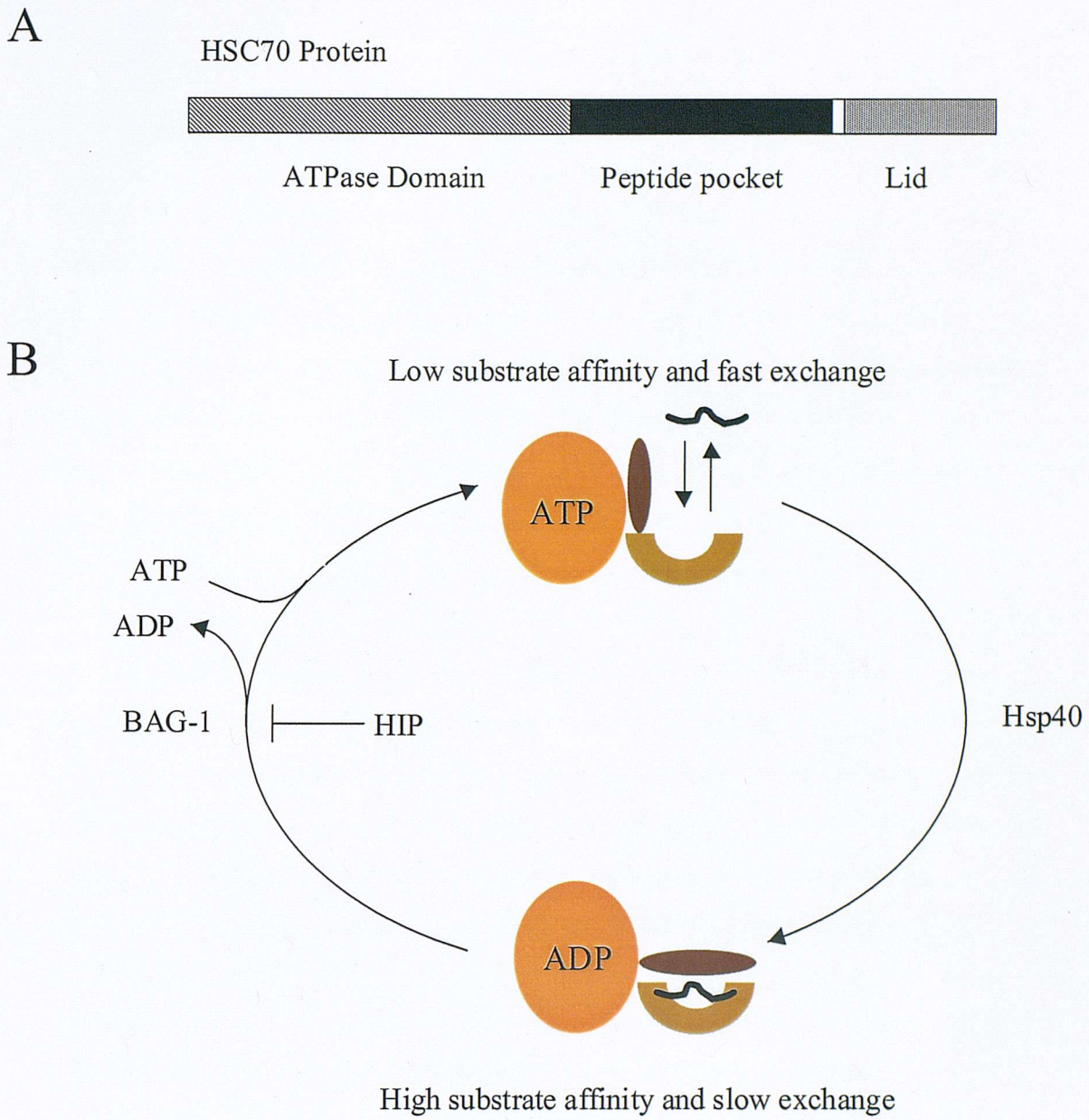


Figure 1.4: HSC70 structure and the HSC70/HSP70 chaperone cycle
The structure of the HSC70 protein is shown schematically (A), and the chaperone refolding cycle diagramatically (B). In the ATP bound form of the chaperone the peptide binding pocket is open and binding of misfolded substrate occurs. ATP hydrolysis catalysed by the cochaperone Hsp40 causes the lid to close over the pocket. Subsequent nucleotide exchange allows refolding and then release of substrate. Nucleotide exchange is stimulated by the co-chaperone BAG-1 and this is opposed by the co-chaperone Hip

HSC70/HSP70 range from 5 - 50 μM whilst the levels of BAG-1 isoforms range from 0.1 - 2 μM (Nollen et al. 2001). Sedimentation equilibrium analysis involves the separation of proteins by mass down a density gradient using centrifugation, and gel filtration chromatography involves the chromatographic separation of proteins by mass down a column of porous (agarose, polyacrylamide or dextran) beads. These techniques have established that BAG-1 exists as a monomer in solution (Stuart et al. 1998). Circular dichroism is a means of determining protein structure by measuring the optical properties of a protein. This technique has shown that BAG-1 is highly helical containing 67% α-helix and 33% random coil with no β-sheet (Stuart et al. 1998). It has been established that BAG-1 and HSC70 interact with a 1:1 stoichiometry. The dissociation constant (Kd, the concentration of BAG-1 at which half of the HSC70 in solution is bound to BAG-1) of the BAG-1:HSC70 interaction has been determined by both isothermal titration calorimetry and surface plasmon resonance. Isothermal titration calorimetry involves the precise measurements of energy absorbed or generated when two molecules interact and surface plasmon resonance involves measurements in changes in refractive index when two molecules interact. From these measurements various properties of the interaction, including the Kd can be determined. By isothermal titration calorimetry the Kd of the BAG-1:HSC70 interaction is 100 nM (Stuart et al. 1998) or 1-3 μM (Sondermann et al. 2001). By surface plasmon resonance the Kd is 500 nM (Stuart et al. 1998). Other BAG family proteins also bind to HSP70 with a similar affinity as BAG-1 (Takayama et al. 1999). The measurements are broadly similar given the different experimental approaches and techniques and demonstrate that BAG-1 and HSC70 bind directly and reversibly with a physiologically competitive affinity.

X-ray crystallography has been performed on a co-crystal of the human BAG-1 BAG domain in complex with the ATPase domain of bovine HSC70 (Sondermann et al. 2001), and the mouse BAG-1 BAG domain has been studied by nuclear magnetic resonance (Briknarova et al. 2001). Helices 2 and 3 of the BAG domain are involved in binding to the HSC70 ATPase domain (Figure 1.2). The carboxyl-terminal lobe of the ATPase domain of HSC70 has been identified as the minimal region of HSC70 sufficient for binding to BAG-1 (Brive et al. 2001). Essential

charged residues in the BAG domain and the HSC70 ATPase domain have been identified and mutation of these residues has been confirmed to abrogate binding. These important residues are also well conserved across species and different BAG family proteins further supporting the model (Briknarova et al. 2001; Sondermann et al. 2002). Helix 1 of the BAG domain is not involved in binding to HSC70 but it has been suggested that it may play a role in maintaining the overall structure of this domain (Briknarova et al. 2001), and it is involved in binding to Raf-1 (Song et al. 2001). The BAG domain of BAG-4 like BAG-1 is made up of three helices, although each helix is three to four turns shorter than its counterpart in BAG-1 thus reducing the domain length by one third. The BAG domain of BAG-4 therefore possibly represents the minimal functional domain required to bind to and modulate HSC70 function.

Since BAG-1 interacts with the ATPase domain of HSC70 and the HSC70 peptide-binding domain remains available for binding substrate proteins. Trimeric complexes have been detected, for example between BAG-1M, HSC70 and c-Jun and HSC70/HSP70 may be an essential intermediate in the binding of BAG-1 to BCL-2 (Takayama et al. 1997; Zeiner et al. 1997). Also far-western blot analysis to determine BAG-1 direct interaction partners do not generally show multiple bands (Zeiner et al. 1997). It is therefore possible that some of the many reported interactions of proteins with BAG-1 may in fact be indirect and mediated through HSC70 or HSP70. Indeed it has been shown that BAG-1M interacts with HSP70 from other species so it is possible that interaction cloning experiments could demonstrate indirect interactions through yeast or insect HSC70 (Zeiner et al. 1997). This does not necessarily mean that these interactions are irrelevant however, and many of the diverse functions of BAG-1 may be dependent on the ability of HSC70/HSP70 to regulate the function of other proteins.

1.5.3 BAG-1 mediated regulation of HSC70/HSP70 function

Since BAG-1 interacts with the ATPase domain of HSC70/HSP70 the effects of BAG-1 on chaperone nucleotide exchange have been extensively investigated (Figure 1.4). BAG-1M alone does not increase the ATPase activity of HSC70 in a

purified in-vitro system (Hohfeld & Jentsch 1997). If however the co-chaperone Hsp40 is added then ATP hydrolysis increases forty fold indicating that BAG-1 accelerates a step in the cycling reaction. Under steady state conditions HSC70 is largely bound to ATP, and this is unaffected by BAG-1. Addition of Hsp40 produces an increase in the ADP bound form of HSC70 demonstrating that Hsp40 stimulates ATP hydrolysis to ADP, but that nucleotide release does not occur. Addition of BAG-1 returns HSC70 to the ATP bound form and thus demonstrates that BAG-1 stimulates ADP release and HSC70 recycling (Hohfeld & Jentsch 1997). Additional cofactors add a further level of complexity to the control of HSC70 ATPase activity. Hip/p48 for example, binds competitively to the ATPase domain of HSC70, and acts to counter BAG-1 function and inhibit nucleotide exchange (Gebauer et al. 1997; Hohfeld & Jentsch 1997; Luders et al. 1998). A further co-chaperone, the HSP70/Hsp90 organising protein (Hop) also binds to the carboxy-terminus of HSC70 and recruits Hsp90 to HSC70 but does not affect ATP hydrolysis (Demand et al. 1998). There therefore exists a rich network of co-chaperones that regulates and controls HSC70 ATPase function.

In vitro assays have been extensively used to assess HSC70 refolding function. The refolding of a thermally or chemically denatured reporter protein, such as luciferase or β-galactosidase can be measured since unfolded reporter lacks enzymatic activity, and on refolding the reporter will regain the ability to utilise substrate. In general addition of BAG-1S (Takayama et al. 1997) or BAG-1M to such in-vitro systems inhibits HSC70 mediated substrate refolding (Gebauer et al. 1997; Zeiner et al. 1997). This is not always found however. When BAG-1S and BAG-1M were compared in a single assay both isoforms stimulated ATPase activity and substrate release but BAG-1M inhibited HSC70 mediated refolding whilst BAG-1S stimulated refolding (Luders et al. 2000b). These results illustrate the difficulties of in-vitro assays since it was subsequently demonstrated that BAG-1M can either stimulate or inhibit reporter refolding dependent on concentration and the concentration of nucleotide used (Gassler et al. 2001). In contrast, in intact cells both BAG-1S and BAG-1M overexpression inhibited firefly luciferase refolding (Nollen et al. 2000). BAG-1L had no effect in these assays but it is possible that this is because

BAG-1L is expressed in the cell nucleus whilst luciferase is expressed in the cytoplasm.

It is not clear why stimulation of nucleotide exchange by BAG-1 results in decreased refolding. It has been suggested that accelerated cycling of HSC70 by BAG-1 stimulates the release of substrates from HSC70, and that this can leads to release of substrate prior to complete folding (Hohfeld 1998; Luders et al. 1998). The effects of BAG-1 on HSC70 ATPase activity and substrate refolding are now reasonably well understood but the possibility that BAG-1 may also regulate many other of the myriad of functions of these chaperones has not been explored. Indeed this may provide a basis for the multiple functions ascribed to BAG-1 (Figure 1.3). In particular it should be noted that the BAG domain of BAG-1 is sufficient for the regulation of refolding by the chaperones, but many other BAG-1 functions require additional domains or parts of BAG-1.

1.6 BAG-1, ubiquitin and the proteasome

ULD-containing proteins are often involved in regulating the ubiquitin system which performs various biological functions, including targeting proteins for degradation via the proteasome (Hershko & Ciechanover 1998; Pickart 2001). Since BAG-1 isoforms contain a ULD it therefore seems likely that some functions of BAG-1 are related to the ubiquitin/proteasome system. Interestingly, in addition to their role in protein refolding, HSC70/HSP70 also play a direct role in the ubiquitylation of some proteins (Bercovich et al. 1997), and it is possible that these functions are linked. The precise function of the ULD in BAG-1 is unknown, and although BAG-1 itself can be ubiquitylated (Alberti et al. 2002; Sourisseau et al. 2001), BAG-1 isoforms are stable proteins with a half life from pulse chase experiments of around 8 hours (Luders et al. 2000a). This suggests that they are generally not themselves targets for rapid degradation by the ubiquitin/proteasome system, although this can occur in certain circumstances (Sourisseau et al. 2001). Interestingly, the BAG-1 ULD contains a conserved lysine residue (K80 in human BAG-1S); the equivalent residue in ubiquitin is critical for covalent attachment of

ubiquitin moieties to form multiubiquitin chains, but the possibility of a potential role of this residue in BAG-1 has not been addressed.

Both BAG-1S and BAG-1M interact with the C-8 and S-1 subunits of the proteasome (Hohfeld et al. 2001; Luders et al. 2000a). This interaction appears to involve the ULD since it was not apparent with a BAG-1 carboxy-terminus fragment that retained the ability to bind HSC70/HSP70 (Luders et al. 2000a). The exact residues of BAG-1 required for this interaction has not been investigated, and in particular it is not known if conserved residues such as the conserved K80 residue is required. The interaction between BAG-1 and the proteasome is ATP dependent and appears to link heat shock proteins to the proteasome, since overexpression of BAG-1S in HeLa cells increases greatly the amount of proteasome C-8 subunit immunoprecipitated with HSP70/HSC70. BAG-1 therefore appears to act as a bridge linking heat shock proteins, via its carboxy-terminal BAG domain, to the proteasome via its amino-terminal ULD (Luders et al. 2000a). Therefore BAG-1 like RAD23 contains a ULD at its amino-terminus, and like RAD23 this mediates an interaction with the proteasome. Unlike RAD23 however BAG-1 interacts with subunits of the main 20S component whilst RAD23 interacts with the S5a subunit of 19S proteasome cap suggesting that these two ULD family proteins have evolved to perform divergent functions.

BAG-1 also binds two molecules with E3 activity, Siah and CHIP (Demand et al. 2001; Matsuzawa et al. 1998). CHIP is a co-chaperone that possesses E3 activity due to a RING finger domain, characteristic of E3 ligases, at its carboxy-terminus (Lorick et al. 1999). CHIP also interacts with 70 and 90 kDa heat shock proteins via three tandem thirty-four amino acid repeats (tetraricopeptide repeats, TPR's) at its amino-terminus (Hohfeld et al. 2001). CHIP appears to target proteins that are irreversibly damaged (and hence that the chaperones are unable to renature) for degradation by the proteasome through substrate ubiquitylation (Connell et al. 2001; Hohfeld et al. 2001). BAG-1 appears to co-operate with CHIP, and target proteins (such as the GR) for degradation by linking the CHIP-substrate-HSC70 complexes to the proteasome (Demand et al. 2001). Whilst this is a compelling model the exact details of the interplay between BAG-1 and CHIP are still unclear. BAG-1 for

example might interact directly with CHIP, rather than through HSP70/HSC70 since the interaction can also be detected under conditions that dissociates BAG-1 from the chaperones. The regions of BAG-1 and CHIP that might mediate this interaction have not been mapped however. BAG-1 itself also appears to be ubiquitylated in a CHIP-dependent manner and this appears to stimulate association of BAG-1 with the proteasome (Alberti et al. 2002). It is not known what residue(s) of BAG-1 are ubiquitylated, and the ubiquitin chain that is formed is atypical in that the individual ubiquitin moieties are linked through lysine eleven of ubiquitin (Alberti et al. 2002). Taken together, these findings suggest that at least some BAG-1 functions are dependent on its ability to coordinate the activity of the chaperone and proteasome systems, and to facilitate or control protein refolding/turnover.

Siah-1, another E3 ligase, was identified as a BAG-1 interaction partner in a yeast two hybrid assay (Matsuzawa et al. 1998). Siah-1, like CHIP, also contains a RING finger domain characteristic of E3 ligases, and Siah-1 is induced by p53 resulting in growth arrest of cells. BAG-1 interacts with Siah-1 proteins and prevents Siah-1 and p53 induced growth arrest (Matsuzawa et al. 1998). The significance of the ubiquitin ligase activity of Siah-1 was not addressed in this study, and interestingly the interaction with Siah-1 required the carboxy-terminus (but not the ULD) of BAG-1.

1.7 BAG-1 and transcription

1.7.1 Nuclear hormone receptors

Hormones are chemical messengers that act at a distant site of action and control many of the basic processes of life, including metabolism, reproduction, fluid balance and growth. Hormones act on either cell surface receptors or on nuclear hormone receptors. Nuclear hormone receptors (NHR) are classically ligand dependent transcription factors and are key regulators of gene transcription and protein synthesis. They therefore control many basic cellular processes including proliferation, differentiation, and apoptosis. It is therefore unsurprising that a wide range of diseases are due to aberrant function and regulation of hormone receptors,

and of interest that many of these hormone receptors are subject to regulation by BAG-1 (Table 1.1) (Cato & Mink 2001). A number of common epithelial cancers, such as breast and prostate, are at least initially dependent on steroid hormones and hormone receptors for cell survival and proliferation and translocations involving the retinoic acid receptor (RAR) are commonly found in promyelocytic leukemia. These NHR are therefore important therapeutic targets, and the rational design of anti-hormone agents targeting these NHR in hormone dependent cancers is dependent on understanding their mechanisms of action and function.

1.7.2 Mechanism of oestrogen receptor function

One of the best understood NHR is the oestrogen receptor (ERα). The gene for ERα was first cloned and sequenced from MCF7 cells and is located on chromosome 6. It encodes a protein 595 amino acids in length with a molecular weight of 66 kDa. The ERα protein contains six functional domains conventionally labelled A to F (Figure 1.5). In addition to binding 17 β-estrodiol (the major secreted oestrogen) through region AF2, and DNA through the DNA binding domain (DBD), the receptor also contains regions that participate in receptor dimerisation (within regions C and E), and that interact with heat shock proteins (also within regions C and E) (MacGregor & Jordan 1998).

Although many NHR such as the GR shuttle between the nucleus and cytoplasm ERα is found within the cell nucleus (Greene et al. 1984). NHR within the cell nucleus are generally found in association with heat shock proteins, which are generally thought to maintain hormone receptors in a conformation suitable for ligand binding and may also play a role in preventing free receptor from binding DNA. During receptor maturation HSP70 and Hsp40 are thought to bind to the receptor and Hop acts as an adaptor protein to recruit a dimer of Hsp90 to the complex (Figure 1.5). Hsp40, HSP70 and Hop then dissociate and a co-chaperone of Hsp90, p23, and further proteins known as immunophilins join the complex (Cato & Mink 2001; Nollen & Morimoto 2002). This allows the receptor to bind to oestrogen, and release the complex of Hsp90, p23 and immunophilins (Nollen & Morimoto 2002). The activated receptor subsequently dimerises and can then bind to DNA oestrogen

Receptor	Effect	Isoform	Reference
Androgen Receptor	Activates	BAG-1L	Froesch et al. 1998
Vitamin D Receptor	Activates	BAG-1L	Guzey et al. 2000
	Inhibits	BAG-1L	Wichter et al. 2001
Glucocorticoid Receptor	Inhibits	BAG-1M, BAG-1L	Kanelakis et al. 1999; Kullmann et al. 1998
Mineralocorticoid receptor	No effect	BAG-1M	Schneikert et al. 1999
Retinoic acid receptor*	Inhibits	BAG-1S	Liu et al. 1998
Retinoid X Receptor*	No effect	BAG-1S	Liu et al. 1998
Thyroid hormone receptor	Inhibits	BAG-1S	Liu et al. 1998

Table 1.1: BAG-1 mediated modulation of nuclear hormone receptors

*BAG-1 inhibits retinoic acid receptor/retinoid X receptor heterocomplexes

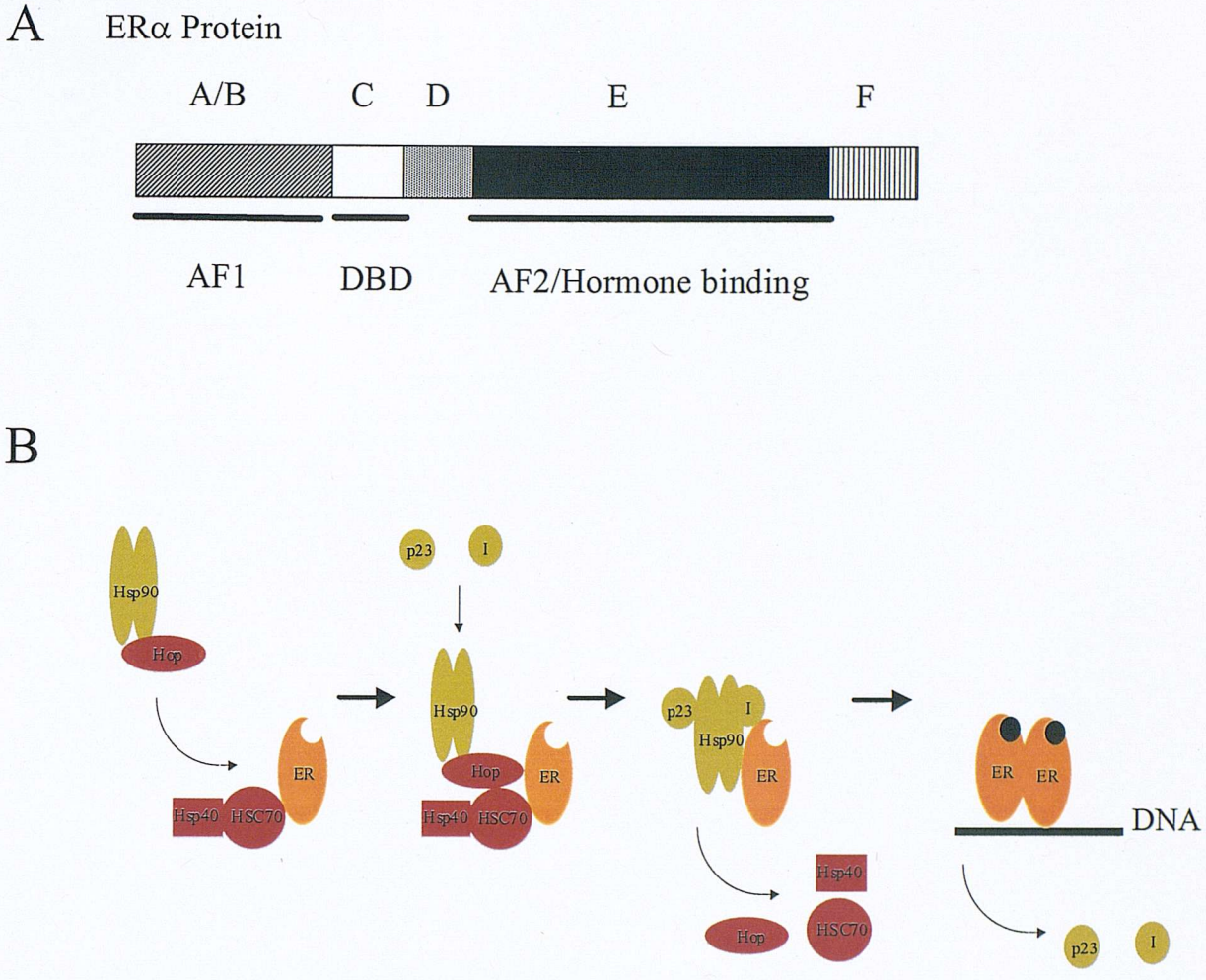


Figure 1.5: ERα domain structure and receptor maturation

The domain structure of the oestrogen receptor is shown schematically (A) and receptor maturation diagrammatically (B). Transcriptional activation of ERα is mediated through domains AF 1 (transactivating function 1) and AF2. AF1 is constitutively active and AF2 is oestrogen inducible and contains the hormone binding domain. The DNA binding domain is situated in region C, and region D contains sequences that direct nuclear localisation. Heat shock proteins (HSC70 and Hsp90), cochaperones (Hsp40 and Hop) and immunophilins are involved in ERα maturation.

response elements (ERE). Since BAG-1 regulates HSP70 this provides a mechanism whereby BAG-1 might modulate the function of hormone receptors. Although BAG-1 does modulate the function of a variety of receptors (Table 1.1) the exact details of how this is achieved by BAG-1 are controversial (Cheung & Smith 2000).

The classical ERE is a 13 base pair palindrome consisting of two five base pair sequences separated by a three base pair spacer. ERα interacts with this through the DBD, the sequence of which is highly conserved between species and NHR family members, and contains two zinc fingers, which mediate the ERα DNA interaction. ERα can alternatively interact with other less classical response elements including AP1 and other sites that bind members of the Jun/Fos family of transcription factors (Kushner et al. 2000). Once this has occurred a large number of proteins, "the preinitiation complex", are recruited and transcription of oestrogen regulated proteins such as the progesterone receptor, cathepsin D and pS2 begins. ERα activity is not necessarily exclusively ligand dependent however and the AF1 domain may initiate a degree of background transcription independent of ligand. This activity is subject phosphorylation at serine 118 through the MAP kinase pathway (Chen et al. 2002a; Kato et al. 1995), and is of interest since BAG-1 can activate the MAP kinase pathway through activation of Raf-1.

1.7.3 BAG-1 modulation of nuclear hormone receptor function

BAG-1M was initially identified as a glucocorticoid receptor (GR) binding protein (RAP46) (Zeiner & Gehring 1995). BAG-1M was also found to bind in vitro to ERα, and an ERα deletion mutant lacking the AF1 domain (but retaining the heat shock protein binding regions), and to the progesterone receptor (PgR), androgen receptor (AR) and thyroid hormone receptor (TR). In all cases binding to receptor was independent of the presence of ligand but required "activation" of the receptor by treatment in high salt concentrations. This strips the receptor of associated heat shock protein complexes mimicking the activated receptor state achieved prior to dimerisation and DNA binding. This is not however incompatible with the BAG-1M:receptor interaction occuring through heat shock proteins intermediates however since although large complexes were stripped off the receptors heat shock proteins

were still present in solution. In addition in most cases, binding to receptor requires the BAG-1 carboxy terminus (containing the BAG domain) and is thus likely to be mediated via, or involve, HSC70/HSP70.

In the prostate gland the luminal secretory epithelial cells are dependent on testosterone for their function, growth and survival, and in the absence of androgens undergo apoptosis. The stimulatory effects of androgens appear to drive many prostatic cancers and androgen ablation, for example with cyproterone acetate (an antiandrogen), is the mainstay of non-surgical treatment for prostate cancer. In addition high levels of BAG-1L are frequently detected in prostate cancer cells (Takayama et al. 1998). Coimmunoprecipitation experiments have demonstrated an interaction between endogenous BAG-1L (and not BAG-1M or BAG-1S) and the AR in the prostate derived cell line LN-CaP (Froesch et al. 1998). This was dependent on the presence of ligand, although BAG-1L did not alter the affinity of the receptor for ligand (Froesch et al. 1998; Knee et al. 2001).

Reporter assays in several different cell lines with BAG-1L overexpression have shown increases in AR transcriptional activity up to four times those obtained with control transfections (Froesch et al. 1998). BAG-1L overexpression correspondingly reduced the effectiveness of cyproterone acetate to inhibit AR transcriptional activity. A carboxy-terminal deletion mutant of BAG-1L failed to potentiate androgen dependent transcription and even acted as a trans-dominant inhibitor suggesting that the association between BAG-1L and heat shock proteins is important for this BAG-1 function. Indeed in-vitro the carboxy-terminus containing the BAG domain is sufficient for binding to the AR, and specific BAG-1L point mutants that do not bind HSC70/HSP70 are not functional in the transcription assays (Briknarova et al. 2001; Knee et al. 2001). Deletion of the BAG-1 ULD had no effect on transcriptional activity however (Knee et al. 2001). BAG-1L does not act by increasing the translocation of the AR from the cytoplasm to the nucleus (Froesch et al. 1998), but both the location of BAG-1 itself within the cell and unique amino-terminal sequences present within BAG-1M and BAG-1L are required for BAG-1 function since redirection to the nucleus via fusion to a heterologous NLS enabled only BAG-1M, but not BAG-1S, to regulate AR function.

The vitamin D receptor (VDR) appears to be regulated by BAG-1L in a similar way to the AR. The VDR is another member of the NHR family and is involved in the regulation of calcium homeostasis and absorption in the intestine, and bone formation. Vitamin D has also been implicated in stimulating differentiation of skin keratinocytes and immune cells and its differentiation and growth inhibitory effects have shown some promise as anti-tumour and psoriasis treatments. Only BAG-1L binds to the VDR and this was dependent on the presence of ligand (Guzey et al. 2000; Witcher et al. 2001). In reporter assays with U87 glioblastoma cells BAG-1L inhibited transcriptional activity by 50-60% in the presence of vitamin D, and this was associated BAG-1L mediated inhibition of DNA binding by the receptor in electrophoretic mobility shift assays (EMSA) (Witcher et al. 2001). In another study in several other cell lines including prostate and kidney cell lines BAG-1L produced up to two fold increases in transcriptional activity (Guzey et al. 2000). The reasons for the differences in the two studies are unclear although they may be cell type specific. BAG1-L also enhanced hormone dependent activation of the promoter for the $p21^{waf-1}$ cyclin dependent kinase inhibitor in kidney cell lines and expression of the endogenous p21 protein in PC-3 stably transfected prostate cell lines (Guzey et al. 2000). This finding is important since it demonstrated effects of BAG-1 on an endogenous gene known to be a target of the VDR in addition to results from reporter assays. Similar to results with the AR a carboxy-terminal deletion mutant of BAG-1L acted as a transdominant inhibitor suggesting that as with the AR interaction with HSC70/HSP70 is important for this function of BAG-1.

Unlike the AR and the VDR both the BAG-1L and BAG-1M, but not BAG-1S isoforms inhibit glucocorticoid receptor (GR) but not (MR) mineralocorticoid receptor function (Kullmann et al. 1998). Both glucocorticoids and mineralocorticoids are secreted by the adrenal cortex; glucocorticods have widespread effects on the metabolism of carbohydrate and protein and mineralocorticods are essential for the maintenance of fluid balance and fluid volume. Surface plasmon resonance studies provide evidence that interaction of BAG-1M to the GR occurs only in the presence of HSC70/HSP70 (Schneikert et al. 2000), and point mutants that fail to bind chaperones fail to repress transcription (Schmidt et al.

2003). Deletion studies show that the interaction requires the hinge region (which separates the DBD from the hormone binding domain) of the GR (Schneikert et al. 2000). BAG-1M appears to be translocated with the receptor and chaperones into the nucleus and the hormone binding domain of the receptor is required for this (Schneikert et al. 1999). The affinity for hormone of GR is unaffected by BAG-1M at ratios of BAG-1M to HSP70 of 1:10 (Schneikert et al. 1999). However with higher levels of BAG-1M inhibition of the hormone binding properties of the receptor were observed (Connell et al. 2001; Kanelakis et al. 1999). It is likely that these differences in receptor hormone binding affinity are due to differences in receptor folding mediated through BAG-1 control of chaperone refolding function during receptor maturation (Cato & Mink 2001). Similar to results obtained with the VDR EMSA studies also demonstrate that BAG-1M appears to interfere with DNA binding (Schneikert et al. 1999), suggesting that BAG-1 functions through at least two distinct mechanisms on GR function (Cato & Mink 2001; Cheung & Smith 2000).

It is not clear whether the frequent specificity for NHR regulation of the larger BAG-1 isoforms stems from the nuclear localisation of these proteins alone or from a requirement for this and additional HSC/HSP70 independent functions encoded by the amino-termini of these isoforms. The acidic-rich repeat has been suggested to be important for conferring GR-regulating activity on BAG-1L and M (Schneikert et al. 1999). Recent work however has demonstrated that eight amino acids proximal to the repeats in BAG-1M, which include basic amino acids that form part of the BAG-1L NLS, are sufficient for non-specific DNA binding (Schmidt et al. 2003). In addition deletion of the repeats alone did not prevent DNA binding, and these basic amino acids were necessary for the inhibition of BAG-1 on GR transactivition, and the repeats were not (Schmidt et al. 2003). How this DNA binding may mediate BAG-1 transactivating activity is unclear, but it is possible that it plays a role in stabilising receptor:DNA complexes through chaperone intermediates and/or recruiting cooperating transcription factors (Zeiner et al. 1999). As discussed there may not be a single mechanism to account for BAG-1's modulation of NHR activity and BAG-1 can also inhibit GR maturation by promoting Hop release from the assembly process in in-vitro systems (Kanelakis et al. 1999; Kanelakis et al. 2000).

Unlike the AR, GR and VDR the effects of BAG-1 on the retinoic acid receptor (RAR) and related thyroid receptor (TR) are exceptional in that they are mediated by the smaller BAG-1S isoform. The RAR inhibits proliferation and induces differentiation and apoptosis. Retinoids are of interest clinically for the treatment of certain leukaemias and epithelial malignancies. 9-cis-retinoic acid is a ligand for both RAR and retinoid X receptor (RXR) whilst all-trans-retinoic acid is a ligand for only RAR. RAR and RXR primarily function as heterodimers, and TR can also interact with the RXR to form heterodimers. BAG-1S binds to the RAR and TR, but not the RXR, and interferes with DNA binding and transcriptional activation by TR:RXR and RAR:RXR complexes (Liu et al. 1998). The BAG-1S:RAR interaction is also unique because the BAG-1S carboxy-terminus was not required for interaction implying that this particular interaction is not mediated through heat shock proteins (Cato & Mink 2001). BAG-1 isoforms therefore modulate the function of several, but not all NHR and as a generalisation BAG-1 isoforms appear to inhibit growth inhibitory receptors and stimulate growth stimulatory receptors.

1.7.4 BAG-1 and receptor independent transcription

Both BAG-1L and BAG-1M interact with DNA in a non-sequence specific manner via basic residues in the amino-terminal ten amino acids of BAG-1M (MKKKTRRRST) (Zeiner et al. 1999). These form part of the amino terminal NLS of BAG-1L and are the same residues required for the binding of BAG-1M to the glucocorticoid response element. They form two clusters of positively charged residues and substitution of either cluster of three lysines or three arginines for three alanines prevents DNA binding. Consistent with DNA binding BAG-1L is able to constitutively activate transcription from a range of promoters (Niyaz et al. 2001). BAG-1M also possesses transcriptional enhancing activity but this requires activation by heat shock and is associated with heat shock induced relocalisation of BAG-1M to the nucleus (Zeiner et al. 1999). DNA binding is however unaffected in a BAG-1M mutant lacking the BAG-1 domain and this deletion even enhances transcription stimulating activity suggesting that chaperone binding is not required and may negatively regulate BAG-1 stimulated receptor independent transcription (Zeiner et

al. 1999). Although transcriptional activity was reported from a range of reporter genes it remains to be determined whether these effects of BAG-1 on transcription are truly global or whether there are specific target genes modulated by BAG-1.

1.8 BAG-1 and cell signalling

1.8.1 The MAP kinase pathway

Mitogen activated protein kinase (MAPK) cascades are signal transduction networks that provide signals controlling important cellular processes including cell proliferation, differentiation and survival. There are three principal cascades in mammals although there is considerable cross-talk and interplay between cascades. The extracellular signal regulated kinase (ERK) cascade signals cell growth and differentiation from cell surface receptors, and the c-Jun N terminal (JNK) kinase cascade and the p38 MAPK cascades signal in stress responses such as apoptosis and inflammation (Schaeffer & Weber 1999). Raf-1 is a serine/threonine kinase involved early in the ERK cascade and phosphorylates and activates MEK1/2 which leads to phosphorylation and activation of ERK1/2 resulting in subsequent phosphorylation and activation of other kinases and transcription factors thus producing growth and proliferation signalling. Raf-1 is classically known to be activated by RAS, a key signaling molecule which is activated by a many receptor linked transduction systems. BAG-1 however presents an alternate and independent mechanism for the activation of Raf-1 (Wang et al. 1996).

1.8.2 BAG-1 binds to and activates Raf-1

BAG-1 binds to the catalytic domain of Raf-1 and activates Raf-1 in vitro and when overexpressed in cells (Song et al. 2001; Wang et al. 1996). Activation of Raf-1 occurs by phosporylation of Raf-1, although how BAG-1 brings this about is unclear, but it is conceivable that BAG-1 stimulates autophosphorylation. Since Raf-1 binds directly to helices one and two of the BAG-1 domain at an overlapping but distinct binding site to HSC70 and HSP70, it is possible to separate binding of HSP70 to BAG-1, from Raf-1 binding to BAG-1 with point mutants (Song et al. 2001). In general however most functional studies have relied on deletions of the BAG domain

which would be expected to prevent both Raf-1 and chaperone binding. A consequence of the overlapping binding sites is that the binding is competitive and high levels of HSP70 displace Raf-1 from BAG-1 (Song et al. 2001). Overexpression of HSP70 prevents activation of Raf-1, whist overexpression of a dominant negative RAS construct in intact cells has no effect on the activation of Raf-1 by BAG-1, although it prevents activation of Raf-1 by RAS in EGF treated cells (Song et al. 2001). Consistent with these results overexpression of BAG-1 has been associated with the pro-survival activity and activation of MAP kinase pathways (Kermer et al. 2002). It has been suggested that during cellular stress increases in HSP70 displace Raf-1 from BAG-1. This then prevents activation of Raf-1 by BAG-1 and HSP70 thereby acts as a negative regulator of cell growth during conditions of stress (Song et al. 2001).

1.8.3 BAG-1 and other signalling pathways

An interaction cloning approach taken to identify binding partners of growth factor receptors has identified BAG-1 as a binding partner of the hepatocyte growth factor receptor (HGFR) (Bardelli et al. 1996). In an independent study BAG-1 was also identified as a binding partner of the membrane bound form of the heparin-binding-epidermal growth factor (EGF)-like growth factor (HB-EGF) (Lin et al. 2001). HB-EGF is a member of the EGF family that was initially identified from conditioned medium and activates two EGF receptor subtypes HER1 and HER4. It is proteolytically processed from a larger membrane anchored precursor that acts as a paracrine growth and adhesion factor, and that is also the receptor for diptheria toxin (Raab & Klagsbrun 1997). BAG-1 interacts with the cytoplasmic tail of this membrane bound form, but not the free diffusable form (Lin et al. 2001). Deletion analysis mapped the binding site to the amino-terminus of BAG-1 demonstrating that the interaction was not mediated through HSP70/HSC70. The HB-EGF:BAG-1interaction is decreased with the induction of apoptosis in both LNCa-P and PC-3 cells and overexpression of both molecules alters cellular morphology in Chinese hamster ovary cells. BAG-1 overexpression also increased the secretion of the processed diffusable form of HB-EGF suggesting that BAG-1 may play a role in

paracrine signalling to other growth factor receptors through modulation of HB-EGF processing (Lin et al. 2001).

Hepatic growth factor (HGF) is a polypeptide that elicits mitogenic, motogenic, morphogenic and survival signalling. It stimulates proliferation of epithelial and endothelial cells and triggers the "scatter" effect by inducing cell dissociation and migration. HGF functions via the HGF receptor (HGFR), a receptor with integral tyrosine kinase activity. Unlike HB-EGF the interaction between BAG-1S and HGFR is mediated by the carboxy-terminus of BAG-1S (Bardelli et al. 1996), and so might be mediated through the chaperones HSP70/HSC70. Overexpression of BAG-1 increases HGF mediated protection from apoptosis, but in contrast to HB-EGF the BAG-1 HGFR interaction is increased with induction of apoptosis (Bardelli et al. 1996). BAG-1 in addition also interacts with the platelet derived growth factor (PDGF) receptor (Bardelli et al. 1996) suggesting a broader role for BAG-1 in facilitating or regulating signal transduction pathways.

1.9 BAG-1 Cell survival and apoptosis

1.9.1 Apoptosis, BAG-1 and BCL-2

Apoptosis is an active cellular process that enables organisms to remove unwanted cells. This process is distinct from pathological necrotic cell death and is the result of an underlying genetically controlled and evolutionarily conserved cell death program. Since many chemotherapeutic and hormonal therapies act by inducing apoptosis, and resistance to apoptosis is a key event in the development of may tumours, understanding these processes is clearly important. BCL-2 family proteins contain conserved BCL-2 homology (BH) domains and are key regulators of this cell death program. BCL-2, the prototypical member of this family, was isolated as a gene involved in follicular B-cell lymphoma. In this disease a specific chromosomal translocation occurs linking the immunoglobulin heavy chain promoter on chromosome 14 to the BCL-2 gene on chromosome 18 resulting in the constitutive expression of the BCL-2 protein. High levels of BCL-2 expression result in a relative resistance to apoptosis in these cells, and enables their continued survival. BCL-2

contains a carboxy-terminal hydrophobic membrane insertion region and primarily localises to the outer mitochondrial membrane where, in conjunction with other BCL-2 family members, it appears to regulate the release of pro-apoptotic factors in particular cytochrome c (Green & Reed 1998; Reed 1998). Release of cytochrome c is important for activation of caspases, highly conserved cysteine proteases that initiate and execute apoptosis in response to many apoptotic inducers.

BAG-1 was initially identified as a BCL-2 interaction partner (Takayama et al. 1995), and derives its name from this. In addition BAG-1 co-operates with BCL-2 in various cellular systems in preventing apoptosis (Table 1.2). BAG-1 itself has no homology to BCL-2 and is not a BCL-2 family protein, but it is possible that some of the anti-apoptotic effects of BAG-1 stem from its targeting and interaction with BCL-2. There is however little direct evidence demonstrating that BCL-2 is an obligate effector of BAG-1's mediated cell survival. It also appears that the interaction between BAG-1 and BCL-2 is indirect and mediated through HSC70/HSP70. BAG-3 and BAG-4 also contain a BAG domain and interact with BCL-2 (Antoku et al. 2001). The BAG-1:BCL-2 interaction has never been demonstrated with purified proteins or between endogenous BAG-1 and BCL-2, and although binding between BAG-1 and BCL-2 has been detected using a range of techniques HSC70/HSP70 may have been present in these assays. In addition ATP (10mM) increases binding whilst depletion of ATP abolishes binding implying a dependence on the chaperones for binding (Takayama et al. 1997).

1.9.2 BAG-1 mediated protection from apoptosis

Two major pathways initiate apoptosis in mammalian cells. In response to internal signals such as DNA damage and consequent p53 induction apoptosis is initiated from the mitochondria under the regulation of the BCL-2 family of proteins. In response to external signals however activation of various cell surface receptors such as CD95 (Fas) and tumour necrosis factor receptor (TNFR) 1 initiate apoptosis. Activation of caspases through these receptors is much more direct and does not require mitochondrial release of cytochrome c. Whilst under most conditions the death receptor and mitochondrial pathways operate independently cross talk can

Table 1.2: Suppression of apoptosis by BAG-1

Apoptosis inducer	Cell Type/Line(s)	Apoptosis/ Viability assay	BAG-1 isoform(s)	Notes	Reference
Cisplatin/ Doxrubicin/	C33A (hu cervical carcinoma)	DE	Hu BAG-1L	Both carboxy and amino terminus required	Yang et al. 2000
Etoposide/		DE, AV	Hu BAG-1L/M/S	Rare p29 isoform inactive	Chen et al. 2002
UV radiation	CHO cells (hamster ovary)	DE	Mo BAG-1S	Cooperation with HB-EGF	Lin et al. 2001
Heat shock	NIH3T3 (mo fibroblasts)	DE	Hu BAG-1L	-	Niyaz et al. 2001
	DU145 (hu prostate cancer)	VCC	Hu BAG-1M	Associated with relocalisation to nucleus	Zeiner et al. 1999
	GM701 (mo fibroblasts)	DE	Mo BAG-1S	-	Takayama et al. 1997
Anti-Fas/	Jurkat (hu T-cells)	DE	Mo BAG-1S	Cooperation with Bcl-2	Takayama et al. 1995
Staurosporine	MLP-29 (mo liver progenitor)	DE	Mo BAG-1S	Cooperation with HGF or PDGF.	Bardelli et al. 1996
	C33A (hu cervical carcinoma)	DE	Hu BAG-1L	Carboxy terminus required for binding to receptors, whole molecule for suppression	Yang et al. 1999
Serum/ Growth factor withdrawal	ZR-75-1 (hu breast cancer)	MX, DE, CA	Hu BAG-1S and BAG-1L	Dominant negative effects of BAG-1 mutants on survival	Kudoh et al. 2002
	NIH3T3 (mo fibroblasts)	DE	Mo BAG-1S	-	Takayama et al. 1995
	MKN74 (hu gastric carcinoma)	MX, DE	Mo BAG-1S	-	Yawata et al. 1998
	BaF3 and BaF-BO3 (mo pro-B-cells)	DE	Mo BAG-1S	-	Clevenger et al. 1997; Sekiya et al. 1997
	PC12 (hu phaeochromacytoma)	DE	Mo BAG-1S	-	Schulz et al. 1997

Continued					
Inducer	**Cell Type/Line**	**Assay**	**Isoform**	**Notes**	**Reference**
	B16-BL6 (mo melanoma)	MX, CA	Mo BAG-1S	-	Takaoka et al. 1997
	CSM14.1 (rat neuronal cells)	DE	Mo BAG-1S	Enhanced MAP kinase activation	Kermer et al. 2002
Dopamine	13.S.1.24 (rat neuronal olfactory)	AV	Hu BAG-1S and BAG-1M	-	Sourisseau et al. 2001
All-trans retinoic acid	MCF7 and ZR-75-1 (hu breast cancer)	DE	Hu BAG-1S	Probably through inhibition of RAR	Liu et al. 1998
Dexamethasone	S49.1 (mo thymoma)	AV	Hu BAG-1M	Probably through inhibition of GR	Kullmann et al. 1998
Anti-CD3	Mixed rat lymphocytes	AV	-	Antisense oligonucleotides used to reduce BAG-1 expression.	Sawitzki et al. 2002

Table 1.2: Suppression of apoptosis by BAG-1

Studies that demonstrate suppression of apoptosis by BAG-1 are summarised.

BAG-1 isoform constructs: Hu: human; Mo: mouse;

Apoptosis Assays: DE: Dye exclusion, MX: mouse Xenograft, CA: caspase activation, VCC: viable cells counted, AV: Annexin V

occur via death receptor mediated cleavage of Bid, a pro-apoptotic BCL-2 family member that following death receptor activation is cleaved by caspase 8, and translocates to the mitochondria where it promotes cytochrome c release (Hengartner 2000). As BAG-1 is able to inhibit the activation of apoptosis that occurs through both mitochondrial and Fas receptor signalling pathways (Takayama et al. 1995) the mechanism of action of BAG-1 can not be limited to modulating mitochondrial function through BCL-2. It is also interestingly that the two can also act synergistically. In Jurkat T cells BAG-1 or BCL-2 alone were relatively ineffective at protecting from apoptosis mediated by CD95 activating antibodies, the protein kinase inhibitor staurosporine, or cytolytic T cells, whilst co-transfection of the two proteins rendered the cells much more resistant (Takayama et al. 1995).

Induction of apoptosis is one of the principle mechanisms of action of chemotherapeutic drugs. An understanding of the factors that may allow tumour cells to become resistant to apoptosis induced by these drugs may therefore provide strategies to counter such resistance and lead to the design of improved chemotherapeutic agents. An in-vitro model of resistance to cisplatin demonstrating that multidrug resistant clonal cell lines of the human endocervical cell line (HEN-16-2) upregulate BAG-1L and BAG-1S provides evidence that BAG-1 upregulation may be involved in acquiring resistance to chemotherapy (Ding et al. 2000). Consistent with this overexpression of BAG-1 isoforms protects C33A cervical carcinoma cells from a range of chemotherapeutic agents (Chen et al. 2002b; Yang et al. 2000). There were however differences between the different agents and different BAG-1 isoforms. BAG-1S protects from cisplatin and staurosporin but not paclitaxel and doxorubicin whereas BAG-1M and BAG-1L protects from all four in C33A cervical carcinoma cells (Chen et al. 2002b). Cisplatin is an alkylating agent that cross links DNA and doxorubicin acts by intercalating adjoining nucleotide pairs on the same strand of DNA, whilst Paclitaxel is an antitubulin agent. It is possible that the different action of the different BAG-1 isoforms may be related to the different mechanisms of action of the different drugs. Protection from staurosporine mediated apoptosis required both the carboxy- and amino-termini of BAG-1L (Yang et al. 2000), and was associated

with decreased caspase activation C33A cervical carcinoma cells (Chen et al. 2002b), suggesting that BAG-1 acts upstream of caspases.

BAG-1 regulates the function of HSC70 and HSP70, two chaperones that are intimately involved in heat shock mediated stress responses. Heat shock has therefore been used as a model system to study the effects of BAG-1 overexpression on BAG-1 mediated protection from apoptosis. BAG-1S protects GM701 fibroblasts but not 293 epithelial cells from heat shock mediated apoptosis (Takayama et al. 1997), and BAG-1M overexpression protects human prostate cancer DU145 cells (Zeiner et al. 1999). Heat shock is associated with relocalisation of BAG-1M to the nucleus and it has been suggested that this might be important for suppression of heat shock induced apoptosis (Zeiner et al. 1999). As described BAG-1L is able to constitutively activate transcription from a range of promoters. The transcriptional enhancing activity of BAG-1M is activated by heat shock and the global repression of endogenous transcription in heat shocked cells is reversed by BAG-1M overexpression and its consequent relocalisation to the nucleus (Zeiner et al. 1999).

Tumours often exist in nutrient deprived microenvironment therefore growth factor withdrawal is also used as a model system for apoptosis studies. In murine pro-B cells IL-3 induces BAG-1 expression and overexpression of BAG-1 can compensate for the absence of IL-3 (Clevenger et al. 1997; Jeay et al. 2000; Sekiya et al. 1997). BAG-1 also abrogates the effects of serum starvation in B16-BL melanoma cells, MKN-74 gastric cancer cells and ZR-75-1 breast cancer cells (Kudoh et al. 2002; Takaoka et al. 1997; Yawata et al. 1998). In ZR-75-1 breast cancer cells the protective effect of BAG-1 was associated with reduced caspase cleavage. Deletion mutants lacking the BAG-1 carboxy-terminus acted in a dominant negative fashion.

In addition to the protective effects of BAG-1 overexpression some negative effects of BAG-1 on cell proliferation and survival have also been described. Overexpression of any of the three human BAG-1 isoforms modestly enhance the sensitivity of C33A human cervical carcinoma cells to N-(4-hydroxyphenyl)retinamide (4-HPR), a synthetic retinoid (Yang et al. 2000). Overexpression of BAG-1S also slows cell growth and reduces clonogenicity in human glioblastoma cell lines (Roth et al. 2000) and this is reversed by co-

overexpression of BCL-2. The significance of these results is unclear and they are difficult to reconcile with the generally protective effect of BAG-1 overexpression.

Whole animal xenograft studies are important because they demonstrate that BAG-1 is able to regulate the function of cells in-vivo as well as in cell culture. BAG-1S overexpression enhances pulmonary metastasis 1.4-fold in B16-BL6 murine melanoma cells and enhances peritoneal dissemination 3.3-fold in MKN74 human gastric cancer cells (Takaoka et al. 1997; Yawata et al. 1998). Subcutaneous tumour growth rates were identical between BAG-1-overexpressing cells and control cells in both model systems, suggesting that the increased metastasis/dissemination did not stem from increased cell proliferation. Enhanced metastatic/dissemination potential did however correlate with resistance to apoptosis induced by serum-starvation, limiting dilution or detachment in BAG-1 overexpressing cells.

ZR-75-1 breast cancer cells injected into the mammary fat pads of female nude mice form tumours 1.4 to 1.6 times larger when transfected to overexpress BAG-1S or BAG-1L compared to control transfected cells (Kudoh et al. 2002). Similar to cell culture models with ZR-75-1 cells, deletion mutants lacking the BAG-1 carboxy-terminus acted in a dominant negative fashion and tumours reached sizes less than one third of control transfected cells. This demonstrates that the endogenous BAG-1 protein in these cell lines is important for growth of these tumours cells in both cell culture and in the xenografts. It also shows that the protective effect of BAG-1 is not merely an artefact of overexpression.

Transgenic animals provide opportunities for investigating how genes function in the context of whole organisms and can thus provide important information that can not be gained from a study of single cells. There have been no reported transgenic studies investigating the effects of deleting the BAG-1 gene (knockout), or of overexpressing the gene under a promoter that directs expression to tissue where BAG-1 is commonly overexpressed in tumours (for example breast). The only reported BAG-1 transgenic model is in the eye (Eversole-Cire et al. 2000). In this model murine BAG-1S was placed under the control of the murine opsin gene to target its expression specifically to photoreceptor cells. Surprisingly BAG-1 expression alone led to retinal degeneration. This was not just due to overexpression

of an ectopic protein because BCL-2 transgenic mice showed no effect. Crossing BCL-2 transgenic mice with BAG-1 transgenic could not rescue from photoreceptor degeneration but did provide resistance to degeneration in a retinitis pigmentosa model. It has been suggested that the degenerative effects of BAG-1 are due to aberrant eye development due to BAG-1 mediated stimulation of retinoid signalling as the RAR is known to be important for eye development. These unexpected results illustrate the importance of transgenic models and inducible systems may help obviate some of the difficulties.

1.10 BAG-1 and human Cancer

1.10.1 Breast Cancer, hormone receptor status and hormone therapy

Although the mortality rate from breast and lung cancer is similar in women, breast cancer is by far the most common malignancy; approximately one in nine women develop breast cancer and breast cancer comprises nearly thirty percent of all new female cancers. In the United Kingdom the age standardised incidence and mortality from breast cancer is the highest in the world, and breast cancer is the single commonest cause of death amongst women aged forty to fifty (McPherson et al. 2000; www.cancerresearchuk.org/statistics).

Breast cancer is generally initially treated by a combination of local treatments to control local disease and adjuvant systemic therapy for any possible micrometastatic disease. Local treatment includes surgery and radiotherapy whilst systemic therapy includes hormone therapy and cytotoxic chemotherapy. The benefits of hormone therapy both in an adjuvant setting and as treatment for metastatic disease have been clearly demonstrated. A meta-analysis has been performed of trials of Tamoxifen as adjuvant therapy, involving approximately thirty thousand women of unknown or ER positive status (it is estimated that twenty-six thousand of these would be ER positive), with five years of treatment with Tamoxifen and up to ten years of follow up. This analysis demonstrates a proportional reduction in recurrence by 47% and a proportional reduction in mortality by 26% (EBCTCG 1998). Benefit was largely resricted to those who were ER positive. It is recognised that hormone

receptor status predicts response to hormone therapy in both an adjuvant and metastatic setting (ASCO 1996). Increasingly PgR status, or the status of other ER transcriptional target genes such as pS2, is also measured to further refine the ability to predict response to hormone therapy. A second ER known as ER beta (ERβ) has also been described but the prognostic significance of expression of this receptor in breast cancer has still not been fully defined (Speirs 2002).

In addition to Tamoxifen other strategies of hormone therapy are also available. In pre-menopausal women medical oopherectomy with leutinising hormone releasing hormone agonists such as Goserelin has proven effective in the treatment of metastatic or locally advanced disease and are showing promise in the adjuvant setting in ongoing trials (Jonat 2001). Post-menopausal women depend on the enzyme aromatase to synthesise oestrogen in peripheral tissues such as adipose tissue. Aromatase inhibitors block the enzyme and so prevent the production of oestrogen in these tissues in post-menopausal women. In a trial of over nine thousand postmenopausal women the aromatase inhibitor anastrozole (Arimadex) demonstrated superiority over Tamoxifen in an adjuvant setting (The ATAC Trialists' Group 2002), and is now licensed for use as a second line agent for adjuvant therapy in post-menopausal women. Interestingly Tamoxifen in combination with anastrozole produced an inferior outcome when compared to Tamoxifen alone and it has been suggested that Tamoxifen acts as a partial agonist and produces some oestrogenic signalling which mitigates the effects of the complete blockade of oestrogen production caused by inhibition of aromatase. Pure antioestrogens such as ICI 182 780 (Faslodex) bind non-competitively to ERα and in contrast to Tamoxifen increase ERα degradation and posses no agonist activity (Robertson 2001). ICI 182 780 has been demonstrated to be as effective as anastrozole for second line therapy in post-menopausal women with advanced disease in phase three trials (Robertson 2001).

Reductions in incidence of contralateral breast cancers in those treated by hormone therapy has led to several large trials to test the effectiveness of Tamoxifen as a chemopreventative agent in women with a high risk of breast cancer. The large American NSABP P1 trial obtained impressive results demonstrating a 49% reduction in incidence of invasive breast cancer in women at increased risk of breast cancer

with five years prophylactic Tamoxifen (Fisher et al. 1998). The risk reduction was found wholly in ERα positive cancers such that the risk of ERα positive cancers was reduced by 69% whilst the risk of ERα negative cancers remained unchanged (Fisher et al. 1998). These results have not however been replicated by two smaller European trials (Kinsinger & Harris 2002). Additionally whilst the more recent IBIS-1 trial demonstrated a 32% reduction in breast cancer incidence with Tamoxifen in women at increased breast cancer risk, there was also a corresponding increase in all cause mortality associated with an increased rate of thromboembolic events (I.B.I.S.Investigators 2002). The overall risk benefit ratio for Tamoxifen therefore remains uncertain and further trials, including those with other antioestrogens including Raloxifine and Anastrozole, are ongoing.

1.10.2 BAG-1 expression and breast cancer

Given the impact of BAG-1 overexpression on multiple growth control pathways, there has been considerable interest in studying the significance of BAG-1 in human cancer. Four large immunohistochemical studies of the expression and clinical significance of BAG-1 in breast cancer have been reported and these are summarized (Table 1.3) (Sjostrom et al. 2002; Tang et al. 1999; Townsend et al. 2002; Turner et al. 2001). Some of the results from these studies are inconsistent and further studies are required to fully understand the role of BAG-1 expression in breast cancer. A consistent finding is that relatively high levels of cytoplasmic BAG-1 expression are detected in two thirds or more cases of breast cancer. Changes in BAG-1 expression can be detected in benign lesions such as sclerosing adenosis, and in ductal carcinoma in situ suggesting that they might represent a relatively early event in breast cancer development (Brimmell et al. 1999). By contrast, the extent of nuclear BAG-1 expression differs widely in these studies, possibly for reasons discussed below, ranging from as low as 20% to almost 70%. Moreover, the proportion of tumours with both nuclear and cytoplasmic expression varies widely from just 1% to more than 60%.

Table 1.3: A summary of major studies of BAG-1 expression in breast cancer

	Tang et al. 1999	**Turner et al. 2001**	**Sjostrom et al. 2002**	**Townsend et al. 2002**
Number of Patients	140	122	126	159
Median Age (years)	63	54 (Mean)	Not Stated	47
Median Follow up (years)	8	12.1	Time to progression 4.2 months Overall survival 9.6 months	12.8
Cohort Characteristics	Mixed	Early Stage (I/II) - All wide excision and radiotherapy	Patients with advanced cancer entered into chemotherapy trial	Mixed (Pre- and post-menopausal subgroups)
Antibody Used	C16 (Santa Cruz Inc.)	KS-6C8	Clone not stated	C16 (Santa Cruz Inc) (AlsoTB2)[c]
Type	Affinity purified rabbit Polyclonal	Mouse monoclonal	Mouse monoclonal	Affinity purified Rabbit Polyclonal
Immunogen	C-terminal peptide of mouse BAG-1S[a]	GST fusion protein containing C-terminal 170 amino-acids of human BAG-1S	GST fusion protein containing C-terminal 170 amino-acids of human BAG-1S	C-terminal peptide of mouse BAG-1S[a]
Antigen Retrieval method	Not Stated	Not Stated	Boiling in citrate buffer	Pressure Cooker (citrate buffer)
Scoring System used	Intensity	H score	Percentage of positive cells	Intensity

Continued	Tang et al. 1999	Turner et al. 2001	Sjostrom et al. 2002	Townsend et al. 2002
BAG-1 positivity (%)				
Nuclear Only	18.2	23[b]		5
Cytoplasmic Only	57.1	65[b]		25
Nuclear & Cytoplasmic	1.4			62
Total (Nuclear or Cytoplasmic)	77.1		Median 60% positive cells	92
Correlations between BAG-1 expression and clinico-pathological parameters[d]	Tumour Differentiation (Nuclear and overall BAG-1)	BCL-2 (Cytoplasmic BAG-1)	Bcl-2, Bax, FasL	Grade (Nuclear BAG-1) ERα status in pre-menopausal (Cytoplasmic BAG-1)
Correlations between BAG-1 expression and clinical outcome[d]				
Univariate analysis	None	DFS,OS (Cytoplasmic BAG-1)	None	None (Trend: improved survival, Nuclear BAG-1)
Multivariate analysis	DFS, OS (BAG-1 expression correlated with poor outcome)	DFS, OS (Cytoplasmic BAG-1)	None	None

Table 1.3: A summary of major studies of BAG-1 expression in breast cancer

[a]Differs from human BAG-1S sequence by a single amino acid; [b]Nuclear ± Cytoplasmic/Cytoplasmic ± Nuclear; [c]Rabbit polyclonal produced using a human BAG-1S-GST fusion protein as the immunogen; similar staining results were obtained as with C16; [d]Correlations included are those with p values <0.05

A strong relationship between nuclear BAG-1 expression and tumour grade/differentiation has been identified in two studies, with relatively high levels of nuclear BAG-1 expression in low grade tumours (Tang et al. 1999; Townsend et al. 2002). By contrast, Turner et al. (Turner et al. 2001) reported no correlation between tumour grade and BAG-1 expression. Correlations with other clinical markers, such as oestrogen receptor alpha (ERα) and BCL-2 expression have been reported in various studies, but these appear to be not as strong as the correlation with grade and are more variably detected.

Turner et al. reported an overall 10-year survival for women with early stage breast cancer of 82% with high cytoplasmic BAG-1 levels versus 42% survival with low BAG-1 levels (Turner et al. 2001). Cytoplasmic BAG-1 status predicted outcome in both univariate and multivariate analyses, and also retained predictive value in a subset of their patients with pathologically negative lymph nodes. This is particularly interesting since it was suggested that this might provide a means by which node negative patients with a relatively poorer prognosis could be selected for further adjuvant therapies, and conversely enable better prognosis node negative patients to avoid such therapies with their concomitant side effects. This exciting finding awaits confirmation (Tang et al. 1999; Townsend et al. 2002). Tang et al found no correlations between BAG-1 expression and outcome in univariate analysis and conversely reported that increased BAG-1 expression correlated with decreased disease free and overall survival in a multivariate analysis controlled for tumour differentiation (Tang et al. 1999). In a further study no correlations were found between cytoplasmic BAG-1 and outcome (Townsend et al. 2002). A tendency was however observed for patients with nuclear BAG-1 expression to have slightly (but not statistically significantly) improved outcomes, consistent with the correlation also found in this study with low tumour grade.

The ability of BAG-1 expression to predict response to adjuvant therapy has currently only been assessed in one study. Sjostrom et al. (Sjostrom et al. 2002) found that BAG-1 expression did not predict time to progression or overall survival in patients with advanced breast cancer entered into a randomised controlled trial

comparing docetaxel with sequential methotrexate and 5-fluorouracil. BAG-1 status therefore appears not to predict response to chemotherapy, but, unlike other studies demonstrating prognostic significance, BAG-1 subcellular localisation was not analysed. Further work is required to assess the ability of BAG-1 to predict response to adjuvant therapy, in particular given the role of BAG-1 in modulation of NHR function, and the importance of adjuvant hormonal therapy in breast cancer.

1.10.3 BAG-1 and other malignancies

BAG-1 expression has also been studied in a range of other cancer types (Table 1.4). Breast, lung, prostate and colorectal cancer account for over half the incidence of cancers in the United Kingdom (52%) and almost half the cancer mortality (47%), and there is evidence that BAG-1 may be involved in all four of these cancers. Despite the described effects of BAG-1L on AR function and possible resistance to anti-androgen tharapy no detailed expression analysis has been performed in prostate cancer however. A microarray analysis has recently identified BAG-1 as a candidate tumour progression gene overexpressed in mouse lung cancers (Yao et al. 2002). Lung cancer is the most common cancer in men and the third most common in women, and since survival rates are very low it is the largest cause of cancer mortality in the United Kingdom. Consistent with the microarray analysis, Rorke et al (Rorke et al. 2001) have studied the expression of BAG-1 protein by immunohistochemistry in non-small cell lung cancer. Similar to studies in other tumour types they found that approximately two thirds of tumours expressed high levels of BAG-1. In particular they found no correlations between BAG-1 and clinico-pathological parameters, but did find that, similar to the study of Turner et al in breast cancer, cytoplasmic expression of BAG-1 independently correlated with improved overall survival.

Colorectal cancer is the third most commonly diagnosed cancer in the United Kingdom, and comes second to lung cancer as the next most common cause of cancer mortality. In a study of BAG-1 expression in 86 colorectal carcinomas approximately two thirds of colorectal carcinomas exhibited a cytoplasmic staining pattern, whilst 24% exhibited a nuclear staining pattern, and no correlations were identified between

Tumour	Reference	Notes
Glioblastoma	Roth et al 2000	BAG-1 expression was detected in 11/12 human glioma cell lines. 15 out of 19 glioblastomas were positive for BAG-1 by immunohistochemistry. In contrast only single neurones and astrocytes were positive in normal brain parenchyma.
Cervical Carcinoma	Yang et al 1999	BAG-1 overexpression in human cervical carcinoma biopsies compared to normal tissue or normal cell lines.
Leukaemia	Kitada et al 1998	Higher levels of BAG-1 in chronic lymphocytic leukaemia (CLL) were associated with failure to achieve complete remission ($p= 0.04$).
Lymphoma	Xerri et al 1999	BAG-1 was detected by immunoblotting in 33/33 biopsy samples from B cell non-Hodgkin's lymphoma. Differences in expression levels were found between lymphoma subtypes.

Table 1.4: Studies of BAG-1 expression in glioblastoma, cervical carcinoma, leukaemia and lymphoma.

Studies included are those involving primary material.

BAG-1 expression and clinicopathological variables examined (Kikuchi et al. 2002). Importantly, nuclear BAG-1 immunostaining was associated with distant metastases and with a worse overall survival in both univariate and multivariate analysis (Kikuchi et al. 2002).

Several groups have studied expression of BAG-1 in human squamous cell carcinomas. Yamauchi et al (Yamauchi et al. 2001) found that, in contrast to breast and lung cancer, but similar to colorectal cancer, nuclear expression of BAG-1 in laryngeal tumours conferred a worse disease-free survival after radiotherapy. In oral squamous cell carcinomas, Shindoh et al. (Shindoh et al. 2000) demonstrated increased BAG-1 expression in tumour tissue relative to adjacent normal epithelium in 60-80% of samples. By contrast, a study of 64 oral squamous cell carcinomas and 17 samples of normal oral epithelium, revealed reduced nuclear BAG-1 expression in oral squamous cell carcinomas ($p=0.036$) compared to normal oral epithelium (Hague et al. 2002).

BAG-1 enhances metastasis in experimental models (Takaoka et al. 1997; Yawata et al. 1998) and Shindoh et al. demonstrated that BAG-1 was expressed in 89% of primary tumours with nodal metastasis compared to 38% of tumours without. BAG-1 expression levels were determined by densitometry and although this alleviates the subjectivity of scoring BAG-1 labelling intensity to some extent it does not give information on whether the immunoreactivity is nuclear or cytoplasmic. BAG-1 expression by immunohistochemistry in paired samples of primary tumour and matched lymph node metastasis demonstrated statistically significant increased cytoplasmic expression in 8 of 13 metastatic tumours relative to the corresponding primary tumour ($P=0.021$) (Hague et al. 2002). However, in contrast to the results of Shindoh et al., no significant difference in BAG-1 expression was detected in the primary tumours between patients with and without associated lymph node metastases. Hague et al. (Hague et al. 2002) determined the predominant BAG-1 immunostaining intensity and it is possible that the maximal intensity may provide improved prognostic value for metastatic potential. Alternatively, the environment at the site of the metastasis may induce BAG-1 expression. The presence or absence of

lymph node metastases is of strong prognostic significance and evaluation of these events in relation to prognosis in larger cohorts is warranted.

1.11 Outline of work

There is compelling evidence that BAG-1 expression is altered in cancer, and that these changes relate to clinical parameters and patient outcome. BAG-1 may regulate crucial growth control pathways important for the development and progression of cancer, and for cancer therapy. Significant questions remain however. The results of the immunohistochemical studies of BAG-1 expression are inconsistant, possibly because of differences in antibodies, staining protocols and the use of heterogeneous patient cohorts. The initial set of experiments were therefore to characterise antibodies to BAG-1, and to determine the clinical significance of BAG-1 in breast cancer, in particular in the context of hormone signalling in a homogenous group of patients all treated with adjuvant hormone therapy. I also determined whether patients with breast cancer had immunological responses to BAG-1, similar to heat shock proteins.

Given the significance of oestrogen receptors in breast cancer it is important to determine whether BAG-1 regulates oestrogen receptors directly, and experiments in chapter four demonstrate that the nuclear BAG-1 isoform binds to and stimulates ERα and ERβ. Finally the role of the cytoplasmic BAG-1 isoform in protecting breast cancer cells from stress-induced apoptosis was investigated. It was demonstrated that BAG-1 can interfere with p53 function, and the regulation of specific gene expression by BAG-1 was analysed by microarray experiments. These results support the hypothesis that BAG-1 is an important molecule in breast cancer, and might prove to be a novel target for breast cancer therapy.

2 Materials and Methods

Merck Ltd (BDH Laboratory Supplies, UK) supplied all chemicals and solvents unless otherwise stated. Laboratory manuals (Ausbel et al. 2003; Sambrook et al. 1989) were used as a reference source for methodological techniques.

2.1 Solutions and Buffers

Solutions were sterilised by autoclaving or filtration (0.2 μm filter) as required.

2.1.1 General Reagents

Phosphate Buffered Saline (PBS)

125 mM Sodium Chloride
16 mM Na_2HPO_4
10 mM NaH_2PO_4
HCl to pH 7.2

Tris (Tris[hydroxymethyl]aminomethane)-Buffered Saline (TS)

10 mM Tris-HCl pH 8.0
150 mM Sodium Chloride

Tris-EDTA (TE)

10 mM Tris-HCl pH7.6
1 mM Ethylenediaminetetra-acetic acid (EDTA)

SSC buffer 20x

3 M Sodium Chloride
0.3 M Na_3citrate.$2H_2O$

Lysozyme solution

50 mM Glucose

10 mM EDTA

25 mM Tris-HCl pH 8.0

ONPG (o-nitrophenyl-beta-D-galactopyranoside) reagent

120 mM $Na_2HPO_4.12H_2O$

80 mM $Na_2H_2PO_4.2H_2O$

2 mM $MgCl_2.6H_2O$

pH to 7.5 with 10 N NaOH then add:

100 mM 2 Mercaptoethanol

1.33 g/L ONPG

2.1.2 Protein Analysis Reagents

Protein Running Buffer

25 mM Tris Base

200 mM Glycine

0.1% (w/v) SDS (sodium dodecyl sulfate)

RIPA Lysis Buffer

150 mM Sodium Chloride

1% (v/v) IPEGAL (Nonidet P-40)

0.5% (w/v) Sodium Deoxycholate

0.1% (w/v) SDS

50 mM Tris-HCl pH 8.0

HEPES Buffered Saline (HBS) 2x

280 mM Sodium Chloride

10 mM Potassium Chloride

1.5 mM Na_2HPO_4

12 mM Dextrose

50 mM HEPES (4-(2-Hydroxy-ethyl)-piperazine-1-ethane-sulfonic acid)
pH to 7.05 with 0.5 M Sodium Hydroxide

HMKEN Buffer

10 mM HEPES pH 7.2
5 mM Magnesium Chloride
142 mM Potassium Chloride
2 mM EGTA (Ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid)
0.2% (v/v) IPEGAL
1% (v/v) Protease Inhibitor Cocktail (Sigma, UK)

Fix

10% (v/v) Glacial Acetic Acid
45% (v/v) Ethanol
45% (v/v) Distilled Water

Coomassie Blue — 0.05% (w/v) Coomassie blue powder in Fix

2.1.3 DNA and RNA Electrophoresis Reagents

Agarose Gel Loading Buffer 6x

0.25% (w/v) Bromophenol Blue
0.25% (w/v) Xylene Cyanole
15% (w/v) Ficoll 400

RNA Gel loading buffer 1.1x

50% deionised formamide
1 x 2(n-morpholino)ethane sulphonic acid (MOPS; Sigma)
2.15 M formaldehyde
5% (v/v) Glycerol

Tris-Borate-EDTA Buffer (TBE)

80 mM Tris Base

80 mM Orthoboric Acid

1 mM EDTA

2.1.4 Bacteriology Reagents

2x Tryptone-Yeast Extract (2x TY) media

16 g/L Tryptone (Becton Dickinson, UK)

10 g/L Yeast Extract (Becton Dickinson, UK)

5 g/L Sodium Chloride

pH to 7.4 with 4M Sodium Hydroxide

Luria Bertani (LB) media

10 g/L Tryptone

5 g/L Yeast Extract

10 g/L Sodium Chloride

pH to 7.4 with 4M Sodium Hydroxide

LB plate media

10 g/L Tryptone

10 g/L Yeast Extract

5 g/L Sodium Chloride

pH to 7.4 with 4 M Sodium Hydroxide

15 g/L Agar (Becton Dickinson, UK)

Where indicated: 100 μg/ml Ampicillin or 100 μg/ml Kanamycin

SOC Media

20 g/L Tryptone

5 g/L Yeast Extract

0.5 g/L Sodium Chloride

2.5 mM Potassium Chloride

10 mM Magnesium Chloride

20 mM Glucose

2.2 Gel Electrophoresis

2.2.1 Agarose Gel Electrophoresis

Agarose gels were made in TBE with 0.05 μg/ml ethidium bromide. Gels were made to 1, 2 or 2.5% (w/v) agarose (Invitrogen, UK). Samples were mixed with one sixth volume agarose gel loading buffer, loaded onto gels and electrophoresed in TBE at 10 V/cm. Promega DNA ladders were used as molecular weight standards. Gels were visualised under long wave ultraviolet light and images captured using a digital camera (Kodak DC 120; Kodak, UK) and Kodak KDS 1D software.

2.2.2 Agarose/formaldehyde gel electrophoresis

RNA agarose/formaldehyde gel electrophoresis was essentially as for DNA agarose gel electrophoresis but with the following exceptions. Gels were made under RNase free conditions. 1% (w/v) agarose gels were made in MOPS buffer with 0.66 M formaldehyde in a fume hood since formaldehyde is toxic by inhalation. 1 μg RNA was added to the RNA gel sample loading buffer and ethidium bromide added to a concentration of 0.1 mg/ml. The samples were then heated at 65 ^{o}C for 15 minutes and cooled on ice prior to loading. Gels were viewed and images captured as for DNA agarose gel electrophoresis. Intact total RNA appears as a smear of poly(A)$^{+}$ RNA and two bright bands representing 28S and 18S ribosomal RNA.

2.2.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to analyse proteins from cell extracts, immunoprecipitations and in vitro translation products. Cells were trypsinised, collected by centrifugation at 300 g and washed once in ice cold PBS. Cells were then lysed in 1x RIPA buffer supplemented with 1% (v/v) mammalian protease inhibitor

cocktail (Sigma, UK) added immediately prior to use. Cell lysates were incubated on ice for 30 minutes and then clarified by centrifugation in a refrigerated microfuge at 13000 rpm for 15 minutes at 4 °C. Protein assay reagent (Bio-Rad, UK) was used to determine the protein content of the clarified lysate. Bovine serum albumin was used as a standard for the assay. Protein sample buffer (New England Biolabs, UK) supplemented with 0.1 M Dithiothreitol (DTT; New England Biolabs) was added and protein extracts normalised to 2 μg/μl final concentration. The samples were then heated at 95 °C for 3 minutes following which, unless otherwise stated, 20 μg was loaded onto pre-cast 4-15% gradient Tris HCl SDS-polyacrylamide gels (Bio-Rad, UK). Broad range protein markers (New England Biolabs, UK) were used as molecular weight standards. Proteins were separated electrophoretically using mini-protean III cells (Bio-Rad, UK) in 1x protein running buffer at 200 Volts. Protein from the polyacrylamide gel was then transferred onto nitrocellulose membrane (Schleicher and Schuell, Germany). Alternatively protein was stained with Coomassie blue, or in the case of ^{35}S labelled products of in vitro translation dried onto Whatmann 3MM (Whatmann, UK) filter paper using a gel drier (Bio-Rad, UK) at 80 °C for 1 hour. ^{35}S Labelled protein were then visualised directly on a phosphor imager (Bio-Rad, UK). Coomassie blue staining was performed by immersing the polyacrylamide gel in Coomassie blue solution for 1 hour following which the gel was destained three times in fix for 20 minutes, and then dried as described.

Transfer onto nitrocellulose membrane was performed in 1x protein running buffer containing 25% (v/v) ethanol in a mini-protean II transfer unit (Bio-Rad, UK) at 100 Volts for 1 hour. The nitrocellulose membrane was then incubated in 2% (w/v) non-fat milk (Marvel) in TS for 30 minutes to block non-specific protein binding sites. Following this the membrane was incubated with the primary antibody at the appropriate concentration in 2% (w/v) non-fat milk/TS at 4 °C overnight unless otherwise stated. Membranes were washed three times in TS with 0.05% (v/v) Tween (TST) for 5 minutes, after which the membranes were incubated in the appropriate horseradish peroxidase (HRP) -conjugated secondary antibody (Amersham, UK) for 1 hour at room temperature at 1/5000 dilution in 2% (w/v) non-fat milk/TS. The membranes were then washed as before and bound immunocomplexes detected using

Supersignal West Pico Chemiluminescent Substrate (Pierce, UK) on a "Fluor-S-MultiImager" (Bio-Rad, UK) using "Quantity One" software (Bio-Rad, UK).

2.3 DNA Manipulations and RNA isolation

2.3.1 Overview

Restriction endonucleases and other enzymes were obtained from New England Biolabs, UK, unless otherwise indicated. Primers for PCR were synthesised by Invitrogen, UK. DNA inserts were obtained by PCR or restriction endonuclease digestion of plasmids as indicated. Plasmid vectors were either obtained commercially linearised with covalently bound topoisomerase I (PCR TOPO TA; Invitrogen UK), or linearised and dephosphorylated prior to ligation. Following ligation or "quikchange" mutagenesis, DNA was transformed into competent bacteria and clones analysed by restriction mapping, and commercial sequencing (MWG Biotech, Germany) of small-scale "miniprep" DNA preparations. Plasmids were purified by centrifugation on caesium chloride gradients containing ethidium bromide.

2.3.2 Restriction Endonuclease Digestion

A typical restriction endonuclease digestion reaction would be:

DNA	1 μg
Restriction Endonuclease	1 μl
10x Buffer	2 μl
10x BSA (1 mg/ml)	2 μl
Water	to 20 μl

Reactions were incubated at the temperature recommended for the restriction enzyme for 1-4 hours. Occasionally reactions were incubated overnight to ensure complete digestion, for example if linearising a vector prior to ligation. The digestion products were assessed by agarose gel electrophoresis.

2.3.3 Phenol:Chloroform Purification and Ethanol Precipitation

For Phenol:chloroform purification an equal volume of water saturated phenol chloroform (1:1 v/v) was added to an aqueous DNA sample and vortexed. The phases were separated by centrifugation in a microfuge at 13000 rpm for 3 minutes and the top aqueous phase collected. Residual phenol was extracted from this with an equal volume of water saturated chloroform as above. DNA was then recovered from the upper phase by ethanol precipitation. This was performed by addition of one tenth volume of 3 M Sodium acetate pH 4.8 and 2.5 volumes 100 % (v/v) ethanol. The DNA solution was then vortexed and incubated at -20°C for 30 minutes. The solution was microcentrifuged at 13000 rpm for 10 minutes at 4°C and the pellet washed with ice cold 70 % (v/v) ethanol and dried briefly under vacuum, or air dried. The DNA pellet was finally resuspended in distilled water or TE.

2.3.4 Gel Purification

To gel purify vectors and insets from digestion or PCR reactions the DNA was electrophoresed on an agarose gel and the appropriate band excised. The excised gel was then purified using Freeze and Squeeze extraction columns (Bio-Rad, UK) according to the manufacturer's instructions.

2.3.5 Polymerase chain reaction (PCR)

Clontech (UK) supplied components for PCR reactions. GC melt, Dimethyl Sulfoxide (DMSO) and Advantage GC Taq polymerase were used when amplifying sequences with rich GC content such as the N terminus of BAG-1L. A typical PCR reaction mixture would be:

DNA plasmid template	5 µl (50 pg – 10 ng)
Oligonucleotide Primers	2 µl (25 pmol each)
10x Reaction Buffer	5 µl
Taq Polymerase	1 µl
(Clontech Advantage mixture)	
Clontech dNTP mixture	1 µl

GC Melt (if used)	5 µl
DMSO	2.5 µl
Water to	50 µl

Control reactions were also prepared with the above mixture replacing the template with water to control for contamination. Typical cycling parameters using a DNA Thermal Cycler (Geneamp 9700, Perkin-Elmer, Belgium) were:

Initial Denaturation	94 °C for 1 minute	1 cycle
Denaturation Annealing and Extension	94 °C for 30 seconds 2 minutes	5 cycles
Denaturation Annealing and Extension	94 °C for 30 seconds 2 minutes	30 cycles
Final Extension	10 minutes	

Primer sequences, names and specific annealing and extension temperatures are described in table 2.1. After amplification the PCR product was confirmed by restriction endonuclease digestion and agarose gel electrophoresis. The PCR product was then gel purified and concentrated by ethanol precipitation.

2.3.6 Subcloning into PCRII TOPO TA

The PCRII TOPO TA (Invitrogen, UK) vector was used to subclone PCR amplified inserts. This vector is supplied linearised with overhanging 3' thymidines and topoisomerase I covalently attached to the ends. Since the proof reading polymerases used for PCR remove the overhanging 3' Adenine overhangs required for the TA cloning it was first necessary to tail the PCR products. This was performed by addition of 1 µl of 5 U/µl Taq polymerase, 5 µl 10x Taq polymerase buffer, 4 µl 2.5 mM dNTP's, and 3 µl 25 mM $MgCl_2$ to 37 µl of gel purified PCR product. The reaction was then incubated on a hot block at 72 °C for 15 minutes. Cloning was

Plasmid	Primer Name	Primer Sequence (5' to 3') and Characteristics	Annealing and Extension Temperatures
pGEX-2TK	pGex 5' Sequencing Primer	GGGCTGGCAAGCCACGTTTGGTG	N/A
	pGex 3' Sequencing Primer	CCGGGAGCTGCATGTGTCAGAGG	
pGEX-2TK-BAG-1L$^{1-71}$	5' GST BAG-1L$^{1-71}$	CGC GGATCC GCTCAGCGCGGGGGGGCG BamHI BAG-1L sequence	Combined annealing and extension 77°C for the first 5 cycles
	3' GST BAG-1L$^{1-71}$	CCG GAATTC TCA CCGCGGCCTGCGAGCGCC EcoRI Stop BAG-1L sequence	followed by 72°C for the following 30 cycles
pcDNA3- BAG-1S^{NLS}	p36-NLS	CCC AAGCTT GAATTC GAAGAGATG HindIII EcoRI Kozak Consensus Sequence CCAAAAAAGAAGAGAAAGGTA AATCGGAGCCAGGAGGTG SV40 T-antigen NLS sequence BAG-1S sequence	Combined annealing and extension 73°C for the first 5 cycles followed by 68°C for the following 35 cycles
	BAG-1 C term (XhoI)	CCG CTCGAG TGCTACACC TCA CTCGGCCAGGGC XhoI Stop BAG-1S sequence	

Table 2.1: Primers used for production and sequencing of pGex-BAG-1L$^{1-71}$ and production of pcDNA3- BAG-1S^{NLS}
Key features within the sequence are indicated. Annealing and extension temperatures used for PCR with each primer are also described

subsequently carried out according to the manufacturer's instructions by incubating the following typical reaction mixture for 5 minutes at room temperature:

Tailed PCR product	1 µl
TOPO Vector	1 µl
5x Salt Solution (as supplied)	1 µl
Water	to 5 µl

The cloned vector was then transformed and plated. If the initial insert was produced by PCR from a plasmid with ampicillin resistance selection for transformed clones was performed using kanamycin to ensure the template was not carried through.

2.3.7 Vector Dephosphorylation and Ligation

Following linearisation the following typical reaction mixture was used to remove 5' phosphate groups from the vector:

Shrimp Alkaline Phosphatase (SAP) 1 U/µl	2 µl
10x SAP buffer	4 µl
Linearised vector	20 µl
Water	to 40 µl

The reaction mixture was incubated at 37 °C for 30 minutes following which the enzymes were heat inactivated at 65 °C for 10 minutes and the vector then purified by phenol chloroform and ethanol precipitation.

Agarose gel electrophoresis and comparison to DNA standards (100 base pair or 1Kb ladder according to size, Promega, UK) was used to estimate DNA concentrations of prepared vector and insert. Multiple ligation reactions were then typically prepared in a 3:1, 1:1 and 1:3 vector to insert molar ratio. The following typical ligation reaction was then incubated at 16 °C overnight:

Prepared Vector	1 µl
Prepared Insert	1 µl
3 U/µl T4 DNA Ligase	1 µl
10x T4 Ligase Buffer	1 µl
Water	to 10 µl

Two control reactions were also prepared, the first replacing the ligase with water to control for undigested unlinearised vector, and the second replacing the insert with water to control for self-ligation of the vector. The ligated vector was then transformed.

2.3.8 Bacterial Cell Strains

The E.coli strain DH5α (Subcloning efficiency, Gibco, UK) was used for plasmid DNA preparations, and the E.coli strain BL21 (Competent BL21 Gold, Stratagene, Holland) for recombinant protein production.

2.3.9 Transformations

1-2 µl of plasmid was added to a 50 µl aliquot of competent bacteria and incubated on ice for 30 minutes. The bacteria were then heat shocked for 45 seconds at 42 °C and allowed to recover on ice for 5 minutes. 400 µl of SOC was then added and bacteria incubated with shaking at 37 °C for 1 hour. 25 µl or 125 µl of the bacterial suspension were spread on to 90 mm prepared LB agar plates supplemented with the appropriate selection antibiotic. Plates were then inverted and incubated at 37 °C overnight prior to selection of colonies.

2.3.10 "Quikchange" mutagenesis

Point mutagenesis was performed using the "quikchange" kit (Stratagene, UK) according to the manufacturer's instructions. Essentially the complimentary primers containing the desired mutation are extended to produce a plasmid containing the desired mutation. *Dpn* I, which is specific for methylated or hemimethylated DNA, is then used to digest methylated parental plasmid. Remaining unmethylated

DNA containing the desired mutation is transformed and colonies selected for small-scale preparation of plasmid DNA.

2.3.11 Small scale preparation of plasmid DNA (minipreps)

Small scale preparation of DNA (minipreps) from colonies for verification of cloning by endonuclease digestion and sequencing was performed using Promega Wizard Miniprep kits (Promega, UK) according to the manufacturer's instructions. This kit utilises a proprietary alkaline lysis technique (see 2.3.12) followed by purification by binding and elution from a resin.

2.3.12 Large scale preparation of plasmid DNA (maxipreps)

Transformed bacteria were grown overnight in 500 ml of media at 37 °C. The cells were collected by centrifugation at 5000 rpm for 10 minutes (Sorvall RC5C Centrifuge, F-16/250 rotor), and then resuspended in 20 ml lysosyme solution freshly supplemented by 1 mg/ml lysozyme. Following lysis for 5 minutes, 40 ml of freshly made 0.2 M sodium hydroxide, 1% SDS (w/v), was added. The solution was gently mixed and left for 5 minutes at room temperature to allow protein and DNA to denature. 20 ml of 3 M sodium acetate pH 4.8 was added to precipitate protein and DNA, and the resultant debris was removed by centrifugation at 6300 rpm for 20 minutes (Sorvall RC5C Centrifuge, SS34 rotor). The supernatant was collected, filtered through muslin, and 40 ml of propan-2-ol added. The DNA was collected by centrifugation at 7000 rpm (Sorvall RC5C Centrifuge, F-16/250 rotor) for 20 minutes, and resuspended in 20 ml of water. 25 g of caesium chloride and 0.7 ml of 10 mg/ml of ethidium bromide was added. Any remaining debris was cleared by centrifugation at 10000 rpm (Sorvall RC5C Centrifuge, F-16/250 rotor) for 10 minutes, following which the plasmid DNA was concentrated within a density gradient by ultracentrifugation at 50000 rpm for 48 hours (Sorvall OTD55B Ultracentrifuge, TFT 50.38 rotor). The upper plasmid DNA band was collected using a peristaltic pump, and the ethidium bromide removed by repeated extraction with water saturated butan-1-ol. The purified plasmid DNA was then concentrated by ethanol precipitation, resuspended in water and concentration measured by absorbance at 260 nm.

2.3.13 RNA isolation

RNA was extracted from cell pellets under RNase free conditions using TRIZOL reagent (Invitrogen, UK), a solution of phenol and guanidine isothiocyanate that disrupts and dissolves cell components whilst leaving the RNA intact. TRIZOL was used according to the manufacturer's instructions. Essentially following incubation of the cell pellet with TRIZOL, chloroform is added which following centrifugation separates the solution into an aqueous and an organic phase. RNA remains in the aqueous (upper) phase and is precipitated by addition of isopropanol and washed with 70% (v/v) ice cold ethanol, air dried and resuspended in 50 μl RNase free water at 55 ^{o}C for 10 minutes. RNA was quantified by measuring absorbance at 260 nm and RNA quality was assessed by agarose/formaldehyde gel electrophoresis.

2.4 Plasmid Constructs

Plasmids used are summarised in table 2.2. The following plasmids were produced in addition to those in table 2.2. Plasmids produced were verified by restriction mapping and commercial sequencing (MWG Biotech, Germany).

2.4.1 pGex-2TK-BAG-1L$^{1-71}$

The plasmid pGex-2TK-BAG-1L$^{1-71}$ was used to produce a recombinant fusion protein of GST and the unique N terminus (amino acids 1-71) of human BAG-1L. This was used for immunisation to produce a BAG-1L specific polyclonal antibody. An insert produced by PCR amplification of plasmid pcDNA-BAG-1L using primers 5' and 3' GST BAG-1L$^{1-71}$ (Table 2.1) was cloned into PCRII-TOPO. The resultant PCRII-TOPO-BAG-1L$^{1-71}$ plasmid was digested with *Eco*RI and *Bam*HI and the fragment cloned into the *Eco*RI and *Bam*HI sites of pGex-2TK. The sequence of the cloned product was confirmed using pGex sequencing primers (Table 2.1).

Plasmid	Source/ kind gifts from	Reference	Notes
pcDNA3-BAG-1S	Dr G Packham	Townsend et al. 2003a	Optimised for expression of Hu BAG-1S
pcDNA3-BAG-1M	Dr G Packham	Townsend et al. 2003a	Optimised for expression of Hu BAG-1M
pcDNA3-BAG-1L	Dr G Packham	Townsend et al. 2003a	Optimised for expression of Hu BAG-1L
pcDNA3-BAG-1S$^{1-155}$	Dr G Packham	Townsend et al. 2003a	Hu BAG-1S with the BAG domain (amino acids 156-230) deleted[a]
pcDNA3-BAG-1S$^{89-230}$	Dr G Packham	Townsend et al. 2003a	Hu BAG-1S with the Ubiquitin like domain (amino acids 1-88) deleted[a]
pcDNA3-BAG-1S$^{1-130}$	Dr G Packham	Townsend et al. 2003a	Hu BAG-1S with the C terminal 100 amino acids (amino acids 131-230) deleted
pcDNA3-BAG-1S^{K80A}	Dr G Packham	Townsend et al. 2003a	Hu BAG-1S point mutant: Lysine 80 within the ULD replaced by Alanine
pGex-2TK-BAG-1S	Dr G Packham	Brimmell et al. 1999	Produces a recombinant GST-BAG-1S fusion protein in bacteria
pcDNA3-BAG-1-RAP46	Dr U Gehring Universitat Heidelberg, Germany	Zeiner et al. 1995	Expresses BAG-1S and BAG-1M together through natural start sites
pcDNA3-mtserBAG-1L	Dr A Cato, Forschungszentrum Karlsruhe, Germany	Schneikert et al. 2000	Hu BAG-1L mutant: All 9 Serines within the acidic repeats replaced by Alanines
ERE3-TKluc	Dr S. Ali, Imperial College, London	Catherino and Jordan 1995	Luciferase reporter: Three copies of vitellogenin consensus estrogen response element cloned upstream of the minimal HSV-1 TK promoter
pSG5-HEGO	Dr S. Ali	Levenson and Jordan 1997	Wild type Hu ERα expression plasmid
pSG5-HE457	Dr S. Ali	Chen et al. 2002	Hu ERα point mutant: serine 118 changed to alanine
pSG5-HE458	Dr S. Ali	Chen et al. 2002	Hu ERα point mutant: serine 118 changed to glutamic acid
pSG5-ERβ1	Dr S. Ali	Pace et al. 1997	Hu ERβ tagged with the FLAG epitope
Bax-luc	Dr X Lu, Ludwig Institute, London	Friedlander et al. 1996	The p53 binding site of the Hu Bax promoter cloned into pGL3-luc
Mdm2-luc	Dr X Lu	Friedlander et al. 1996	The p53 binding site of the Hu mdm-2 promoter cloned into pGL3-luc
cmv bam neo SN-3	Dr X Lu	Baker et al. 1990	Hu p53 expression plasmid
CMV-β-gal	Invitrogen	-	β-galactosidase reporter plasmid

Table 2.2: Plasmids and proteins encoded

[a] Proteins encoded by these constructs are represented diagrammatically (Figure 3.1)

Hu: Human, DS: Data sheet available from supplier

2.4.2 pcDNA3- BAG-1S ^{NLS}

The plasmid pcDNA3-BAG-1S^{NLS} encodes the sequence for a protein consisting the nuclear localisation signal (NLS) from the simian virus 40 T-antigen (PKKKRKV) fused to the amino-terminus of BAG-1S. The plasmid was made by PCR amplification of the plasmid pcDNA3-BAG-1S with primers indicated (Table 2.1) to produce an insert that was cloned into the *Hind*III and *Xho*I sites of pcDNA3.

2.4.3 BAG-1 domain point mutants

The plasmid pcDNA3-BAG-1L^{C330A} is a point mutant of BAG-1L. The equivalent mutation, Cysteine to Alanine at amino acid 204 within the BAG domain in mouse BAG-1S prevents binding of HSC70 (Song et al. 2001). This mutant was produced by "quikchange" mutagenesis using primers indicated in table 2.3.

Additional mutations were also made by "quikchange" mutagenesis within the BAG domain of both BAG-1S and BAG-1L using primers indicated in Table 2.3. BAG-1 proteins were produced that do not bind HSC70 or HSP70 (pcDNA3-BAG-1S^{Q169A,K172A}, pcDNA3-BAG-1L^{Q227A,K231A}, pcDNA3-BAG-1S^{Q201A}, pcDNA3-BAG-1L^{Q316A}, pcDNA3-BAG-1S^{D208A,Q212A} and pcDNA3-BAG-1L^{D323A,Q327A}) (Briknarova et al. 2001). Mutations were also made in BAG-1S and BAG-1 L by "quikchange" mutagenesis within helix one of the BAG domain that is not involved in binding to HSC70 or HSP70 (pcDNA3-BAG-1S^{E112A,K116A} and pcDNA3-BAG-1L^{E227A,K231A}).

2.5 Antibodies for Western Blotting

The primary antibodies used for Western blotting are listed in table 2.4.

2.6 Protein production and binding assays

2.6.1 Small scale recombinant protein production and purification

Small-scale protein preparations were made to test inducibility and protein production. BL21 cells transformed with the appropriate pGex-2TK construct were cultured overnight at 37°C in 200 µl of LB media supplemented with ampicillin at

Plasmid	Primer Name	Primer Sequence (5' to 3') and Characteristics
pcDNA3-BAG-1L^{C330A}	C215A(F)	GTGGAGCAGAACATC GCC CAGGAGACTGAGCGG C → A
	C215A(R)	CCGCTCAGTCTCCTG GGC GATGTTCTGCTCCAC C → A
pcDNA3-BAG-1S^{E112A,K116A} and pcDNA3-BAG-1L^{E227A,K231A}	H1(F)	GAACAGTCCACAGGAAGCGGTTGAACTAGCGAAGTTGAAACATTTGG E → A K → A
	H1(R)	CCAAATGTTTCAACTTCGCTAGTTCAACCGCTTCCTGTGGACTGTTC E → A K → A
pcDNA3-BAG-1S^{Q169A,K172A} and pcDNA3-BAG-1L^{Q284A,K287A}	H2(F)	GGAGAGTAAAAGCCACAATAGCGCAGTTTATGGCGATCTTGGAGGAG Q → A K → A
	H2(R)	CTCCTCCAAGATCGCCATAAACTGCGCTATTGTGGCTTTTACTCTCC Q → A K → A
pcDNA3-BAG-1S^{Q201A} and pcDNA3-BAG-1L^{Q316A}	H3A(F)	GGCTTGGTAAAAAAGGTTGCGGCATTCCTAGCCGAGTG Q → A
	H3A(R)	CACTCGGCTAGGAATGCCGCAACCTTTTTTACCAAGCC Q → A
pcDNA3-BAG-1S^{D208A,Q212A} and pcDNA3-BAG-1L^{D323A,Q327A}	H3B(F)	GCCGAGTGTGCCACAGTGGAGGCGAACATCTGCCAG D → A Q → A
	H3B(R)	CCTGGCAGATGTTCGCCTCCACTGTGGCACACTCGG D → A Q → A

Table 2.3: Primers used for production of BAG domain point mutants

The underlined codons are those containing the mutations. The amino-acid substitutions made are indicated below the sequences.

Antibody to	Name/Clone	Supplier/ kind gift from	Reference	Type	Antigen (human unless specified)	Concentration/Dilution
Human BAG-1	191 TB2	Dr G Packham	Brimmell et al. 2002	Ra Po	GST-BAG-1S	Serum at 1/1000 dilution
Human BAG-1	3.10 G3E2	Dr G Packham	Brimmell et al. 2002	Mo Mo	GST-BAG-1S	Hybridoma Supernatant
Mouse BAG-1	m10	Dr G Packham	Packham et al.1997	Ra Po	GST-mouseBAG-1S	Serum at 1/1000 dilution
p53	DO1	Santa-Cruz, Autogen Bioclear UK Ltd	Bonsing et al. 1997	Mo Mo	Recombinant wild type p53	0.2 μg/ml
p21	SX118	Dr Xin-Lu, Ludwig Institute, UK	Fredersdorf et al. 1996	Mo Mo	GST-p21	Ascites at 1/2000 dilution
PCNA	PC10	Dr Xin-Lu	Hall et al. 1990	Mo Mo	Rat PCNA	Ascites at 1/1000 dilution
Ubiquitin	FK2	Affiniti Bioreagents, UK	-	Mo Mo	Polyubiquitinylated lysosome	10 μg/ml
HSC70	B6	Santa-Cruz	-	Mo Mo	HSC70 carboxy-terminus peptide	0.2 μg/ml
HSP70	C92F3A5	Stressgen, Bioquote Ltd, UK	-	Mo Mo	Recombinant HSP70	1 μg/ml
Oestrogen Receptor	6F11	Novacastra, Vector Laboratories Ltd, UK	Bevitt et al. 1997	Mo Mo	Recombinant ER alpha	Reconstituted hybridoma supernatant at 1/100 dilution
p27	SX53G8	Dr Xin-Lu	Fredersdorf et al. 1997	Mo Mo	GST-p27	Ascites at 1/1000 dilution
Bax	N20	Santa-Cruz	-	Ra Po	Peptide mapping to Bax C-terminus	0.5 μg/ml
DCC	A20	Santa-Cruz	-	Go Po	Peptide mapping to DCC C-terminus	1 μg/ml
β-catenin	C18	Santa-Cruz	-	Go Po	Peptide mapping to β-catenin C-terminus	1 μg/ml
FLAG tag	M2	Sigma,UK	Brizzard et al. 1994	Mo Mo	Flag epitope: DYKDDDDK	4 μg/ml

Table 2.4: Primary antibodies used for Western blotting

Ra Po = Rabbit Polyclonal, Mo Mo = Mouse Monoclonal, Go Po = Goat Polyclonal

100 μg/ml. The following day 2 ml of LB media supplemented with 100 μg/ml ampicillin was added, and this was cultured for a further 2 hours. isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.3 mM to induce recombinant protein production and the bacteria allowed to grow for a further 2-3 hours. Cells were then collected by centrifugation in a microfuge at 6000 rpm, resuspended in 100 μl of PBS containing 10 mM DTT and disrupted by sonication at 23 kHz and approximately 100 W for 10 seconds on ice (MSE Soniprep 150). 7 μl of 20% (v/v) Triton X100 was added and samples centrifuged at 13000 rpm for 10 minutes to remove insoluble debris and the supernatant collected. 30 μl of washed glutathione sepharose bead (Amersham Pharmacia Biotech, UK) slurry was then added and allowed to bind for 30 minutes. The beads were then collected by centrifugation at 1000 rpm for 1 minute and washed twice with 250 μl of PBS prior to boiling in 50 μl of 1x protein sample buffer and analysis by SDS PAGE.

2.6.2 Large scale recombinant protein production and purification

Large-scale preparations of fusion protein were required for polyclonal antibody production, binding studies and serological studies. BL21 cells transformed with the appropriate pGex-2TK construct were cultured overnight at 37°C in 50 ml of LB media supplemented with ampicillin. 500 ml of LB media supplemented with ampicillin was added the following day and cultured for a further 1-2 hours. IPTG was added to a final concentration of 0.3mM to induce recombinant protein production and the bacteria allowed to grow for a further 1-3 hours. Cells were then collected by centrifugation at 6000 rpm for 10 minutes (Sorvall RC5C centrifuge, F-16/250 rotor), resuspended in 50 ml of ice cold PBS resuspension solution supplemented with 10 mM DTT, 50 mM EDTA, 1 mM Phenylmethylsulfonyl fluoride (PMSF) and bacterial protease inhibitor cocktail (Sigma, UK). The suspension was then sonicated for approximately 5 minutes (23 kHz, approximately 200 W) on ice. 710 μl of 20% (v/v) Triton X100 was added and samples centrifuged at 13000 rpm for 10 minutes (Sorvall RC5C centrifuge, SS34 rotor) to remove insoluble debris and the supernatant collected. 500 μl of washed glutathione sepharose bead slurry was then added and left rotating to bind for 1 hour at room

temperature. The beads were then collected by centrifugation at 1000 rpm for 1 minute and washed six times for 10 minutes with 10 ml of PBS resuspension solution prior to elution. Recombinant protein was then eluted in fresh 50 mM Tris HCl containing 50 mM reduced Glutathione solution made up to pH 8 with 4 M sodium hydroxide. Three elutions were performed by addition of 1 ml of this solution and rolling on a spiramix (Spiramix 10, Thermo Life Sciences, UK) for 10 minutes at room temperature. This was followed by an overnight elution at 4 °C. Eluted fractions were then assayed for protein concentration using protein assay reagent (Bio-Rad). A sample was also subjected to SDS-PAGE and Coomassie blue staining to ensure production of the correct protein and assess degree of degradation and purity. Protein was concentrated and purified using Amicon YM3 spin columns (Millipore, UK) according to the manufacturer's instructions. Protein was then divided into aliquots and stored at –80 °C.

2.6.3 In-vitro translation

T7 TNT Quick rabbit reticulocyte lysate (Promega) was used to produce in-vitro translated proteins. This system contains the RNA polymerase, nucleotides, salts, and ribonuclease inhibitor within the reticulocyte lysate mastermix. For ^{35}S labelled in-vitro translations 5 µl translabel (70% methionine, 30% cysteine, 1000 Ci/mmol, 14.3 mCi/ml; ICN, UK) was added to 80 µl of mastermix, which was then divided into 8 µl aliquots. Alternitively for "cold" in-vitro translations 5 µl of 1 mM Methionine was added to 200 µl of the mastermix, which was then divided into 8 µl aliquots. For typical reactions, 1 µl of DNA plasmid template (0.4 µg) was added to 8 µl of mastermix. The reaction mix was incubated at 30°C for 90 minutes and the in-vitro translation product analysed as required.

2.6.4 Co-Immunoprecipitation

Cells were typically plated at 1 x 10^6 per 90 mm dish or at 5 x 10^6 per 150 mm dish, and transfected if required. The following day cells were harvested. Cells were resuspended in HMKEN buffer by trituration through a 21 g needle, lysed on ice for 30 minutes and clarified by centrifugation (12000 rpm in a microfuge for 10 minutes).

The remaining sample was precleared using 25 µl bead volume of protein G sepharose beads (Amersham Pharmacia Biotech, UK) for 30 minutes at 4 °C. Protein G sepharose beads were removed by centrifugation. 10% of the lysate was retained as a whole cell lysate. The remaining lysate was divided and incubated with the BAG-1-specific rabbit polyclonal antibody, 191 TB2 (5 µl per 900 µl lysate) or with pre-immune control serum (5 µl) at 4 °C for 16 hours. The lysate was incubated with 20 µl bead volume protein G sepharose beads for 1 hour and immunocomplexes collected by centrifugation. The beads were washed five times using HMKEN, resuspended in SDS-PAGE sample buffer and heated at 95 °C for 5 minutes prior to Western blotting. To investigate the effect of DNAse on interactions, 5 µl of RQ1 DNAse (Promega) was added and the lysates were incubated at 37 °C for one hour prior to addition of 191 TB2 or pre-immune serum.

2.6.5 GST pulldown assay

Cell lysate was produced as for co-immunoprecipitation assays except pre-clearing was with glutathione sepharose beads. GST-BAG-1S or GST (generally 1 µg), was added to cell lysate or to rabbit reticulocyte lysate containing in-vitro translated proteins (generally 5 µl for each 1 µg of GST-BAG-1S) and incubated for 1 hour at 4 °C. 20 µl bead volume glutathione sepharose was added and samples incubated for a further hour at 4 °C. Complexes were recovered by centrifugation and beads washed five times using HMKEN, resuspended in SDS-PAGE sample buffer and heated at 95 °C for 5 minutes prior to Western blotting.

For peptide competition assays peptides with sequences corresponding to part of helix 3 of the BAG-1 BAG domain (LKRKGLVKKVQAFLAECDTVE) and part of the Bcl-2 protein (RDPVARTSPLQTPAA) were used. These peptides were incubated at concentrations of 10 µM, 1 µM and 100 nM with rabbit reticulocyte lysate for 30 minutes at room temperature prior to addition of GST or GST-BAG-1S.

2.6.6 Far-western blotting

Cell lysates (40 μg) were separated by SDS-PAGE, transferred onto nitrocellulose membrane and blocked with TS containing 2% (w/v) non-fat skimmed milk. The filter was incubated for 16 hours at 4 °C with GST-BAG-1S (2 μg/ml) or GST (2 μg/ml) and an anti-BAG-1 antibody, at concentrations described for Western blotting. Bound complexes were probed with an HRP conjugated anti-rabbit or anti-mouse secondary antibody (Amersham, UK) and detected by autoradiography as for Western blotting.

2.7 Production of BAG-1L specific antibody

2.7.1 Immunisation protocol

Three New Zealand White Rabbits were used for the production of the BAG-1L specific antisera. These were housed at the University of Southampton Biomedical Research Facility. Staff at the University of Southampton Biomedical Research Facility performed immunisations, test and final bleeds and general care of the rabbits. Test bleeds were taken from an ear vein, and subcutaneous immunisations were given at a maximum of 0.25 ml per site with a maximum of 4 sites per immunisation. 200 μg of GST-BAG-$1L^{1-71}$ protein was given per immunisation per rabbit, mixed in a 50% (v/v) emulsion with Freunds complete adjuvant (Sigma) for the initial immunisation and 50% (v/v) Freunds incomplete adjuvant (Sigma) for subsequent immunisations. The emulsion was prepared by trituration of GST-BAG-$1L^{1-71}$ protein solution (200 μg /ml) made up in sterile PBS with an equal volume of adjuvant for approximately 30 minutes on ice through a 21 g needle followed by trituration through a 25 g needle. The immunisations and test bleeds were performed according to the schedule below.

1 August 2000	Prebleed
2 August 2000	First Immunisation (Freunds Complete Adjuvant)
23 August 2000	First Booster (Freunds Incomplete Adjuvant)
30 August 2000	First Test Bleed

12 September 2000	Second Booster
22 September 2000	Second Test Bleed
5 October 2000	Third Booster
16 October 2000	Third Test Bleed
26 October 2000	Fourth Booster
6 November 2000	Fourth Test Bleed
17 November 2000	Fifth Booster
27 November 2000	Final Bleed

2.7.2 Preparation of sera from blood

Prebleeds and test bleeds (10 ml of blood) were obtained from each rabbit and approximately 100 ml of blood from the final bleed from each rabbit. Blood was allowed to stand overnight at 4 °C for clot to form and retract. The serum was then removed and centrifuged at 2000 g for 10 minutes to remove any remaining clot and debris. The sera were then aliquoted and stored at –20 °C.

2.8 Mammalian Cell Culture and analysis

Griener, UK, supplied all tissue culture plasticware, PAA laboratories, Austria, supplied serum, and Gibco, UK, supplied all other cell culture media and trypsin, unless otherwise stated.

2.8.1 Mammalian Cell Lines

Cell lines used are described in Table 2.5, and were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% (v/v) foetal calf serum, 2 mM L-glutamine, 100 μg/ml penicillin G and 100 μg/ml streptomycin. Cells were cultured in a humidified incubator at 37 °C and 10% (v/v) CO_2. All media was pre-warmed to 37 °C before use. Cells were cryopreserved in 50% (v/v) DMEM, 40% (v/v) foetal calf serum, and 10% (v/v) DMSO. MCF7 clones overexpressing BAG-1 (BAG-1S clones 4, 5, 13 and 19) or containing control pcDNA empty vector clones (pcDNA clones 3, 4, 9 and 11) were from Dr G Packham and Dr P Townsend. Stable

Cell line	Tissue of origin (All human)	Comments
MCF-7	Breast	Express oestrogen receptor alpha (ERα), weakly express oestrogen receptor beta (ER β) and express progesterone receptor (PgR) (Hu et al. 1998; Levenson & Jordan 1997). Contain wild type p53 (Gudas et al. 1995)
ZR-75.1	Breast	Express oestrogen receptor alpha (ERα)
T47D	Breast	Express oestrogen receptor alpha (ERα)
BT-474	Breast	Express oestrogen receptor alpha (ERα)
MDA-MD-231	Breast	Oestrogen receptor negative
SK-Br-3	Breast	Oestrogen receptor negative
CAL-51	Breast	Oestrogen receptor negative (Gioanni et al. 1990)
HEK-293	Embryonal Kidney	Do not contain oestrogen and progesterone receptors. Contain low levels of endogenous BAG-1.
HeLa	Cervix	Contain high levels of BAG-1L. Do not express ERα.

Table 2.5: Cell Lines

All cell lines were obtained from Dr G Packham, and were originally from either the American Type Tissue Collection (ATCC) or the European Collection of Cell Cultures (ECACC) except CAL-51 which were originally from the Dutrillaux laboratory.

transfectants were maintained in media supplemented with Genticin (G418; Promega) 100 μg/ml.

2.8.2 Transfection of Cell lines

Cells were transfected using either the Fugene 6 reagent (Roche, UK) according to the manufacturer's instructions, or by the calcium phosphate technique. 1 x 10^6 cells were typically plated in a 90 mm dish on the day prior to transfection. The media was replaced with fresh media one hour prior to transfection. When using Fugene 6, a total of 1 μg of plasmid DNA was used per 90 mm plate unless otherwise specified. A 3:1 Fugene to DNA ratio was found to be optimal. For calcium phosphate transfection, DNA was added to 200 μl of HBS and gently resuspended. 10 μl of 2.5 M $CaCl_2$ was added, gently mixed, and allowed to precipitate over 20 minutes at room temperature. Following gentle resuspension this was added dropwise to cells. The Fugene/DNA or calcium phospate/DNA complex was removed after 6 hours. Cells were then assayed 24-76 hours following transfection.

2.8.3 Production of BAG-1 overexpressing MCF-7 cell lines

Stable transfectants of BAG-1M and BAG-1L were produced as transfectants of these isoforms had not been previously produced within the laboratory, and further BAG-1S transfectants were made as those available had gradually lost BAG-1S expression. Stable transfectants were therefore produced for each of the BAG-1 isoforms, and for pcDNA3 as empty vector control cell lines. MCF-7 cells were transfected in 90 mm dishes. The media was replaced with media supplemented with G418 at 100 μg/ml and resistant clones allowed to grow out. Cells were maintained in media containing G418 thereafter. When the cells approached confluence they were trypsinised and seeded into three 150 mm dishes at low density. Colonies were allowed to grow from single cells over a period of 3 to 4 weeks. Ten colonies were selected for each construct and trypsinised within cloning rings. The cells were grown in wells of 24 well plates and allowed to grow to confluence, when they were trypsinised and plated into 12 well plates. The procedure was repeated allowing each colony to grow to confluence in wells of 6 well plates and subsequently 90mm dishes.

At this stage stocks of each colony were cryopreserved and clones assayed for BAG-1 expression by Western blotting.

2.9 Long term clonogenic survival assays

MCF7 cells were plated at a density of 2.1 X 10^4 per well of a 24 well plate, with 1 ml of media in each well. Generally duplicate plates were seeded such that one plate was heat shocked and the other plate acted as a control. In addition two wells were plated with MCF7 cells and not transfected to act as a control for G418 selection.

Transfection was as described, with Fugene-6 using 0.5 μg total DNA per well made up to 0.5 μg using pcDNA3 empty vector as 'carrier' DNA if less than 0.5 μg of BAG-1 construct was required. The following day the cells were heat shocked by incubation for 1 hour at 42 °C (Hybaid Micro-4 oven, Hybaid, UK). Following the heat shock the cells were returned to the humidified tissue culture incubator at 37 °C and allowed to recover overnight. The next day the cells were trypsinised and plated into a fresh 24 well plate. Three 1/10 serial dilutions were made from each well such that there was a 1/1, 1/10, 1/100 and 1/1000 dilution of each. Six hours later media was replaced. In all wells except one of the two groups of four wells containing untransfected cells the media added was supplemented with G418. Clones were allowed to grow out and plates re-fed every 4 or 5 days. Clones of a good size normally appeared between 2 and 4 weeks.

To stain and fix the plates media was removed and the plated rinsed with PBS and fixed for 7 minutes with 1ml methanol per well. The methanol was removed and the plates allowed to air-dry for 10 minutes. 0.5 ml of Giemsa (Sigma, UK) stain was then added to each well and left to stain the cells for 3 minutes. The Giemsa was then removed and the plates washed by submersion in distilled water and then allowed to air dry. Colonies were then counted using a colony counter.

2.10 Reporter Assays

2.10.1 Oestrogen dependent transcription

Cells were cultured in phenol red free DMEM supplemented with 10% (v/v) charcoal dextran treated serum (Hyclone, UK) for three days prior to plating in 90 mm dishes at 2 x 10^6 cells per dish. Charcoal dextran treatment removes oestrogens from serum, and phenol red free DMEM was used since phenol red containing DMEM contains a contaminant from the production of phenol red that is a potent oestrogen (MacGregor & Jordan 1998). The following day cells were transfected using the Fugene 6 reagent (Roche). A constant amount of CMV-β-gal (a control reporter plasmid containing the β-galactosidase cDNA under transcriptional control of the CMV IE promoter) was included in each transfection to control for transfection efficiency and non-specific effects on transcription. MCF7 cells were transfected with 3.3 μg of pcDNA3 derived expression plasmid, 1.7 μg of CMV-β-galactosidase expression plasmid and 1.7 μg of luciferase reporter construct. HEK-293 cells were transfected with 3.3 μg of pcDNA3 derived expression plasmid, 0.66 μg of CMV-β-galactosidase expression plasmid, 1.32 μg of luciferase reporter construct and 1.32 μg of oestrogen receptor construct. 16 hours later, transfected cells were trypsinised and plated in 6 well dishes at 2 x 10^5 cells per well. Five hours later the media was replaced with media containing 17-β oestradiol at the appropriate concentration. The following day cells were harvested and analysed for β-galactosidase and luciferase activity. Luciferase activity was measured using LucLite plus reagent (Packard, UK) and a Topcount (Packard, UK) according to the manufacturer's instructions. β-galactosidase activity was measured using ONPG reagent and absorbance read at 405 nm on a plate reader (Dynatech MR-500).

2.10.2 p53 Dependent transcription

Cells were plated in 6 well plates at 2 x 10^5 cells per well on the day prior to transfection. If the calcium phosphate transfection technique was used for transfection a total of 10.6 μg of DNA was used per well comprising 2 μg of reporter plasmid, 0.5

μg of CMV-β-gal, 0.1 μg of CMV-SN3 (a p53 expression plasmid) or pcDNA3, and pcDNA3-BAG-1 or empty pcDNA3 (8 μg). If Fugene 6 reagent was used for transfection the ratio of plasmids was maintained and a total of 2 μg of plasmid per well was transfected. Cells were assayed for β-galactosidase and luciferase activity 48 hours after transfection as described above.

2.11 Fluorescence Microscopy

Cells were plated on coverslips in a 6 well plate and allowed to attach overnight. If required, cells were transfected the following day. Cells were washed twice between each of the following steps with PBS. Cells were fixed for 20 minutes at room temperature in 4 % (w/v) paraformaldehyde, 3 % (w/v) sucrose in PBS and blocked at 4 °C in PBS containing 10% (v/v) new calf serum (NCS) and 0.05% (w/v) sodium azide for 20 minutes to prevent non-specific antibody binding. Cells were then stored at 4 °C in this buffer until ready for staining when they were permeablised with PBS containing 1% (v/v) Triton X-100 for 20 minutes. Cells were labelled for 1 hour at room temperature with primary antibody diluted in PBS containing 10% (v/v) NCS with 1% (v/v) Triton X-100. 191 TB2 antisera and 3.10 G3E2 ascites were used at 1:1000 dilution and purified BAG-1L specific antibody 662 was used at 40 μg/ml. After washing, fluorescein isothiocyanate (FITC)-labelled secondary antibody (DAKO) was added at a 1:500 dilution and incubated for 1 hour. Finally, the cells were incubated in 300 nM DAPI (Molecular Probes) for 10 minutes prior to mounting (DAKO Fluorescent Mounting Media) and visualisation by fluorescence microscopy with a Zeiss Axiovert 200 microscope. Images were captured using Openlab 3.0.8 software (Improvision, UK).

2.12 Immunohistochemistry and tissue labelling assessment

2.12.1 Immunohistochemistry

Initial BAG-1, ERα and PgR immunohistochemistry was performed with Anna Maison (4th year medical student, Southampton University) as part of a medical student project supervised by the author and Dr Adrian Bateman (Department of

Pathology, Southampton General Hospital). Mr Ron Lee (Pathology Department, Southampton University) kindly performed the rest of the Immunohistochemistry. This was performed on 4 micron sections which were dewaxed for 10 minutes in xylene and washed in 100% (v/v) alcohol through to 70% (v/v) for 1 minute in each. Endogenous peroxidase activity was blocked using 3% (v/v) hydrogen peroxide in methanol for 15 minutes. Antigen retrieval was performed using microwave treatment in 0.01 M citrate buffer (pH 6.0) for 25 minutes at medium power for BAG-1 and PgR, or pressure cooker treatment for 2 minutes at full pressure in 0.01 M citrate buffer for ERα. BAG-1 specific antibodies were KS6-C8 (Dako, UK) used at 1/100 dilution and purified 3.10 G3E2 used at 1 μg/ml. ERα and PgR were detected using 1D5 (Dako, UK) and PgR636 (Dako, UK) antibodies both at 1/200 dilution. Sections were incubated in primary antibodies diluted in tris buffered saline (TS) overnight at 4 °C. Sections were warmed to room temperature, washed in TS and incubated with biotinylated goat anti-mouse immunoglobulins (Biogenex, USA) at 1/50 dilution for 30 minutes. The sections were washed in TS and incubated with HRP-conjugated streptavidin (Biogenex, USA) at 1/50 dilution for 30 minutes. Sections were then washed in TS and incubated with diaminobenzidine substrate (Biogenex, USA) for 10 minutes, following which they were washed in tap water and counter stained in Harris's haematoxylin before being differentiated in acid alcohol, blued in tap water, dehydrated and mounted with cover slips. Negative controls lacking primary antibody and batch controls using sections known to give intermediate staining were included on each staining run.

2.12.2 Assessment of immunohistochemical labelling

Assessment of immunohistochemical labelling was performed with Dr Adrian Bateman. An intensity-proportion score (H-score) was used to analyse immunostaining (Kinsel et al. 1989). The labelling intensity of each section was allocated a value of 0 (no labelling), 1 (weak), 2 (intermediate) or 3 (strong) and was multiplied by the percentage of positive cells (nuclear labelling for oestrogen receptor alpha (ERα) and progesterone receptor (PgR); nuclear and cytoplasmic labelling assessed separately for BAG-1) to give a maximum possible score of 300. For

analysis of ERα and PgR an H-score of ≥75 was considered positive since this value has been previously used in the clinical assessment of ERα status. An H-score of > 100 was considered positive for nuclear or cytoplasmic BAG-1. Where immunostaining was patchy the area of most intense labelling was assessed. Benign breast epithelium was used in each section as a positive internal control for all of the antibodies. Sections where the tumour appeared negative but there was also no labelling of the benign tissue were restained.

2.13 Enzyme-Linked Immunosorbent Assay (ELISA)

To test for BAG-1 immunoreactivity in patient sera and monclonal antibody hybridoma supernatant, wells of 96 well plates were coated with 20 ng of GST-BAG-1S or GST in 100 µl PBS supplemented with sodium azide 0.05% (w/v) at 4 °C overnight. The wells were washed three times with 100 µl of PBS with 0.05% Tween 20 (v/v) to remove unbound protein. Non-specific protein binding sites were blocked with 0.1% (w/v) BSA in PBS (0.1% BSA-PBS) for 2 hours at room temperature. 100 µl of antibody at the appropriate dilution, in 0.1% BSA-PBS, was added to wells and allowed to bind overnight at 4 °C. Wells were washed as before and the appropriate HRP conjugated secondary antibody added in 100µl volume in 0.1% BSA-PBS and allowed to bind for 2 hours at room temperature. Wells were washed as before and 100 µl of OPD substrate (Sigma, UK) added. The absorbance was measured on a plate reader at 450 nm. BAG-1 specific immunoreactivity was obtained by subtracting absorbance obtained with the GST signal from absorbance obtained with GST-BAG-1S. Comparisons were made by normalising to common controls on each plate.

For epitope analysis of monoclonal antibodies, hybridoma supernatant or purified antibody was screened by ELISA against a panel of peptides. A series of 28 peptides 20 amino acids in length and staggered by four amino acids scanning the BAG domain of BAG-1 was used. 100 µl of 100 µM peptide was allowed to bind overnight at 4 °C to wells of a 96 well plate. Wells were then washed and blocked as described above and 200 µl of hybridoma supernatant or antibody at a concentration

of 1 μg/μl added. This was allowed to bind overnight at 4 °C and the wells were then washed and bound antibody detected using an HRP-conjugated anti-mouse antibody and OPD substrate as described above.

2.14 Microarray analysis

MCF-7 clonal cell lines pcDNA^{A} and BAG-1S^{D} were plated in 90 mm dishes at 1 x 10^6 cells per dish. The following day cells were heat shocked by replacing the media in each dish with 20 ml of media pre-warmed to 44 °C followed by incubation at 44 °C for one hour. Cells were then returned to 37 °C. For controls media was replaced with 20 ml of media pre-warmed to 37 °C and culture continued at 37 °C. Previous experiments had demonstrated that following heat shock with these conditions pcDNA clones undergo apoptosis whilst BAG-1S clones do not. Twelve hours after heat shock, cells were harvested, snap frozen in liquid nitrogen and stored at –80 °C.

ResGen (Invitrogen) breast specific gene filters (GF225), and the associated probe labelling and purification kit (Invitrogen; GF kit 2) were used. Each array is a 5 x 7 cm nylon filter containing 5184 cDNAs and expressed sequence tags known to be expressed in breast tissue and total genomic DNA controls. Each spot contains 0.5 ng of DNA. Each filter was pretreated by boiling in 0.5 % (w/v) SDS for 10 minutes and prehybridised in a hybridisation oven (Hybaid Micro-4) for 3 hours at 42 °C in 7.5 ml MicroHyb buffer with 7.5 μg Poly dA and 7.5 μg Cot-1 DNA as blocking agents. Radiolabelled cDNA probes were generated and hybridisations performed using supplied reagents and exactly as per the manufacturer's recommendations. Briefly the cDNA probe was produced by reverse transcription of RNA in the presence of ^{33}P dCTP (ICN, UK; 3000 Ci/mM, 10 mCi/ml), and purified by binding, washing and elution from a spin column. The probe was boiled for 2 minutes, chilled on ice for 2 minutes and was then added to the MicroHyb blocking buffer with the pretreated filters. Following hybridisation for 18 hours at 42 °C unbound probe was removed by washing twice with 2 x SSC containing 1% (w/v) SDS and once at 55 °C with 0.5 x SSC containing 1% (w/v) SDS. The filters were then imaged using a "Fluor-S-

MultiImager" (Bio-Rad, UK) and images captured using "Quantity One" software (Bio-Rad, UK).

The images were imported into and analysed with Pathways 4 software (Invitrogen). The alignment of each individual spot was checked manually and corrected where necessary. Spots obscured by "blooming" from adjacent spots were invalidated. Pairwise comparisons were made with control (unheatshocked) pcDNA clonal cell line. Intensities were normalised between filters by comparison of the average intensity of all the spots on each filter, and the Chen test was used to identify spots that were differentially regulated at a 90 % confidence limit.

2.15 Statistical Analysis

All statistical analysis was performed using SPSS for windows version 10 (SPSS inc., Chicago). Unless otherwise indicated data from duplicates within experiments is expressed as mean $\pm$ the standard error of the mean. Paired sample and independent sample T tests are used to assess the significance of differences in means.

For immunohistochemical studies Pearson's chi-squared test and chi-squared test for trend were used to assess associations between BAG-1 status and tumour grade, lymph node status and tumour size. Pearson's correlation coefficient was used to assess correlation between H-scores. Kaplan-Meier plots were used to demonstrate survival. The effect on survival of various prognostic factors including BAG-1 status was assessed using Cox proportional hazards regression. Single and multiple variable analysis of nuclear and cytoplasmic BAG-1 was performed using a cut off H score value of 100. The Wald test was used to determine the statistical significance of survival analysis exploratory variables.

2.16 Sequence Analysis

The human BAG-1 sequence (GenBank accession number: NM_004323) was downloaded from the NCBI website (www.ncbi.nlm.nih.gov). Sequence analysis was performed using Mac-Vector software (Version 6.5, Accelrys, UK).

3. BAG-1 expression and serological responses in breast cancer

3.1 Introduction

BAG-1 expression is frequently altered in breast cancer and patterns of BAG-1 expression may predict clinical outcome (Table 1.3). The published literature on the clinical significance of BAG-1 expression is conflicting and this may in part be due to the use of different techniques, reagents or cohorts (Cutress et al. 2001) therefore a detailed analysis by immunohistochemistry of BAG-1 expression in breast cancer was performed. The specific aims were firstly to characterise monoclonal antibodies to BAG-1 available within the laboratory and to produce BAG-1L specific antisera. Secondly to perform a detailed immunohistochemical analysis of BAG-1 expression and isoforms in breast cancer. The final aim was to determine if, similar to heat shock proteins, breast cancer patients develop immunological responses to BAG-1.

Ten monoclonal antibody hybridomas to BAG-1 were available within Dr Packham's laboratory. These had been produced using GST-BAG-1S as the immunogen, and therefore these antibodies detect all three BAG-1 isoforms. 3.10 G3E2 has been used extensively within the laboratory, generally as hybridoma supernatant, for Western blotting (Brimmell et al. 1999; Packham et al. 1997). The other nine monoclonal antibodies had only previously been characterised to a limited degree, and neither 3.10 G3E2 nor any of the other antibodies had been extensively characterised nor optimised for immunohistochemistry. In addition it was not clear how many of these antibodies represented distinct clones. Since these antibodies represented a potentially valuable research resource their continued characterisation was of importance. For example any differences in epitope location or characteristics, that these antibodies could distinguish such as post-translational modification, might represent useful tools for further studies on BAG-1. In addition it was very important that a reliable "in house" antibody was optimised for immunohistochemical studies of BAG-1 expression in archival formalin fixed paraffin embedded material. The

principle advantage of an antibody that works reliably on archival formalin fixed paraffin embedded material is that it enables potential utilisation of valuable pathology department archives. Staining characteristics can thus be linked to clinico-pathological features and importantly to clinical outcome and follow up.

At the start of the study a detailed analysis of the expression of individual BAG-1 isoforms in human breast cancer had not been previously performed and so a cohort of breast cancer patients was characterised for immmunohistochemical study. Since BAG-1 proteins are differentially localised between the nucleus and cytoplasm, and are likely to possess distinct functions, the expression profile of individual BAG-1 isoforms may be expected to have additional clinical and biological significance. Previous immunohistochemical studies have used pan-BAG-1 antibodies such as the monoclonal antibodies mentioned that recognise epitopes common to all three BAG-1 isoforms, and it is not clear if nuclear localisation of BAG-1 reflects expression of the BAG-1L isoform, and cytoplasmic localisation reflects expression of the BAG-1S and BAG-1M isoforms.

It is possible that whilst nuclear labelling of BAG-1 by immunohistochemistry with a pan-BAG-1 antibody might represent expression of BAG-1L as generally assumed, it could alternatively represent relocalisation of other isoforms to the nucleus within an abnormal tumour microenvironment. It is for example known that the BAG-1S and BAG-1M isoforms relocalise to the nucleus under conditions of stress such as heat shock (Townsend et al. 2003a; Zeiner et al. 1999). Differentiating between these possibilities is important since there are many examples of BAG-1 isoform specific function, in particular that of modulation of ER dependent transcription (Chapter 4). It was anticipated that a BAG-1L-specific antibody would help to address these issues as a comparison of BAG-1L-specific immunostaining with pan-Bag-1 immunostaining might be expected to demonstrate the relative expression and localisation of BAG-1L compared to BAG-1S.

It is impossible to generate a BAG-1S-specific antibody since the BAG-1S isoform sequences are contained wholly within the larger isoforms (Figure 1.1). Relative to BAG-1S, the larger isoforms have additional amino-terminal sequences and so production of a BAG-1L specific antibody is possible if epitopes are

recognised within the unique amino-terminus of BAG-1L not present within the other two isoforms. Since no isoform specific antibody previously existed BAG-1L specific antisera were produced by immunisation of rabbits with a protein containing the unique amino-terminal sequences of BAG-1L.

Heat shock proteins are one of the most highly conserved groups of proteins across species, but despite this immune responses to various heat shock proteins have been reported in a wide range of diseases. BAG-1 binds to and regulates the function of heat shock proteins (Hohfeld 1998; Takayama et al. 1997; Zeiner et al. 1997), but it is not known if auto-antibodies are formed against BAG-1. Autoantibodies have been reported to HSP70 in approximately one third of healthy controls and one third of women with breast cancer (Conroy et al. 1998a). Autoantibodies to HSP27 are also found in approximately one third of women with breast cancer but not in normal controls. In addition the presence of these autoantibodies correlates with increased overall survival (Conroy et al. 1998a). In contrast autoantibodies to HSP90 in breast cancer patients, which are not found in normal controls, correlate with decreased overall survival from breast cancer (Conroy et al. 1998b).

HSP70 has been implicated in antigen presentation, including presentation of mutant p53 (Wells & Malkovsky 2000). Ten to twenty-five percent of breast cancer patients develop antibodies to p53 (Crawford et al. 1982; Lubin et al. 1995; Soussi 2000). In breast cancer tissue HSP70 co-localises with mutant p53 (Iwaya et al. 1995), and some of the mutant p53 forms complexes with HSP70. Patients in whom mutant p53 complexes with HSP70, as demonstrated by immunoprecipitation, have anti-p53 antibodies in their sera, and conversely those with mutant p53 who do not form p53 HSP70 complexes, do not have anti-p53 antibodies in their sera (Davidoff et al. 1992). Since BAG-1 binds to, and regulates HSP70, it is possible that BAG-1 either modulates antigen presentation by heat shock proteins, or perhaps similar to p53, is presented by HSP70 as an antigen in disease states such as breast cancer. The sera of patients with breast cancer, and of healthy controls, was therefore investigated for the possible presence of autoantibodies to BAG-1. If present, such autoantibodies may be of prognostic significance, and may suggest novel functions of BAG-1 in tumour immunity and immunogenicity.

3.2 Characterisation of anti-BAG-1 Monoclonal Antibodies

Ten pan-BAG-1 monoclonal antibodies available within the laboratory were characterised. The ultimate aim was to develop and optimise one of these antibodies for use on archival formalin fixed paraffin embedded material. BAG-1S deletion mutants were initially characterised and then used to characterise the reactivity of the antibodies.

3.2.1 Characterisation of expression of BAG-1S mutants

Two BAG-1S deletion mutants BAG-1S$^{1-155}$, and BAG-1S$^{89-230}$ were used to localise the epitope of the monoclonal antibodies (Figure 3.1). Expression of the mutants was tested initially by Western blotting using polyclonal anti-BAG-1 antisera 191-TB2 (Brimmell et al. 1999)(Figure 3.2A). This demonstrated that this polyclonal antisera detected these proteins, although immunoreactivity to the deletion mutants was less than that to the full-length proteins. Since 191-TB2 was raised against the whole BAG-1S protein it is likely that antibodies within the antisera are directed to different parts of the protein. To confirm this a comparison of the expression levels of the deletion mutants with full length protein was made by performing ^{35}S labelled in-vitro translations. An example is shown (Figure 3.2B). These experiments demonstrated similar expression levels between deletion mutants and full length protein.

3.2.2 Epitope mapping of the panel of anti-BAG-1 monoclonal antibodies

The deletion mutants BAG-1S$^{1-155}$ and BAG-1S$^{89-230}$ were expressed at approximately equal levels by both in-vitro translation and by transfection into MCF-7 and HEK-293 cells, and were used to map the region of the BAG-1S protein containing the epitopes of each of the monoclonal antibodies by Western blotting. As the middle third of the BAG-1S protein is common to both mutants if the epitope of an antibody was located within this region of BAG-1S then both mutants should be detected by Western blotting with that antibody. Antibodies that recognised epitopes within either the carboxyl-terminal or amino-terminal third of BAG-1S unique to

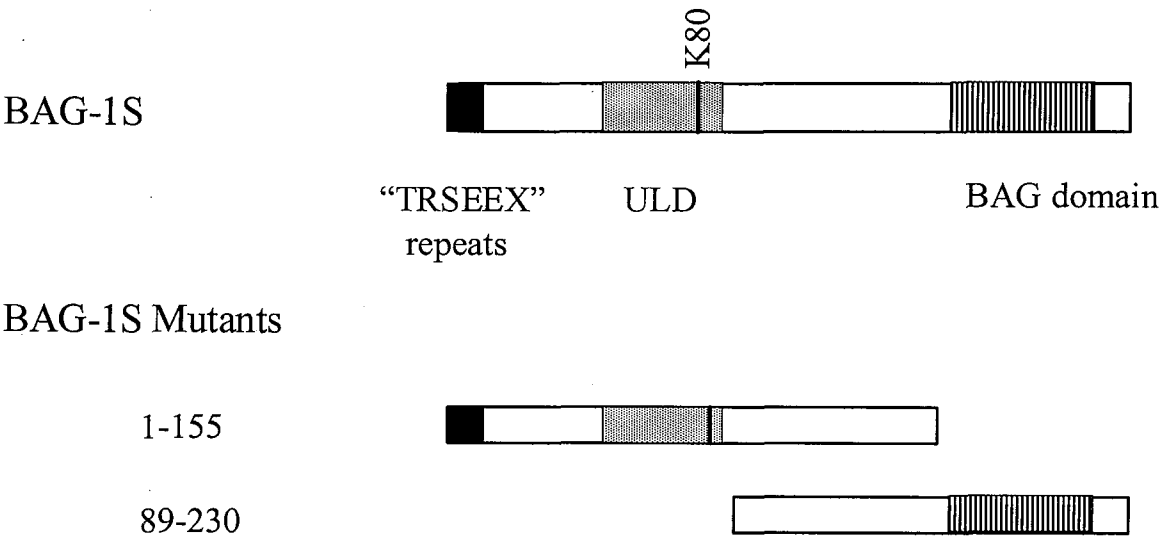


Figure 3.1: BAG-1S and BAG-1S mutants.

The striped region denotes the BAG domain and the dotted region the ubiquitin like domain (ULD). The conserved lysine within the ubiquitin like domain at amino acid 80 in BAG-1S is indicated with a line.

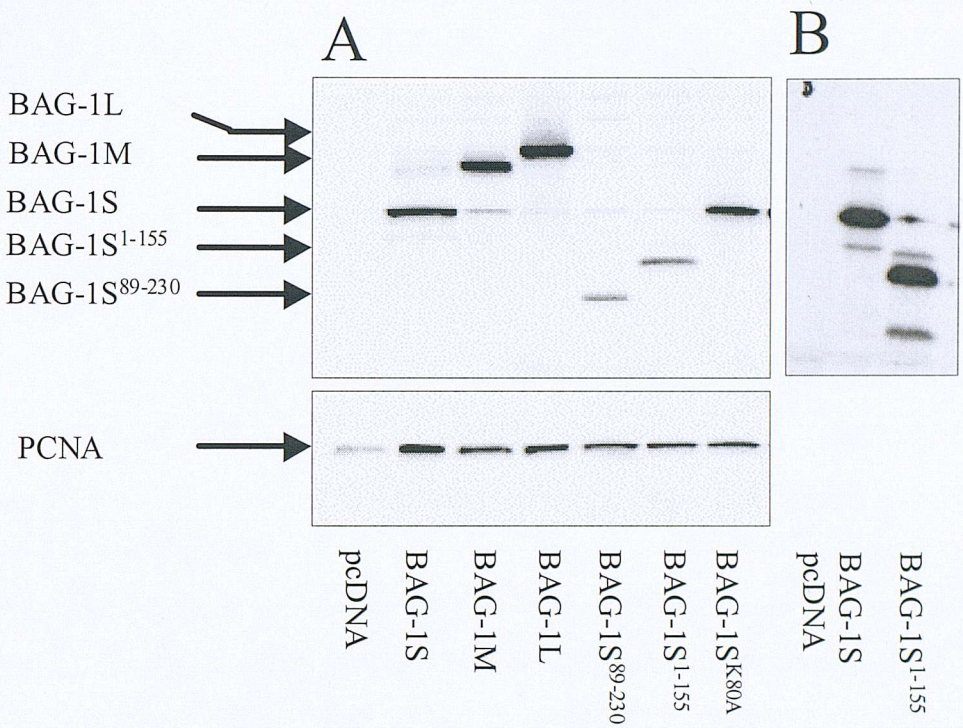


Figure 3.2: Expression of BAG-1S deletion mutants

BAG-1 proteins and deletion mutants were produced by transfection of MCF-7 cells (A) or by ^{35}S methionine labelled in-vitro translation (B). Transfected MCF-7 cell lysate (A) and in-vitro translated proteins (B) were separated by SDS-PAGE. Protein was transferred onto nitrocellulose filters and immunoblotted for BAG-1 with antibody 191-TB2 or as a loading control for PCNA with PC10. Polyacrylaminde gels containing in-vitro translated protein labelled with ^{35}S methionine (B) were dried in a gel drier and analysed on a phosphor-imager.

either the BAG-$1S^{1-155}$ or BAG-$1S^{89-230}$ mutant respectively would recognise only the appropriate mutant. The mutants were produced by in-vitro translation and subjected to SDS-PAGE and transferred onto nitrocellulose filters. In-vitro translations of empty vector (pcDNA3) and full length BAG-1S were also included to act as negative and positive controls. Hybridoma supernatant from each monoclonal antibody was used to probe the filters (Figure 3.3 and summarised in Table 3.1).

No antibody recognised epitopes within the middle third of BAG-1S common to both deletion mutants. Three patterns of reactivity were noted. The epitopes of antibodies 3.10 G3E11 and 3.10 G3F11 are located within the carboxy-terminal third of BAG-1S. The remaining eight of the monoclonal antibodies have epitopes located within the amino-terminal third of BAG-1S. Four of the antibodies detected the BAG-$1S^{89-230}$ in-vitro translated mutant more weakly than they detected the full-length in-vitro translated BAG-1S protein. It is possible that this deletion mutant undergoes differences in post-translational modification compared to the full-length protein. Others have since confirmed the location of the epitope of 3.10 G3E2 by screening a library of overlapping peptides. 3.10 G3E2 was found to recognise the amino acid sequence RSEEVTREEMA, which constitutes the two Serine/Threonine rich repeats closest to the carboxyl-terminus within the BAG-1 isoforms (Petersen et al. 2001).

3.2.3 3.8 D4F4 does not discriminate BAG-1 phosphorylation status

BAG-1 is modified by phosphorylation in vivo at it's amino-terminus (Schneikert et al. 2000; Takayama et al. 1998), and this is the region in which 3.8 D4F4's epitope is located. It was therefore hypothesised that the difference between deletion mutant and full length protein might represent differences in post translational modification of the epitope such as phosphorylation, which this antibody is able to recognise. Two approaches were taken to test this hypothesis.

Lymphocytes from patients with B-CLL contain a second slightly higher molecular weight form of BAG-1S, which reports have suggested might represent a phosphorylated form of this isoform (Kitada et al. 1998). If 3.8 D4F4 preferentially recognises either wild type or phosphorylated forms then one might expect a change in the ratio between this higher molecular weight form of BAG-1S and the usual form

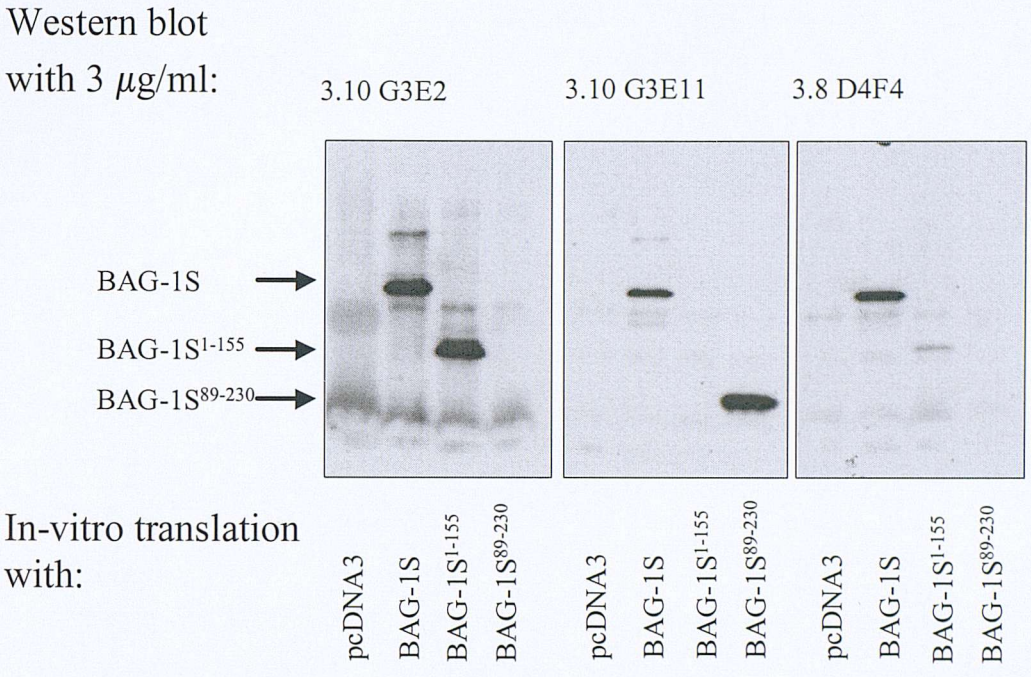


Figure 3.3: Epitope mapping of anti-BAG-1 monoclonal antibodies by Western blotting

In-vitro translated proteins were separated by SDS-PAGE, transferred onto nitrocellulose membrane and probed with monoclonal antibody hybridoma supernatant to locate the approximate part of the BAG-1S protein within which the epitope of each antibody is located. Examples of all three patterns of reactivity found are illustrated. 3.10 G3E2 detects the BAG-1^{1-155} mutant approximately equally to full-length protein, whereas 3.8 D4F4 detects the full-length BAG-1S protein better than it detects the BAG-1^{1-155} deletion mutant. 3.10 G3E11 detects the BAG-$1S^{89-230}$ deletion mutant. No antibody detected both mutants.

Antibody	BAG-1S mutant recognised by Western blotting	Epitope Location (amino acids)	
		1-89	155-230
3.8 D4E9	BAG-1S$^{1-155}$ *	+	-
3.8 D4F4	BAG-1S$^{1-155}$ *	+	-
3.8 D4G4	BAG-1S$^{1-155}$ *	+	-
3.8 D4G5	BAG-1S$^{1-155}$ *	+	-
3.9 F1E11	BAG-1S$^{1-155}$	+	-
3.9 F1F9	BAG-1S$^{1-155}$	+	-
3.9 F1G10	BAG-1S$^{1-155}$	+	-
3.10 G3E2	BAG-1S$^{1-155}$	+	-
3.10 G3E11	BAG-1S$^{89-230}$	-	+
3.10 G3F11	BAG-1S$^{89-230}$	-	+

Table 3.1: Epitope location recognised by anti-BAG-1S monoclonal antibodies. The majority of the monoclonal antibodies have epitopes located within the amino-terminal third of BAG-1S. Four of these antibodies (indicated by an asterisk) demonstrated reduced immunoreactivity to the BAG-1S$^{1-155}$ deletion mutant compared to their immunoreactivity to the full-length in-vitro translated BAG-1S protein. The epitopes of the remaining two antibodies are located within the carboxy-terminal third of BAG-1S.

of BAG-1S when detected by Western blotting with this antibody compared to for example 3.10 G3E2. 3.8 D4F4 however recognised similar ratios of both forms when compared to 3.10 G3E2 (data not shown).

The second approach involved an expression plasmid for BAG-1L, BAG-$1L^{mtser}$. In this plasmid all 9 phospho-acceptor sites have been mutated from serine to alanine, and the resultant protein product is not phophorylated in-vivo (Schneikert et al. 2000). This protein was not recognised at all by antibody 3.8 D4F4 whilst wild type BAG-1L was recognised normally, and by contrast antibody 3.10 G3E2 recognised both BAG-1L and BAG-$1L^{mtser}$ equally (Figure 3.4). This suggests that the epitope for 3.8 D4F4 includes one of the three serines present in the four Serine/Threonine rich repeats contained in BAG-1S, and mutating the serine to alanine destroyed the epitope. This is more likely than the alternative possibility that this clone preferentially recognises phosphorylated protein since the immunogen initially used to produce the hybridomas (GST-BAG-1S) was produced in bacteria, and so was unlikely to have undergone a significant degree of post-translational modification including phosphorylation.

3.2.4 The epitope of 3.10 G3E11 and 3.10 G3F11

The epitope of the antibodies that map to the BAG domain was determined by screening the antibodies by ELISA against a series of overlapping peptides that scanned the BAG domain. Peptides were bound in duplicate to wells of 96 well plates and binding of antibody to peptide assessed. ELISA's were performed twice for each antibody (data not shown). The mean signal from all the peptides was approximately equal to the background signal. Peptide 10 (sequence KELTGIQQGFLPKDLQAEAL) consistently produced the greatest signal with both antibodies. This was approximately twofold that of the background signal obtained, whilst signal from GST-BAG-1 was approximately threefold that of the background. The epitope of these antibodies therefore lies, at least in part, within the sequence of peptide 10. Within the BAG domain (Figure 1.2) this region corresponds to the distal two turns of helix one, the proximal two turns of helix two and the hinge region between.

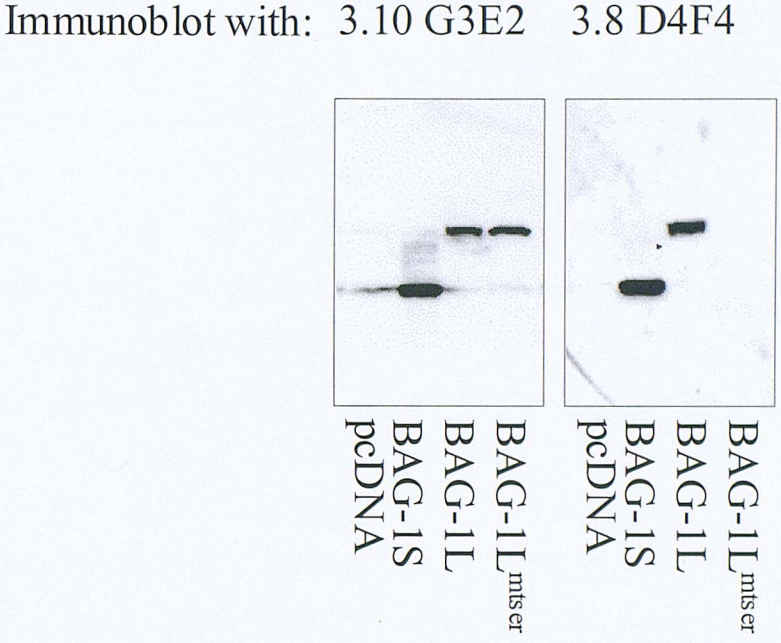


Figure 3.4: Epitope analysis of monoclonal antibodies 3.10 G3E2 and 3.8 D4F4. HEK-293 cells were transfected with expression constructs encoding full length BAG-1S or BAG-1L and BAG-1L^{mtser} and pcDNA. Lysates (20 μg) from these cells were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and probed with monoclonal antibodies 3.10 G3E2 and 3.8 D4F4.

3.2.5 Optimisation 3.10 G3E2 for Immunohistochemistry

Initial attempts to optimise 3.10 G3E2 for immunohistochemistry involved purified antibody. The performance of the antibody was assessed on the strength of specific staining compared to the level of non-specific background staining. Blocking experiments with purified GST-BAG-1S demonstrated that pre-incubation with GST-BAG-1S prevented specific immunoreactivity confirming the specificity of the staining. This preparation produced minimal background and stromal staining on formalin fixed paraffin embedded material at 1 μg/ml. Since another anti-BAG-1 antibody KS-6C8 has been used previously in the literature (Takayama et al. 1998; Turner et al. 2001), BAG-1 labelling with 3.10 G3E2 was compared to labelling with KS-6C8. These two antibodies produced a similar pattern of immunoreactivity to the different BAG-1 isoforms in a panel of breast cancer cell lines (Figure 3.5).

A comparison of labelling of 58 breast cancer sections produced consistent staining patterns (Figure 3.6) and moderate to good correlation of H scores with labelling obtained with KS-6C8 (Nuclear BAG-1 H score: $p<0.001$, $R=0.575$; Cytoplasmic BAG-1 H score: $p<0.001$, $R=0.672$). However staining was inconsistent between batches of purified 3.10 G3E2 and this was demonstrated by differing staining of same batch controls on successive runs. To obtain a high titre preparation of the antibody the hybidoma was sent to a commercial company (Moravian Biotechnology Ltd, Czech Republic) for preparation of ascites. The resultant ascites worked extremely well and produced very clean labelling even at dilutions of 1/8000 to 1/16000, and labelling obtained was extremely consistent between successive runs.

3.3 BAG-1 expression in breast cancer

BAG-1 expression was studied in a cohort of patients with breast cancer to determine the clinical significance of BAG-1. BAG-1 labelling was assessed for associations with clinicopathological features of the tumours and with clinical outcome.

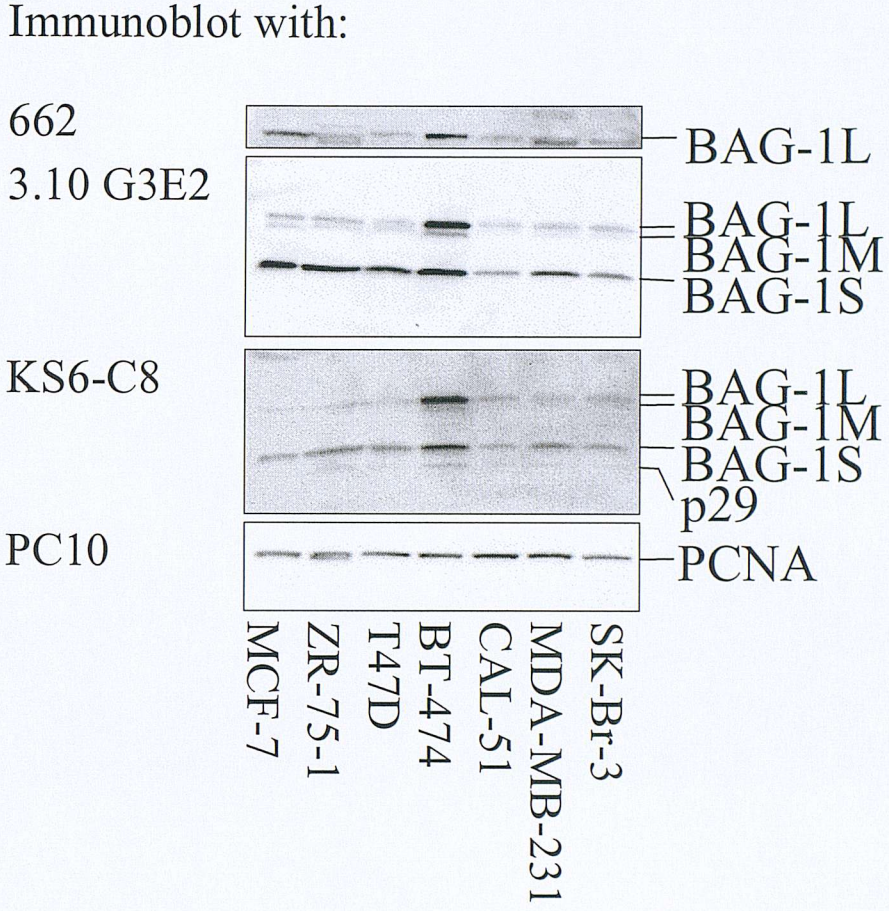


Figure 3.5: BAG-1 Expression in a panel of breast cancer cell lines

RIPA lysate from a panel of breast cancer cell lines was subjected to SDS-PAGE and transferred onto nitrocellulose membrane. Membrane was then blotted with the indicated antibodies. PC10 was used as a loading control to blot for PCNA.

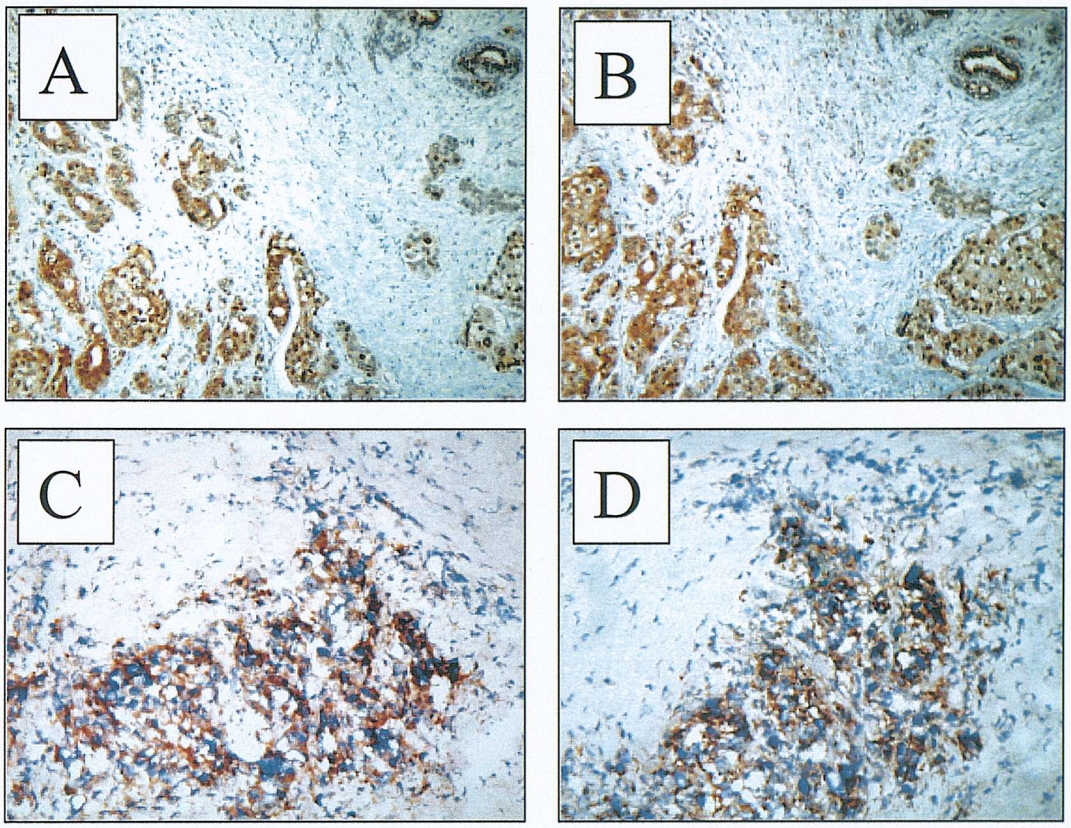


Figure 3.6: Comparison of BAG-1 antibodies for immunohistochemistry in a single tumour

Representative examples are shown from a single tumour known to exhibit nuclear and cytoplasmic labelling. Near consecutive sections were taken from paraffin fixed (A and B) and fresh frozen material (C and D) and used for immunohistochemistry. Paraffin fixed sections were labelled using the anti-BAG-1 monoclonal antibodies KS-6C8 (A) and 3.10 G3E2 (B) and photographed at x10 magnification. Frozen sections were labelled using the anti-BAG-1L specific antibody 662 (C) and pan-isoform antibody 3.10 G3E2 (D) and photographed at x20 magnification.

3.3.1 The patient cohort

Since BAG-1 is multifunctional it is possible that the significance of BAG-1 expression may vary in tumours treated with differing adjuvant therapies. The conflicting reports on the clinical significance of BAG-1 immunohistochemical labelling may stem, at least in part, from studying heterogeneous patient groups with differing treatment regimens. It is therefore important that the impact of BAG-1 expression on survival is analysed in cohorts of patients treated in a relatively homogeneous manner. Since ERα is an important determinant of breast cancer development and progression, and given the importance of adjuvant hormone therapy in breast cancer, a cohort of breast cancer patients treated with adjuvant hormonal therapy was characterised and studied.

Following local Research Ethics Committee approval (Southampton and South West Hants Local Research Ethics Committee; Submission Number: 159/00), 144 patients were studied. The Southampton Breast Unit Database, a prospectively collected database containing information on all breast cancer patients treated in Southampton since 1981, was used to identify patients meeting the inclusion criteria. Consecutive patients with available tissue blocks diagnosed with primary invasive breast carcinoma between 1990 and 1995 were included in the study. Consecutive patients were chosen to minimise the possibility of any selection bias. The Southampton Pathology department records were computerised in 1990 and the department advised that patients treated prior to 1990 not be included. Patients included received surgery, followed by adjuvant hormone therapy but not chemotherapy. Patients who received adjuvant chemotherapy were not included as the aim was to specifically assess the effect of BAG-1 expression in predicting outcome in patients receiving systemic adjuvant hormone therapy. Six cases were excluded since their tissue failed to produce any labelling with any antibody on multiple occasions (including internal positive control). The median follow-up was 5 years 7 months.

The cohort ranged from age 37 to 94 (mean 63; median 62). Fifty-four patients (39%) were treated surgically by mastectomy and 84 patients (61%) by breast conserving surgery. In addition 92 (67%) patients underwent axillary dissection.

Breast radiotherapy was given in the majority of cases treated by breast conserving surgery. The standard first line adjuvant hormone therapy given in the unit was Tamoxifen (20 mg/day), but patients were occasionally changed to Anastrozole (1 mg/day) if Tamoxifen was not tolerated. The histological diagnosis was invasive ductal carcinoma in 112 (81%) patients, invasive lobular carcinoma in 10 (7%) patients and other types of invasive breast carcinoma in 16 (12%) patients. Other cohort characteristics are summarised (Table 3.2).

3.3.2 Expression of BAG-1 oestrogen receptor and progesterone receptor

The tumours were analysed by immunohistochemistry to determine expression of BAG-1. Oestrogen receptor alpha (ERα) and progesterone receptor (PgR) status was not routinely assessed in the unit at the time the patients were treated for their primary cancer. Given the biological and clinical importance of ERα and progesterone receptor PgR status in patients with breast cancer, and in particular in those treated with hormone therapy, ERα and PgR status was also assessed. ERα and PgR labelling was assessed using antibodies 1D5 and PgR636 respectively since those antibodies are currently used to assess ERα and PgR expression clinically. BAG-1 expression was analysed using antibody KS-6C8 because at the time of the study batches of purified 3.10 G3E2 were not staining consistently between runs. A subset of 58 patients was however analysed with antibody 3.10 G3E2 with similar results as obtained with KS-6C8 (section 3.3.6). Examples of BAG-1 immunostaining are shown in Figure 3.7. 15 tumour samples were completely negative (including normal, uninvolved breast epithelium) on repeated analysis for BAG-1 expression although expression of ERα or PgR was detected in these samples. Since normal breast epithelium has been consistently reported to be BAG-1 positive in previous studies (Brimmell et al. 1999; Takayama et al. 1998; Yang et al. 1999), the BAG-1 status of these cases was classified as unknown.

BAG-1 as well as ERα and PgR and labelling was evaluated using H-scores (section 2.12.2). Frequency H-score histograms for BAG-1 demonstrated a bimodal distribution as previously described (Turner et al. 2001). A cutoff H-score of 100 separated these two distinct populations of patients. Overall using this cutoff to

Exploratory variable		N	Deaths (%)	P-value	Hazard Ratio (95% CI)
Age Category (years)	<55	46	9 (20%)		1
	56-75	68	13 (19%)	0.943	0.97 (0.41-2.28)
	76+	24	5 (21%)	0.534	1.42 (0.47-4.24)
Lymph Node Status	Negative	43	6 (14%)		1
	Positive	49	14 (29%)	0.075	2.39 (0.92-6.22)
Tumour Size	T1	57	6 (10%)		1
	T2	63	16 (25%)	0.029	2.86 (1.11-7.34)
	T3	17	5 (29%)	0.048	3.32 (1.01-10.92)
Tumour Grade[a]	1 and 2	89	11 (18%)		1
	3	48	16 (33%)	0.003	3.22 (1.49-6.94)
ERα Status	Negative	29	12 (41%)		1
	Positive	83	10 (12%)	<0.001	0.21 (0.09-0.50)
PgR Status	Negative	48	17 (20%)		1
	Positive	84	7 (15%)	<0.001	0.192 (0.08-0.47)
Nuclear BAG-1 Status	Negative	56	17 (30%)		1
	Positive	66	7 (11%)	0.015	0.33 (0.14-0.81)
Cytoplasmic BAG-1 Status	Negative	95	20 (21%)		1
	Positive	27	4 (14%)	0.600	0.75 (0.26-2.20)

Table 3.2: Single variable predictors of outcome from breast cancer

[a]Tumours were graded using a modification of the Bloom and Richardson system (Bloom & Richardson 1957;Elston & Ellis 1991)

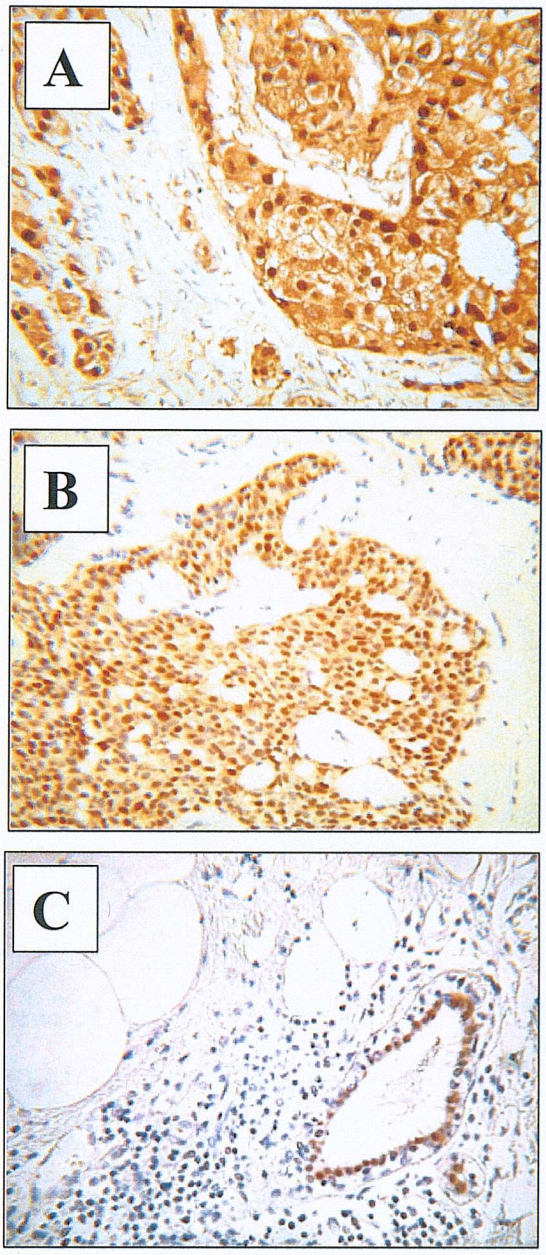


Figure 3.7: BAG-1 expression in human breast cancer.

Representative examples of immunostaining in breast cancer samples using the anti-BAG-1 monoclonal antibody KS-6C8 at x25 magnification. A, nuclear and cytoplasmic BAG-1 expression; B, predominantly nuclear BAG-1 expression; C, negative for both nuclear and cytoplasmic BAG-1 expression in tumour cells but nuclear BAG-1 labelling in adjacent normal epithelium. Labelling of adjacent normal epithelium was used as a positive internal control for each section.

classify patients as positive or negative for BAG-1 expression 54% of patents were classified as positive for BAG-1 labelling in the nucleus of tumour cells and 23% of patients positive for BAG-1 labelling in the cytoplasm. Generally tumours were negative or exhibited nuclear or nuclear and cytoplasmic labelling for BAG-1. Tumours very rarely exhibited cytoplasmic labelling alone. ERα and PgR labelling of tumours was nuclear as previously described (Elashry-Stowers et al. 1988; Greene et al. 1984; Seymour et al. 1990). A cutoff H-score of 75 was used to classify patients as positive or negative for ERα and PgR expression since this value has previously been used for the assessment of ERα and PgR receptor status clinically. 74% of patients were considered positive for ERα expression and 64% of patients positive for PgR expression. These values are similar to previously reported frequencies of hormone receptor status in large studies and indicate that for hormone receptor status this cohort and the assessment techniques used are representative for the disease a whole (Chu et al. 2001; Li et al. 2003).

3.3.3 Association of BAG-1 with clinicopathological features

Information on tumour size, histological grade, and lymph node status was obtained from the pathology department histology reports. These clinico-pathological features, and ERα and PgR status, were studied for association with BAG-1 expression, since any such associations may give clues as to the biological role or importance of BAG-1 expression in these tumours.

There was a strong inverse association between nuclear BAG-1 expression and tumour grade (χ^2 test for trend=16.51, P<0.001), but not lymph node status (χ^2=0.84, P=0.361). This demonstrates that BAG-1 nuclear expression occurs in well-differentiated tumours and is lost in poorly differentiated and more aggressive tumours. There was a borderline significant association between nuclear BAG-1 expression and tumour size (χ^2=3.22, P=0.073). ERα expression correlated moderately with PgR expression (p<0.001, r=0.52), consistent with PgR being an ERα target gene. Nuclear BAG-1 expression was also moderately correlated with PgR expression (p<0.001, r = 0.42) and was also associated, but less strongly, with ERα expression (p=0.02, r = 0.31). Nuclear BAG-1 expression is therefore associated

with expression of an ERα target gene (PgR) and less strongly with the expression of ERα itself. Loss of nuclear BAG-1 expression occurs with loss of ERα expression and loss of PgR expression. There was also a strong correlation between nuclear and cytoplasmic BAG-1 expression ($p<0.001$, $r = 0.74$), but compared to nuclear BAG-1 expression the association between cytoplasmic BAG-1 expression and PgR and ERα was marginally weaker ($p<0.001$, $r=0.32$ and $p=0.003$, $r=0.30$ respectively).

3.3.4 Association of BAG-1 expression with clinical outcome

New prognostic factors that help classify patients into good or bad prognosis groups, and new predictive factors that predict response to treatment, will enable improved selection of treatment regimens for patients, and provide increased information for decisions when the benefits and side effects of treatments are considered. In addition associations of protein expression with outcome may provide indication as to the suitability of the protein as a potential target for therapy. It is therefore important that the expression of proteins such as BAG-1 are examined for associations with patient outcome.

Information on patient survival was obtained from patient records and from the Southampton Breast Unit database. The impact of BAG-1 expression on survival was demonstrated using Kaplin-Meier plots (Figure 3.8). Of the 123 patients with known BAG-1 status 24 patients died of breast cancer and 3 patients died from other causes. Positive (H-score >100) nuclear, but not positive cytoplasmic BAG-1 labelling was associated with significantly increased survival in single variable analysis ($p=0.015$) (Table 3.2). The hazard ratio of one third indicates that at any point in time patients with nuclear BAG-1 expression have one third the probability of dying of breast cancer of those without nuclear BAG-1 expression. Patients with nuclear BAG-1 expression in this study have approximately a 90% probability of ten year survival from breast cancer compared to a 60-70% probability of ten year survival for those who lose nuclear BAG-1 expression.

Surprisingly, axillary status did not reach significance in single variable analysis, although nodal status is widely recognised as the single most informative prognostic factor (Miller et al. 1994). Approximately one third of patients in this

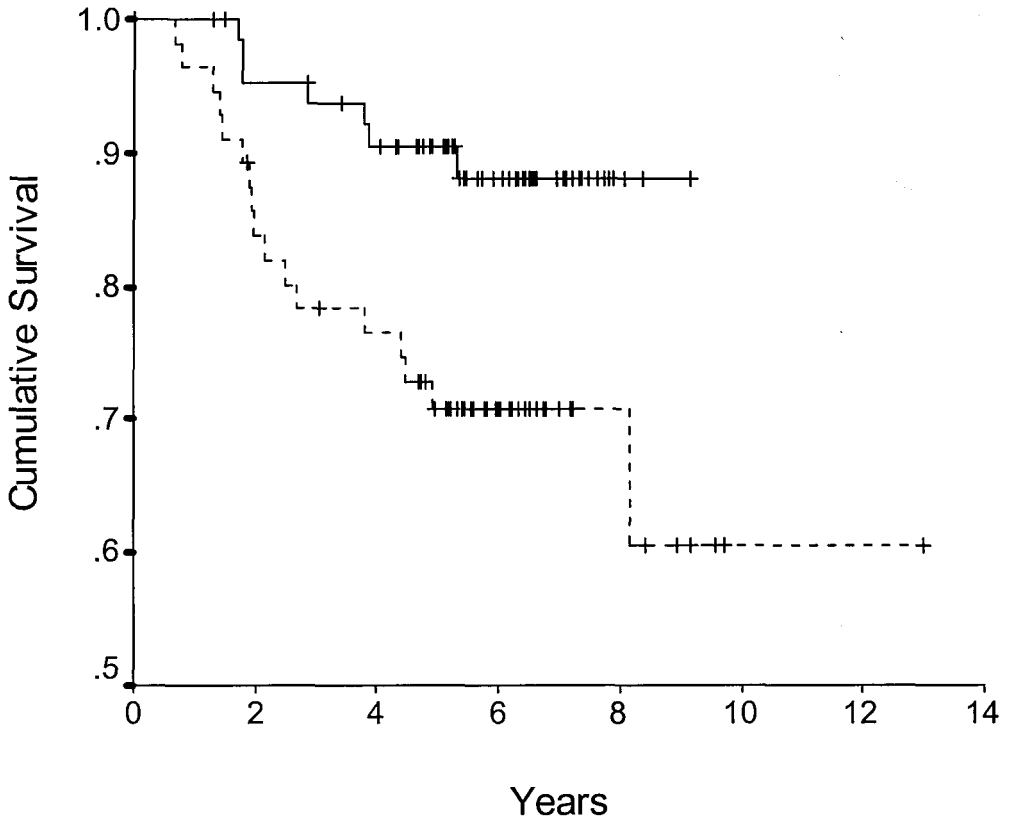


Figure 3.8: Kaplan-Meier plot of the effect of BAG-1 expression on survival. Solid line, nuclear BAG-1 positive (N=66, 7 deaths from breast cancer), broken line, nuclear BAG-1 negative (N=57, 17 deaths from breast cancer). Hazard Ratios: BAG-1 positive: 0.334 (95% Confidence Interval 0.138-0.809), BAG-1 negative: 1; P=0.015).

study did not undergo axillary dissection however, and so axillary status was unavailable in these cases. The protocol within the Southampton Breast Unit at the time the patients were treated advised axillary surgery only for patients with tumours larger than 1 cm and this bias might be expected to further weaken the prognostic power of axillary status in this study. In contrast to lymph node status, other factors including tumour size, tumour grade, ERα status and PgR status all predicted patient outcome demonstrating that in all other ways this cohort behaved as expected for prognostic variables. Loss of ERα and PgR expression was associated with poorer outcome. There was in addition a gradation such that those with loss of both receptors had the poorest probability of survival followed by those with loss of only one of the receptors, and those with expression of both receptors had the highest probability of survival (data not shown). Increasing tumour size was associated with a reduced probability of survival as was increasing tumour histological grade. Grade one and two tumours had to be graded together since there were no patient deaths in those with grade one tumours.

Finally, the significance of BAG-1 expression was determined in a limited multiple variable model including nuclear BAG-1 status, tumour size and tumour grade and patient age (Table 3.3). Factors including tumour size and grade, which should be significant, were not significant if axillary status was included in the model, possibly due to larger numbers of missing cases and subsequently smaller numbers of events (patient deaths) available for analysis. A multiple variable model is therefore presented excluding axillary status. Multiple variable models are important because they demonstrate the additional prognostic information provided by the candidate variable over and above the other known prognostic variables included in the model. Nuclear BAG-1 status predicted patient outcome in this limited multiple variable model with the caveat that this should be interpreted with caution since it was not possible to include nodal status.

Exploratory variable		N	Deaths (%)	P-value	Hazard Ratio (95% CI)
Age Category (years)	<55	40	8 (20%)		1
	56-75	61	12 (20%)	0.742	1.17 (0.46 to 2.96)
	76+	21	4 (19%)	0.770	1.20 (0.35 to 4.15)
Tumour Size	T1	49	6 (12%)		1
	T2	57	13 (23%)	0.026	3.13 (1.15 to 8.55)
	T3	16	5 (31%)	0.039	3.73 (1.07 to 13.01)
Tumour Grade	1 and 2	81	10 (12%)		1
	3	41	14 (34%)	0.018	2.84 (1.20 to 6.75)
Nuclear BAG-1 Status	Negative	56	17 (30%)		1
	Positive	66	7 (11%)	0.022	0.33 (0.13 to 0.85)

Table 3.3: Limited Multiple variable model of outcome from breast cancer (122 patients included with 24 deaths)

3.4 Production of BAG-1L specific antisera

Since nuclear labelling of BAG-1 was found to be associated with ERα and PgR status and patient outcome it became important to determine if this labelling represented expression of BAG-1L or relocalisation of the other isoforms to the nucleus. Therefore to produce antisera specific for BAG-1L, a recombinant protein containing the unique amino-terminus of BAG-1L was initially produced and purified. The purified protein was used to raise polyclonal antibodies in rabbits using standard techniques. The antibody subsequently optimised for use in Western blotting immunoprecipitation, and immunohistochemistry.

3.4.1 Production of the pGEX-2TK-BAG-1L$^{1-71}$ expression construct

The pGEX vectors are designed to enable expression of foreign polypeptides in bacteria in a form that enables purification under non-denaturing conditions. Peptides are expressed as fusions to the carboxy-terminus of glutathione-S-transferase (GST), a 26 kDa enzyme. These fusion proteins are typically soluble, and can be purified by virtue of the high affinity of GST for Glutathione, immobilised on agarose beads. Elution with reduced soluble glutathione enables recovery of the fusion protein.

The specific cloning strategy for the production of the pGex-2TK-BAG-1L$^{1-71}$ expression construct is described in the materials and methods. Initial difficulties were encountered producing the appropriate unique amino-terminal sequence of BAG-1L by PCR due to the extremely high GC content of this region. This was overcome by using proof reading Taq polymerases selected for ability to read through GC rich regions (Clontech Advantage GC Taq), GC melt (a DNA relaxing agent), and DMSO. Finally cloning of the correct insert, free of PCR mutations, was confirmed by commercial sequencing.

3.4.2 GST fusion protein production

The pGex-2TK- BAG-1L$^{1-71}$ plasmid was transformed into the E. Coli strain BL21. This strain is used for recombinant protein production since it is deficient in

OmpT and Lon proteases, which may otherwise interfere with isolation of intact recombinant protein. Induced GST- BAG-$1L^{1-71}$ protein was not visible in crude BL21 bacterial cell lysates compared to uninduced lysates (Figure 3.9). By contrast positive control BL21 bacteria transformed with pGex-2TK to produce GST on induction produced a clear band in the induced cells compared to the uninduced. Subsequently BAG-$1L^{1-71}$ protein was bound to glutathione beads, which were then extensively washed to reduce non-specific protein binding prior to boiling in running buffer. Using this technique it was possible to detect the GST- BAG-$1L^{1-71}$ protein, although it was clearly present in far smaller quantities when induced than GST alone, or GST-BAG-1S.

It rapidly became apparent that the BAG-$1L^{1-71}$ protein was either poorly produced, rapidly degraded or toxic to the bacteria. Since the bacteria grew to good density within the media it appeared that the protein was not toxic. Additional protease inhibitors reduced degradation but did not increase overall yield significantly and so it was concluded that the protein was poorly produced. Further attempts to increase the protein yield by altering the length of time and temperature that the bacteria were allowed to grow and were induced produced moderate improvements in the yield.

The BAG-$1L^{1-71}$ protein was produced initially by binding it to, and eluting it from, glutatione beads in columns. With these protocols significant amounts of protein remained in the flow through not bound to the beads, and a significant amount of protein could be boiled off the beads after glutathione elution, and was therefore not "elutable" from the beads. It was found that for this protein optimal binding and elution occurred when performed with freshly made 50mM Glutathione in 50ml tubes on a spiramix (Figure 3.10A).

Following these optimisation steps a total of 4 litres of bacterial broth was required to produce sufficient GST- BAG-$1L^{1-71}$ protein for the immunistation stages. The combined fractions were then washed with PBS in Amicon YM3 spin columns, These columns retain substances with a molecular weight greater than 3000, and so glutathione was removed and the BAG-$1L^{1-71}$ protein concentrated (Figure 3.10B).

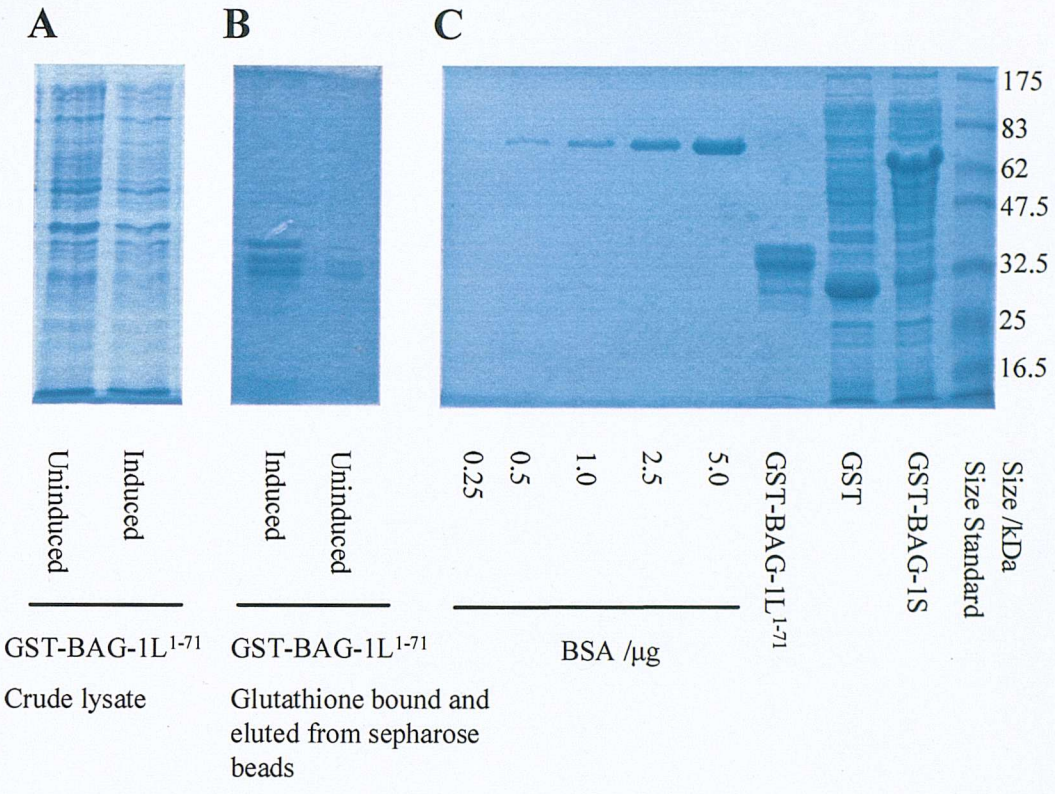


Figure 3.9: Production of GST- BAG-1L$^{1-71}$ protein.

BL21 bacteria were transformed with the pGex-2TK- BAG-1L$^{1-71}$ plasmid. Bacteria were then grown in the presence (Induced) or the absence of IPTG (Uninduced) to induce recombinant protein production. Protein was separated by SDS-PAGE and stained by Coomassie blue. Protein separated included crude bacterial lysates (A) and protein purified by binding to and elution from glutathione beads (B). Bacteria were also transformed with pGEX-2TK and pGEX-2TK-BAG-1S and induced with IPTG as controls (C). Crude lysates are shown for GST and full length GST-BAG-1S which migrate at 26kDa and 62kDa respectively. BSA (bovine serum albumin) was used to quantify yields (C). The GST- BAG-1L$^{1-71}$ protein seen in B is present as three bands whereas in C it is present as two bands following the addition of extra protease inhibitors to the lysis buffer prior to purification with glutathione beads.

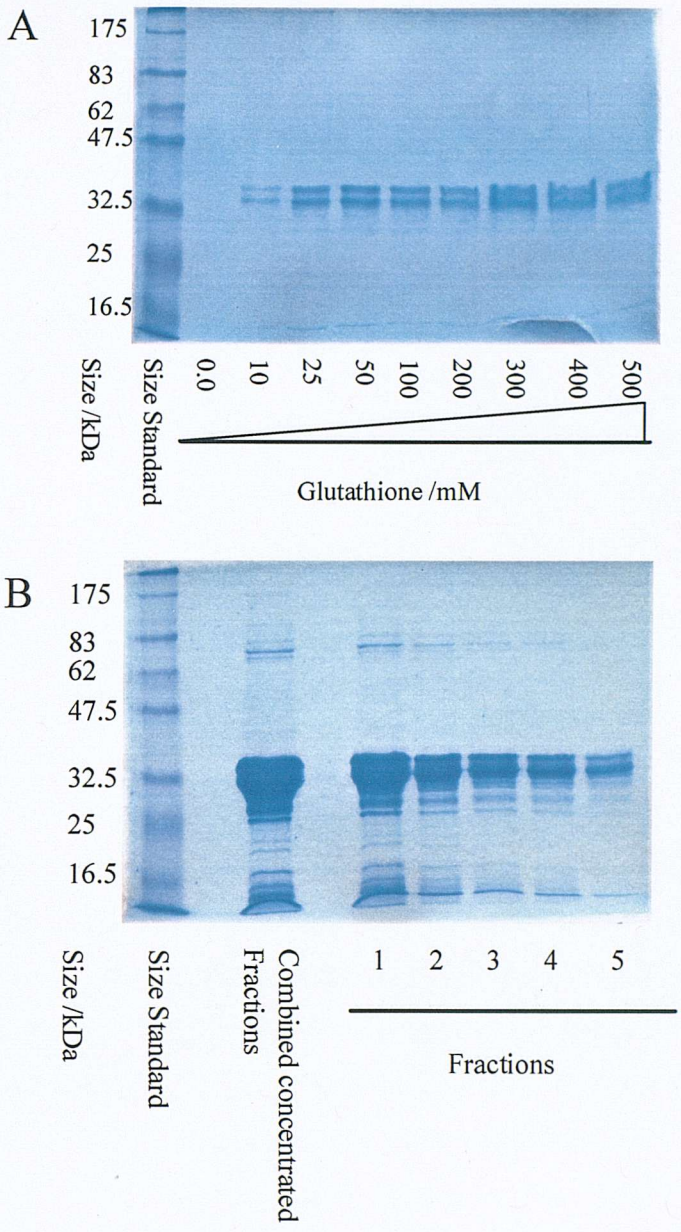


Figure 3.10: Optimisation and large scale production of GST-BAG-1L$^{1-71}$ protein BL21 bacteria transformed with the pGex-2TK- BAG-1L$^{1-71}$ plasmid were induced with IPTG to produce recombinant GST- BAG-1L$^{1-71}$ protein. GST fusion protein was then purified by binding to glutathione beads, washing and elution with glutathione. Protein eluted was separated by SDS-PAGE and stained with Coomassie blue. (A) Elution of GST- BAG-1L$^{1-71}$ with different concentrations of Glutathione to elute GST fusion protein off the beads. (B) Large scale production of GST- BAG-1L$^{1-71}$ for immunisation. Protein was eluted in 50 mM Glutathione in five fractions. These were combined, purified and concentrated, to produce concentrated BAG-1L$^{1-71}$ protein. This was resuspended in PBS prior to immunisation.

3.4.3 Immunisation protocol and Results of test bleeds

Three New Zealand White Rabbits were used for the production of the BAG-1L specific antisera. The results from the prebleed, and the first and last test bleed are shown in figure 3.11, alongside controls blotted with pan-BAG-1 polyconal 191-TB2 (performed at the same time as the prebleed and test bleed 1), and with the pan-BAG-1 monoclonal 3.10 G3E2 (performed with test bleed 4). There was no significant anti-BAG-1 immunoreactivity prior to immunisation in any of the rabbits. Following immunisation specific immunoreactivity to protein at a size corresponding to BAG-1L was detected in sera from all three rabbits.

3.4.4 Specificity of the antisera for BAG-1L

Two approaches were taken to demonstrate the specificity of the sera for BAG-1L. Initially sera were used to immunorecipitate BAG-1L, followed by Western blotting with anti-BAG-1 monoclonal antibody 3.10 G3E2. This was initially in HEK-293 cells transfected with the pcDNA3-BAG-1L expression construct (data not shown). Following the success of these experiments they were repeated on untransfected MCF-7 cells to immunoprecipitate endogenous BAG-1 (Figure 3.12A). The characteristic pattern of the 3 BAG-1 isoforms can be observed in the input MCF7 cell lysate. Only the BAG-1L isoform is seen following immunoprecipitation with the BAG-1L specific antisera. Following immunoprecipitation with the preimmune sera there is minimal background activity, whilst following immunoprecipitation with pan-BAG-1 antisera there is enrichment of all three BAG-1 isoforms. This experiment further demonstrates, for the first time, that BAG-1L does not heterodimerise with the other BAG-1 isoforms, at least under these conditions.

The second approach to demonstrate the specificity of the sera involved immunodepletion of BAG-1 from cell lysate using anti-BAG-1 antibody 3.10 G3E2 (data not shown). Western blotting of a control lysate (beads only depleted), compared to a lysate immunodepleted of BAG-1 isoforms with 3.10 G3E2, with antibody 662 confirmed that the band detected at 50 kDa by this antibody was indeed BAG-1L.

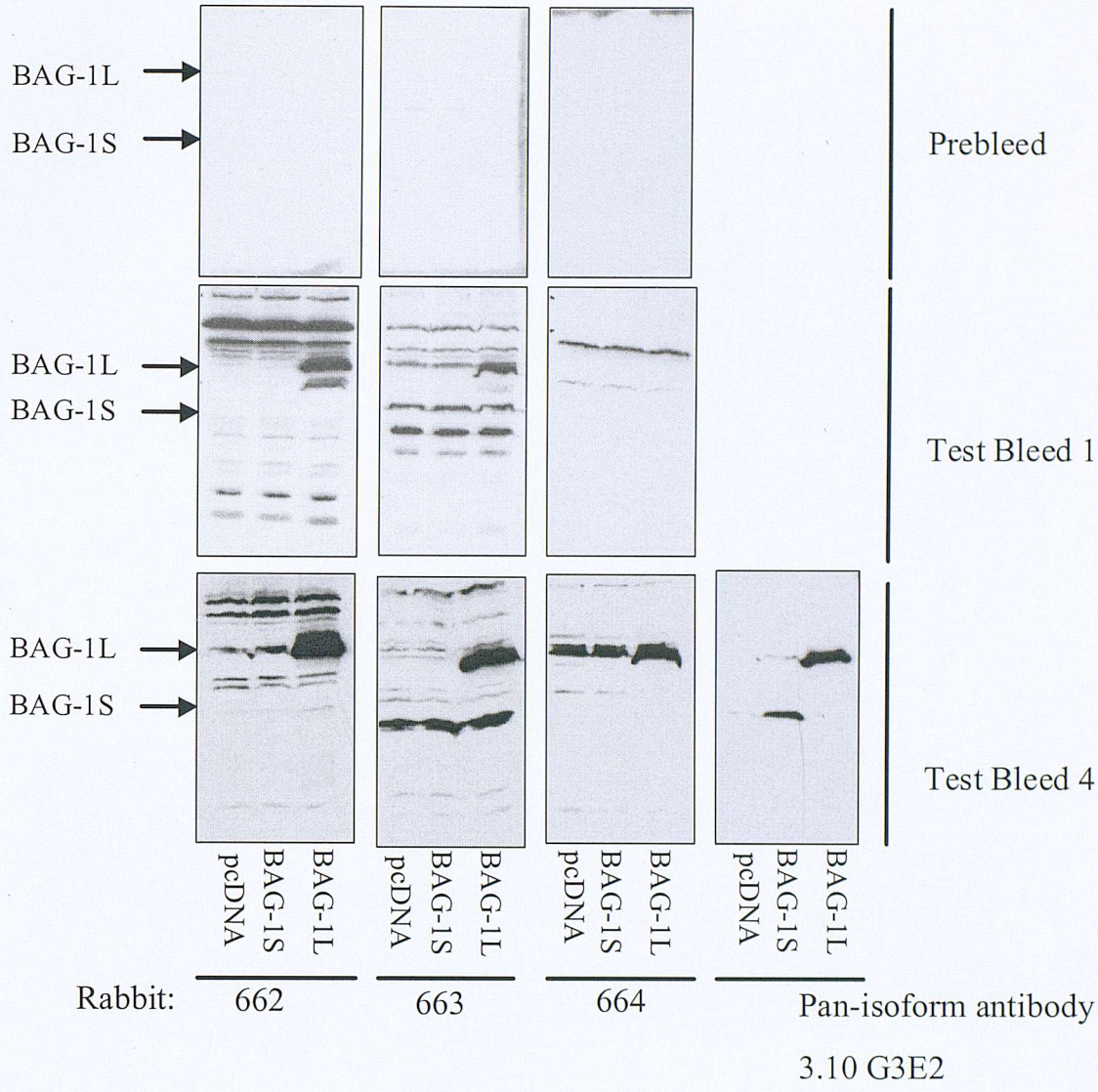


Figure 3.11: Immunoreactivity of prebleed and test bleeds to BAG-1L.
HEK-293 cells were transfected with pcDNA3 (lanes 1), pcDNA3-BAG-1S (lanes 2) or pcDNA3-BAG-1L (lanes 3). Protein was separated by SDS-PAGE and blotted with antibodies as indicated. For the pre-bleed and test bleed 1 40 μg of protein was loaded, and the sera was used at 1/100 dilution in TS. For test bleed 4, 20 μg of protein was loaded and the sera were used at 1/500 dilution in TS. Blots with the pan-BAG-1 isoform antibodies demonstrate the position of BAG-1S and BAG-1L. Specific reactivity to BAG-1L increased with test bleeds, particularly in rabbits 662 and 663. Cross reactivity also increased with 663 whilst in contrast it did not significantly change with 662.

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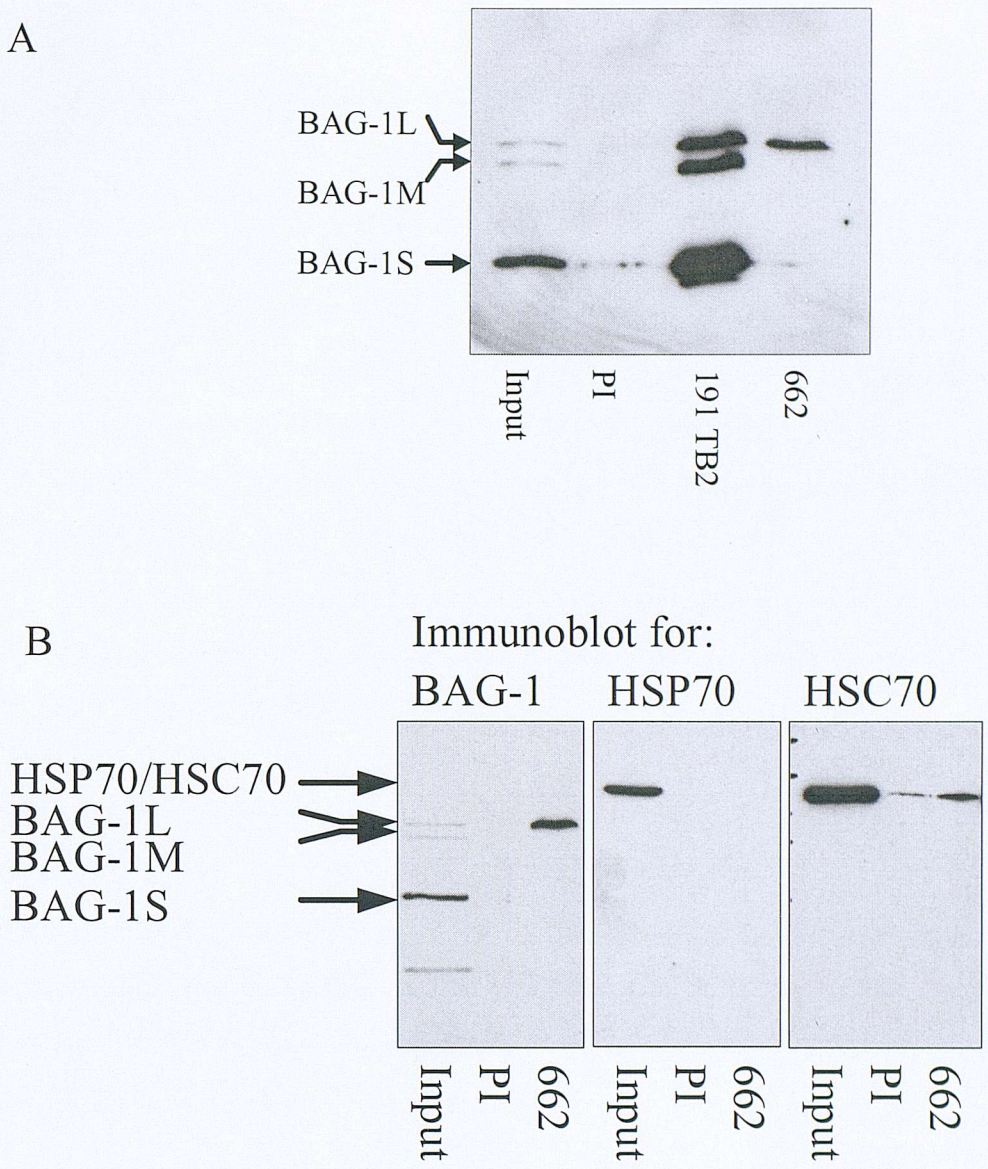


Figure 3.12: Immunopreciptitation with BAG-1L specific antisera 662
(A) Immunoprecipitation of untransfected MCF7 cells with control pre-immune (PI) sera, pan-BAG-1 antibody 191-TB2 and BAG-1L isoform specific antisera (662) followed by Western blotting with anti-BAG-1 antibody 3.10 G3E2. (B) BAG-1L was immunoprecipitated from untransfected MCF7 cells with BAG-1L specific antisera 662, or control pre-immune sera (PI), and Western blotting for BAG-1, HSC70 and HSP70 performed as indicated.

3.4.5 BAG-1L binds to HSC70 but not HSP70 in MCF7 cells

The BAG-1L specific antiserum was used to explore the interaction of BAG-1L with the chaperones HSC70 and HSP70. Co-immunoprecipitation experiments with pan-BAG-1 antibodies including 191-TB2 have previously demonstrated BAG-1 isoforms to be bound to both HSC70 and HSP70 in untransfected unstressed MCF-7 cells (Townsend et al. 2003a; Townsend et al. 2003b). However these immunoprecipitations do not reveal which isoforms of BAG-1 bind to the chaperones. Immunoprecipitation experiments with the BAG-1L specific antiserum demonstrated that under these conditions BAG-1L is bound to the constitutive HSC70 but not the inducible HSP70 (Figure 3.12B). Although HSP70 was present in the input whole cell lysate none of the HSP70 was bound to BAG-1L. In contrast a proportion of HSC70 was bound to BAG-1L. This isoform specific difference has not been previously reported. Low levels of HSC70 were detected in control preimmune immunoprecipitations, but significantly less than in the 191-TB2 immunoprecipitation. Low levels of chaperone were often detected in control immunoprecipitations and this may be due to the chaperone binding to partially denatured immunoglobulin in the serum through the chaperone peptide-binding region. There is however much greater immunoreactivity following immunoprecipitation with 191-TB2 demonstrating the specific interaction between BAG-1L and the chaperone.

3.4.6 Expression of BAG-1L in breast cancer

The BAG-1L specific antisera were initially used for Western blotting to examine BAG-1L expression. Since non-specific bands were present in addition to the band corresponding to BAG-1L at 50 kDa, immunoglobulin was purified from sera by ammonium sulphate precipitation (kindly performed by Maureen Power, Tenovus, Southampton). This resulted in reduction of background signal from antibody 662. Since 662 demonstrated a greater balance of specific BAG-1L immunoreactivity compared to cross-reactivity than 663 and 664 (Figure 3.11) attempts were then made at affinity purification of antibody 662 (Maureen Power). Negative selection to remove anti-GST antibodies produced little appreciable benefit.

In addition unfortunately, attempts to couple the GST- BAG-$1L^{1-71}$ protein to cyanogen bromide columns were unsucessful and so positive selection was not possible. Further work was therefore performed with purified immunoglobulin from antisera 662. Although there were some differences Western blotting of a panel of human breast cancer cell lines overall showed a similar pattern of BAG-1L expression as the monoclonal antibodies 3.10 G3E2 and KS-6C8 (Figure 3.5).

Immunofluorescence microscopy with antibody 662 produced encouraging results. Nuclear labelling in BAG-1L transfected MCF7 cells was much stronger than in pcDNA transfected cells as expected because of the known nuclear localisation of BAG-1L (Brimmell et al. 1999). Nuclear labelling in untransfected BT474 cells which have higher levels of endogenous BAG-1L than MCF7 cells was stronger than in pcDNA transfected MCF7 cells (Figure 3.13), and immunoreactivity in untransfected MCF-7 cells was no different to that in pcDNA transfected MCF7 cells.

Although the intent was to determine expression of BAG-1L in the cohort, unfortunately despite the use of a range of different antigen retrieval techniques, the antibody demonstrated no immunoreactivity on archival formalin fixed paraffin embedded material. Antigen retrieval approaches used included microwave and pressure cooker heat induced antigen retrieval and both EDTA and citrate buffers were tried. Enzymatic pre-treatments with trypsin were also attempted. Primary antibody was used at a range of concentrations (5 μg/ml to 80 μg/ml).

The antibody did produce labelling of frozen tumour material with little background labelling (Figure 3.6). Architectural detail is much less clear with frozen material but it is still apparent from the figure that both this antibody and the pan-isoform antibody 3.10 G3E2 produced only cytoplasmic labelling. This is true even with tumours that are known to produce nuclear labelling with 3.10 G3E2 from paraffin embedded sections. This is surprising and suggests that the fixation procedure with formalin used both for routine clinical specimens and for the flourescence microscopy in some way reveals BAG-1 epitopes that are hidden in fresh frozen sections. In total, adjacent sections from eight tumours were labelled with both 3.10 G3E2 and 662. Of these eight, six were classified as positive from staining of formalin fixed paraffin embedded material for nuclear and cytoplasmic

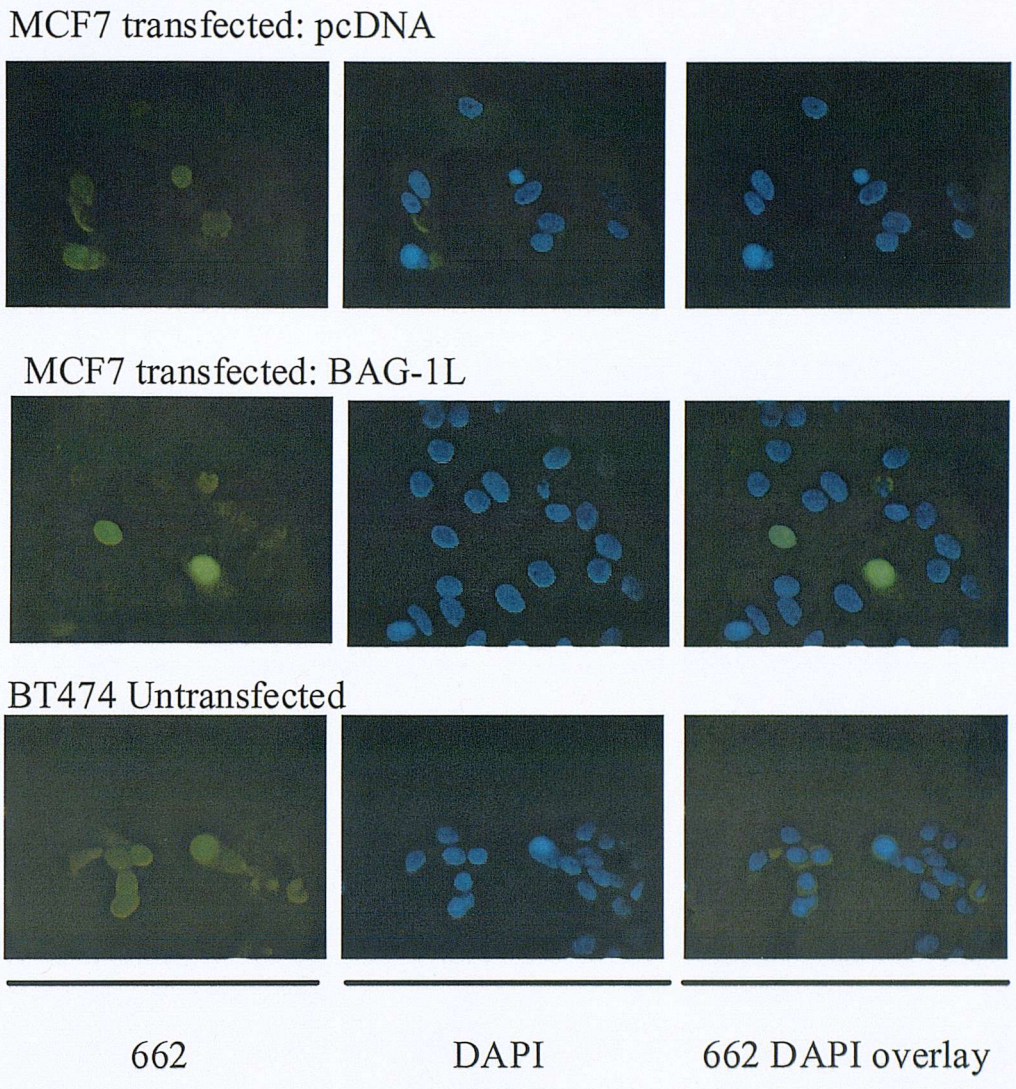


Figure 3.13: Immunofluorescence microscopy with antibody 662

MCF7 cells were transfected with control pcDNA plasmid or BAG-1L expression construct (top and middle rows respectively) and BT474 cells left untransfected. Cells were fixed and labelled with antibody 662 and stained with DAPI.

BAG-1 using the criteria described, and one as positive for nuclear BAG-1 with one classified as negative (data not shown). From frozen material both antibodies produced cytoplasmic staining to a variable degree in all tumours (data not shown).

3.5 BAG-1 autoantibodies in human sera

To determine whether, like heat shock proteins, auto-antibodies to BAG-1 are present in human sera an assay was developed. Patient sera was used to immunoblot for known proteins that had been separated by SDS-PAGE and transferred onto nitrocellulose membrane.

3.5.1 Assay development and optimisation

To develop and optimise an assay capable of detecting auto-antibodies in human a reliable positive control was required. Epstein-Barr virus (EBV) infects over 90% of humans and an immune response to this is readily mounted (Cohen 2000). The EBV nuclear antigen (EBNA1) therefore formed an ideal positive internal control for pilot studies. Known EBV positive sera and negative sera (from the Virology Department, Southampton General Hospital) were used as positive and negative controls. EBV positive sera detected EBNA1 by Western blotting of IB4 cells (an EBV immortalised lymphoblastoid cell line) in five out of five known EBV positive sera samples at a wide range of sera dilutions (1/20 to 1/500). An HRP conjugated anti-human IgG (DAKO) used at 1/1000 dilution was found to be optimal as a secondary antibody.

Breast cancer patient sera were obtained from the CRC Wessex Medical Oncology Unit Tumour Bank, and sera of healthy controls anonymously and unlinked from a local health-screening program. Initial experiments with SDS-PAGE of crude unpurified BL21 bacterial cell lysates, induced with IPTG to express GST-BAG-1S, were not successful (data not shown). This was largely because patient's sera strongly reacted to constituents within the bacterial cell lysate obscuring any possible specific immunoreactivity. Purified preparations of recombinant GST and GST-BAG-1S were therefore produced and used in further assays. Optimal results were obtained at 1/100 dilution of sera. The optimum quantity of protein for SDS-PAGE varied between sera

because of differing background immunoreactivity, and so all sera were tested against two different quantities of GST and GST-BAG-1S (6μg and 1μg).

3.5.2 Breast cancer patients and healthy controls

Sera from 12 patients with breast cancer and from 9 healthy controls were tested. Three different patterns of immunoreactivity were recognised. Positive immunoreactivity was defined as reactivity to GST-BAG-1S but not to GST alone. In a proportion of cases equal immunoreactivity to GST-BAG-1S and GST was found. This was classified as negative since immunoreactivity to the BAG-1 fusion protein was likely directed to the GST part of this protein. The remainder of cases did not show reactivity to either GST-BAG-1S or GST, despite demonstrating other non-specific bands (Figure 3.14). These were classified as negative. Overall sera from 3/12 (25%) patients with breast cancer, and from 3/9 (33%) healthy controls, contained specific antibodies against BAG-1S. In addition there were no obvious associations between tumour stage, grade, ERα status or BAG-1 expression determined by immunohistochemistry or the presence of BAG-1 autoantibodies (Table 3.4). Since specific immunoreactivity to BAG-1 was found in healthy controls this test is unlikely to have utility as a test for breast cancer patients. It is of interest however that in this small study the frequency of immunoreactivity for BAG-1 is similar to that described in studies of autoantibodies to HSC70.

Western blotting was performed to test if easily detectable quantities of BAG-1 protein was present in the sera of breast cancer patients, in particular those with autoantibodies to BAG-1 since p53 protein has previously been found in sera of cancer patients (Soussi 2000). Sera (total protein content 7μg) was loaded from each of seven breast cancer patients and tested for the presence of BAG-1 by immunoblotting with two specific BAG-1 antibodies; 3.10 G3E2 and 191-TB2 (data not shown). BAG-1 isoforms were clearly visible in MCF-7 positive control cell lysate but not within the sera indicating that at least by these techniques BAG-1 is not readily detectable in sera from breast cancer patients.

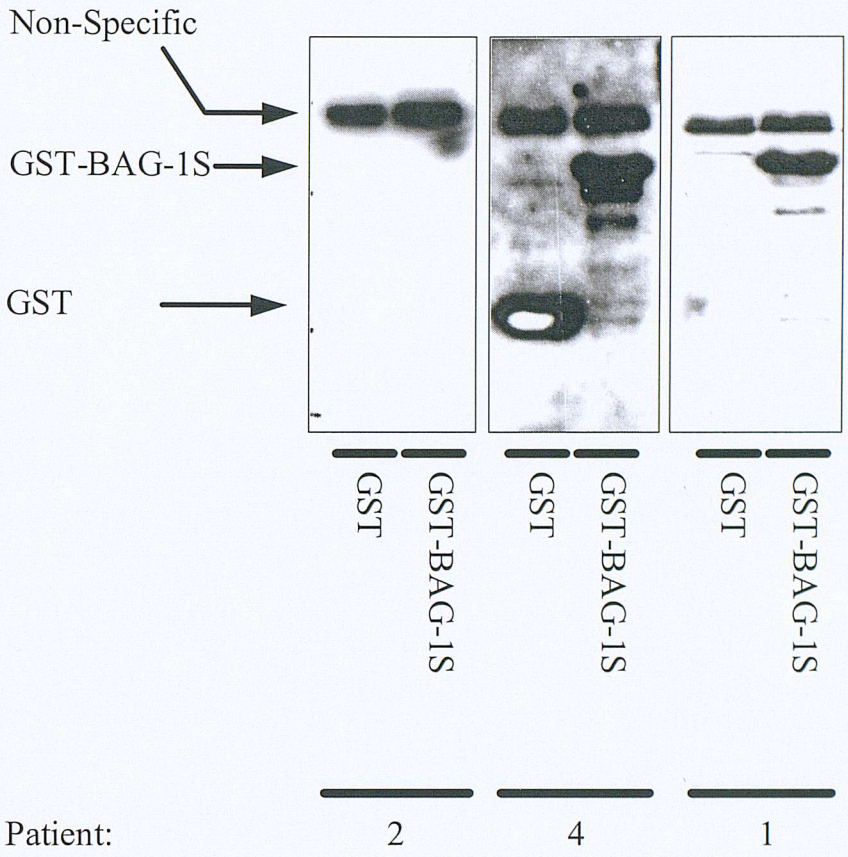


Figure 3.14: Representative examples of sera immunoreactivity to GST and GST-BAG-1S by Western blotting.

GST and GST-BAG-1S (6μg) were resolved on SDS-PAGE, transferred to nitrocellulose and incubated with human sera (dilution 1/100 in TS). Non specific high molecular weight bands that may represent heat shock proteins were seen in most cases. Examples are shown of sera that demonstrated specific immunoreactivity to GST-BAG-1S (Patient 1; Table 3.4), equal immunoreactivity to GST-BAG-1S and GST (Patient 4), and immunoreactivity to neither (Patient 2).

Study Id Number	Tumour Type	Tumour Size	Tumour Grade	Nodal Status	ERα status	BAG-1 Auto-antibodies	Tumour Nuclear BAG-1 labelling	Tumour Cytoplasmic BAG-1 labelling
1	Invasive Ductal[a]	T1	2	N0	Positive	+	nd	nd
2	Invasive Ductal	T2	3	N1	Positive	-	70	0
3	Invasive Ductal	T2	3	N1	Positive	+	100	0
4	Invasive Ductal	T2	3	N1	Positive	-	100	100
5	Invasive Ductal	T1	3	N0	Negative	-	70	70
6	Invasive Ductal	T1	3	N1	Positive	-	225	300
7	Invasive Ductal	T1	1	N0	nd	-	300	100
8	Invasive Ductal	T1	1	N1	Positive	-	50	50
9	Invasive Ductal	T1	3	N0	Negative	-	0	50
10	Mixed	T1	2	N1	Positive	+	100	100
11	Mixed	T1	1	N1	Positive	-	100	100
12	Invasive Ductal	T2	3	N1	Negative	-	100	150

Table 3.4: BAG-1 auto-antibodies in breast cancer patients

Tumour size, grade nodal and oestrogen receptor (ERα) status were obtained from pathology reports. Tumour BAG-1 labelling was obtained by immunohistochemistry with antibody 3.10 G3E2 as described in section 2.12 and H scores are reported. Immunoreactivity of sera were determined as described in section 3.5 by immunoblot analysis. nd = Not determined. [a] mastectomy specimen contained no residual tumour.

3.6 Discussion

An assay was developed to determine immunoreactivity of human sera to BAG-1 proteins. Approximately a third of those tested produced specific antibodies to BAG-1 proteins and there were no differences between breast cancer patients and healthy controls. This almost exactly mirrors what is found with HSP70, to which BAG-1 binds and regulates where one third of breast cancer patients and controls are found to have anti-HSP70 autoantibodies. This is different however to HSP27 and HSP90 where autoantibodies to these proteins are not found in healthy controls and the presence autoantibodies to these proteins in breast cancer patients is associated with improved or worse prognosis respectively (Conroy et al. 1998a; Conroy et al. 1998b). The finding that auto-antibodies to BAG-1 exist in human sera is therefore unlikely to be of clinical significance. It remains possible however that BAG-1 is involved in regulating antigen presentation by heat shock proteins and the finding of auto-antibodies to BAG-1 may be of biological significance as auto-antibodies to various proteins have been previously described in healthy individuals even in the absence of auto-immune disease. These autoantibodies appear to be part of the normal functioning immune system and it has been suggested that they may be involved in clearance of aging or damaged cells or anti-tumoural or anti-viral surveillence (Lacroix-Desmazes et al. 1998).

Ten monoclonal antibodies available within the laboratory were characterised, with the specific aims of determining information immediately useful for further work. The techniques used were readily available within the laboratory and enabled determination of epitope localisation. From an overall consideration of the results it is likely that the antibodies are derived from four distinct clonal hybridomas. Since the hybridomas were initially grown and screened as mixed populations this is possible. 3.10 G3E11 and 3.10 G3F11 both have their epitope located at the junction of helix one and two on the BAG domain and behave with similar relative affinities. Similary 3.8 D4E9, 3.8 D4 F4, 3.8 D4G4 and 3.8 D4G5 all have similar reactivities to the mutants tested and with their epitope located or involving one of the two serines within the repeat sequence of BAG-1S. 3.10 G3E2 also has its epitope mapping to

those repeats but reacts differently to the mutants and so is derived from a different clone. Lastly 3.9 F1E11, 3.9 F1F9 and 3.9F1G10 are likely all derived from the same clone and have their epitope located between amino-acids 1-89 of BAG-1S. Further characterisation and optimisation of 3.10 G3E2 has resulted in a production of a preparation of antibody that works very effectively on formalin fixed paraffin embedded material. The 3.10 G3E2 ascites as a primary antibody for immunohistochemistry is effective reproducible and cheap (our preparation of primary antibody costs of 0.04 pence per slide compared to £1.08 per slide for KS-6C8 obtained commercially). This ascites produces comparable staining results to KS-6C8 as well as comparable results by Western blotting.

A BAG-1L specific antibody was successfully produced. Although the antibody worked well for immunoprecipitation and reasonably well for Western blotting it did not work at all for immunohistochemistry on formalin fixed paraffin embedded tissue. It did work well however on paraformaldehyde fixed cell culture material for immunoflourescence. With frozen tumour material both antibody 662 and 3.10 G3E2 failed to stain BAG-1 in the nuclei of any of the frozen tissue samples tested, even those samples from tumours where formalin fixed paraffin embedded tissue was known to stain for nuclear BAG-1 with 3.10 G3E2. This is an interesting phenomenon, particularly since nuclear BAG-1 staining of paraformaldehyde fixed cell culture material in immunoflourescence studies is exactly as expected with both antibodies. It is possible that BAG-1 epitopes in the cell nucleus are blocked in tumours but some how revealed by paraformaldehyde fixation or antibody retrieval techniques. In addition this also suggests that BAG-1L is present in the cytoplasm in tumour tissue under some conditions. There is evidence for example that BAG-1L interacts with the retinoblastoma protein, and disruption of this interaction results in cytoplasmic relocalisation of BAG-1L (Arhel et al. 2003).

Previous studies of the clinical significance of BAG-1 expression in breast cancer have produced conflicting results (Tang et al. 1999; Townsend et al. 2002; Turner et al. 2001; Cutress et al. 2001). This may stem, at least in part, from differences in the treatment regimens in various patient cohorts. In this study patients treated with hormonal therapy whose tumours expressed nuclear BAG-1 enjoyed

better outcome than those who did not. A strong inverse correlation was found between nuclear BAG-1 expression and tumour grade and a moderate association between nuclear BAG-1 expression and ERα and PgR expression. Therefore, this study suggests that there is a group of ERα and PgR positive tumours with high levels of nuclear BAG-1, and these patients respond relatively well to hormone therapy. Consistent with this, the PgR is a transcriptional target of ERα (Horwitz et al. 1978), and combined with ERα may better predict response to hormone therapy (ASCO 1996; Horowitz & McGuire 1975). BAG-1 is not oestrogen regulated so unlike PgR expression nuclear BAG-1 is not expressed as a consequence of ER signalling (Brimmell et al. 1999), and so it is possible that nuclear BAG-1 is playing a role in the signalling process itself

Although BAG-1 status was predictive of outcome in single variable analysis and predictive of outcome independent of tumour size and grade and patient age in limited multiple variable analysis, this could not be confirmed in multiple variable analysis including all variables. The reduced power of the multiple variable model when all variables were included was due to the fact that a significant proportion of the cohort had not undergone axillary surgery leading to a reduction in the number of evaluable cases and events. This is because treatment protocols were different in the unit when this cohort of patients was treated and not all patients with smaller tumours underwent axillary surgery. Further work is therefore required to confirm if the prognostic value of nuclear BAG-1 status in patients treated with hormonal therapy is independent of other parameters. Also as no studies have examined BAG-1 expression in paired breast tumour and lymph node metastases, and since such information has provided insights into BAG-1 function in the pathogenesis oral squamous cell carcinomas, this should also be addressed (Hague et al. 2002).

The correlation detected here between BAG-1 expression and ERα was found in a subset of patients in an earlier study, where a correlation with tumour grade, and a tendency for improved outcome in patients with high levels of nuclear BAG-1 was also found (Townsend et al. 2002). Tang et al., found in contrast that nuclear BAG-1 expression conferred worse prognosis in multivariate analysis (Tang et al. 1999). In the study described in this chapter the same antibody was used as in the study of

Turner et al. but whereas here nuclear BAG-1 was associated with improved outcome in their study cytoplasmic BAG-1 was associated with improved outcome (Turner et al. 2001). A strong correlation between nuclear and cytoplasmic BAG-1 expression was found in this study however and so it is possible that slight differences in the immunohistochemical and scoring techniques used between these studies account for some of the differences. It is also likely that some of the variation reported for the effect of BAG-1 on survival stems from the different cohorts studied. The patient cohort studied by Turner for example when compared to our cohort here, was younger, less frequently treated with hormone therapy, and a proportion was also treated with chemotherapy. Future studies should focus on the impact of BAG-1 in well-defined patient cohorts or be sufficiently large to allow meaningful subgroup analysis.

It is not immediately apparent why an anti-apoptotic protein might be associated with good prognosis. Such an observation is not unique however, since expression of Bcl-2 is also associated with good prognosis in breast cancer. Multiple alterations contribute to carcinogenesis, and tumours that counter apoptosis by overexpressing Bcl-2 and/or BAG-1 might represent one class of tumours. Other tumours may have disabled apoptotic responses through accumulation of distinct changes (e.g., p53 mutation, ErbB2 overexpression) which might have more profound effects on cell death sensitivity or additionally affect proliferative pathways resulting in more aggressive tumour growth. Effects of BAG-1 on NHR function in hormone sensitive tumours may also play a role.

Some of the inconsistencies between studies will undoubtedly stem from experimental differences and the subjective nature of immunohistochemical analyses. The signal detected in an immunohistological assay is not linear with the antibody concentration, nor with many other of the technical parameters (Wynford-Thomas 1992). In addition such immunohistochemical studies only provide a "snapshot" in time of the characteristics of the tumour, and do not easily appraise tumour heterogeneity. For example in the four published studies of BAG-1 expression in breast cancer, various antibodies, antigen retrieval methods and scoring systems (intensity versus "H-score") were used, all of which might have significantly

influenced the results (Table 1.3). A change in antigen retrieval technique or scoring threshold might alter the proportion of tumours that are considered to be positive for expression and might contribute to the variation in detection of nuclear BAG-1 expression in breast cancer. Such difficulties, amongst others, have been encountered with the immunohistochemical analysis of p53 (Wynford-Thomas 1992). Although BAG-1 is part of a family of related proteins, we have seen no evidence of cross reaction of these antibodies with other BAG family members, but this remains a theoretical possibility, especially in antibodies raised against antigens that contain the conserved BAG domain.

Another important difference between studies is the composition of the patient cohorts, which are likely to differ in many ways e.g., menopausal status and stage and treatment. Since all studies to date are retrospective, the patient selection and exclusion criteria, treatment protocols and outcome measures are both different between studies and not necessarily available in their entirety. Since BAG-1 can impact on multiple cell control pathways, the impact of specific patterns of BAG-1 expression on survival may depend greatly on the treatment applied in different cohorts. For example, only BAG-1L regulates ERα and ERβ function (chapter 4) and the expression of nuclear BAG-1L might be particularly important in determining response to hormonal therapy in hormone sensitive cancers such as breast and prostate cancer. By contrast, all BAG-1 isoforms appear to possess anti-apoptotic activity and therefore cytoplasmic BAG-1S might be particularly important in determining responses to chemotherapy. Therefore, the significance of nuclear or cytoplasmic staining might differ depending on the primary treatment modality and since there is heterogeneity between previous studies as to treatment, the differences between these studies must be interpreted in the light of this.

4. BAG-1 and oestrogen dependent transcription

4.1 Introduction

Oestrogens play an important role in the development of breast cancer (Clemons & Goss 2001; Colditz 1998; Dickson & Stancel 2000), and stimulate both the proliferation and survival of breast cancer cells (Foster et al. 2001; Mandlekar & Kong 2001). Adjuvant hormonal therapies such as Tamoxifen counter the actions of oestrogens and reduce the probability of death and recurrence in those with breast cancer (EBCTCG 1998). The actions of oestrogens and anti-oestrogens are mediated by two receptors, oestrogen receptor alpha (ERα) and the more recently identified oestrogen receptor beta (ERβ). Identifying factors that influence ER function will increase our understanding of key mechanisms involved in the development of breast cancer and may provide strategies to improve hormonal therapy.

BAG-1 isoforms bind and regulate the activity of several nuclear hormone receptors (NHR) (Table 1.1) (Cato & Mink 2001; Froesch et al. 1998; Guzey et al. 2000; Kullmann et al. 1998; Liu et al. 1998; Witcher et al. 2001; Townsend et al. 2003b). In addition BAG-1L activates transcription from a range of promoters apart from those associated with nuclear hormone receptors, and BAG-1M produces similar effects following heat shock (Niyaz et al. 2001; Zeiner et al. 1999). Activation or repression of NHR function by BAG-1 is frequently isoform specific. For example, BAG-1L, but not BAG-1S and BAG-1M increases androgen receptor function (Froesch et al. 1998). By contrast, BAG-1M and BAG-1L but not BAG-1S inhibits glucocorticoid receptor activity (Kullmann et al. 1998). It is thought that, at least in part, BAG-1 functions via HSC70/HSP70 since chaperone molecules are important for NHR function (Cheung & Smith 2000; Pratt & Toft 1997). BAG-1 NHR modulatory function is often (Briknarova et al. 2001; Knee et al. 2001), but not always (Liu et al. 1998), dependent on BAG-1 carboxy-terminal regions and residues important for chaperone binding (Knee et al. 2001). BAG-1 may in such cases act to regulate the refolding of receptors by HSC70/HSP70 required when changing from

ligand bound to ligand free conformations. It has been suggested that BAG-1 mediated modulation of androgen receptor function may be of relevance to mechanisms by which prostate cancers become resistant to hormonal therapies (Froesch et al. 1998), and this may also be the case with the oestogen receptor in breast cancer. BAG-1M has for example been demonstrated to interact with ERα in vitro (Zeiner & Gehring 1995) but it had not been reported whether BAG-1 isoforms bind ERα in cells and whether BAG-1 proteins alter receptor function. Earlier work in Dr Packham's laboratory however had demonstrated that BAG-1 did bind to ERα in untransfected MCF7 cells (Cutress et al. 2003), but did not show which BAG-1 isoforms were responsible for binding to ERα since the BAG-1 antibody used detected all BAG-1 isoforms. The aims of the experiments presented in this chapter were to determine which BAG-1 isoform(s) bind to oestrogen receptors ERα and ERβ, and more importantly to determine if BAG-1 modulates transcription through these receptors.

4.2 BAG-1 and oestrogen dependent transcription in MCF7 cells

Since BAG-1 modulates the function of nuclear hormone receptors, and nuclear BAG-1 expression was shown to be associated with ERα and PgR expression and was predictive of survival breast cancer patients treated with hormone therapy (Chapter 3) experiments were performed to determine if BAG-1 might impact on ER function. The effect of overexpression of the various BAG-1 isoforms on ER dependent transcription was therefore studied.

4.2.1 BAG-1L potentiates oestrogen-dependent transcription

Reporter assays were used as a specific and sensitive technique to determine whether BAG-1 isoform expression alters the ability of 17 β-oestradiol (the major secreted oestrogen) to stimulate ER-dependent transcription. These assays involve transient transfection of reporter plasmids containing DNA binding sites for ERα, a basal promoter to provide binding sites for general transcription factors and to determine correct initiation of transcription, and an easily assayed marker gene. The

first oestrogen response element (ERE) identified was that of the vitellogenin A2 gene of *Xenopus laevis* and for oestrogen dependent transcription in these studies a reporter containing three copies of this response element cloned upstream of the minimal HSV-1 TK promoter and luciferase reporter gene was used. Luciferase is an enzyme derived from the firefly *Photinus pyralis* which in the presence of a suitable substrate will produce light. Various studies have demonstrated that light production in these systems is proportional to transcriptional activity (White & Parker 1999). To control for transfection efficiency and for general effects on transcription a second reporter containing a different marker (the enzyme β-galactosidase) under the constitutive CMV IE promoter was used and cotransfected in the assays described. Reporter assays were performed in MCF-7 breast cancer cells containing endogenous ERα and ERβ, and HEK-293 cells cotransfected with oestrogen receptors. These assays are specific in that other assays of oestrogenic activity, such as breast cancer cell proliferation may, for example be effected by other activities of BAG-1 such as a possible direct mitogenic action through Raf-1. Reporter assays are also very sensitive and concentrations of 17β-estradiol down to 1 pM can be detected in a dose dependent manner (Pons et al. 1990).

Initial experiments were performed to determine the optimal ratios and 17β-estradiol concentrations for further experiments (data not shown). In MCF-7 cells transfection of 50% (3.3 μg) BAG-1 pcDNA based expression construct along with 25% (1.7 μg) of each of the two reporter constructs allowed maximal BAG-1 overexpression with minimal loss in overall assay sensitivity. Dose response experiments demonstrated that maximal responses occurred at 100 – 200 pM of 17β-estradiol. Physiological concentrations of 17β-estradiol are less than 150 pM in post-menopausal women. In pre-menopausal women 17β-estradiol concentrations are less than 110 pM within the follicular phase of the menstrual cycle and range between 300 to 1000 pM during mid-cycle and the luteal phase, demonstrating that the assays reported here are performed at approximately physiological concentrations.

MCF7 cells were cotransfected with the ERE-containing luciferase reporter construct and with expression plasmids engineered to overexpress specific BAG-1 isoforms (Townsend et al. 2003a), and treated with 17β-estradiol. A concentration of

17β-estradiol of 10 pM was chosen since dose response experiments established that this concentration produced half the maximal response from these assays, and so this concentration might be best expected to demonstrate any positive or negative effects of BAG-1 on transcription. Overexpression of BAG-1L, but not BAG-1S or BAG-1M, increased transcriptional responses to 10 pM 17β-oestradiol (Figure 4.1) by approximately 50%. This effect of BAG-1L was specific since neither 17β-estradiol nor any of the BAG-1 isoforms, including BAG-1L, had any effect on the expression of luciferase in cells transfected with a control reporter construct lacking ERE (Figure 4.1). In addition BAG-1L did not increase expression of the CMV IE promoter within the β-galactosidase expression construct used to control for transfection efficiency relative to BAG-1S or BAG-1M transfected cells (data not shown). The lack of effect of BAG-1S and BAG-1M was not due lack of BAG-1 protein expression, since all the BAG-1 isoforms were over-expressed to similar levels in MCF7 cells (Figure 4.2).

The effects of BAG1-L were dose-dependent and BAG-1L mediated potentiation of oestrogen dependent transcription increased with increasing BAG-1L overexpression (data not shown). For example in two experiments, each performed in quadruplicate, where 1.65 μg of BAG-1L and 1.65 μg of empty pcDNA was transfected, transcription was increased by 27% (SEM 17%) when compared with transcription if all the 3.3 μg was empty pcDNA. When 3.3 μg of BAG-1L expression construct was used transcription increased to 54% (SEM14%) when compared with transcription if all the 3.3 μg was empty pcDNA. The fact that this effect is titratable is consistent with a causal relationship between the increase in BAG-1L expression and the increase in transcriptional activity.

Dose response experiments were performed in MCF-7 cells with varying concentrations of 17β-oestradiol. MCF-7 cells overexpressing BAG-1L were compared to MCF-7 cells transfected with the control pcDNA expression construct. In these experiments, MCF7 cells overexpressing BAG-1L were more sensitive to 17β-oestradiol across the whole dose-response range, and also produced a greater maximal response to 17β-oestradiol. In the presence of BAG-1L five times less hormone was required to produce a twofold increase in transcription (Figure 4.3).

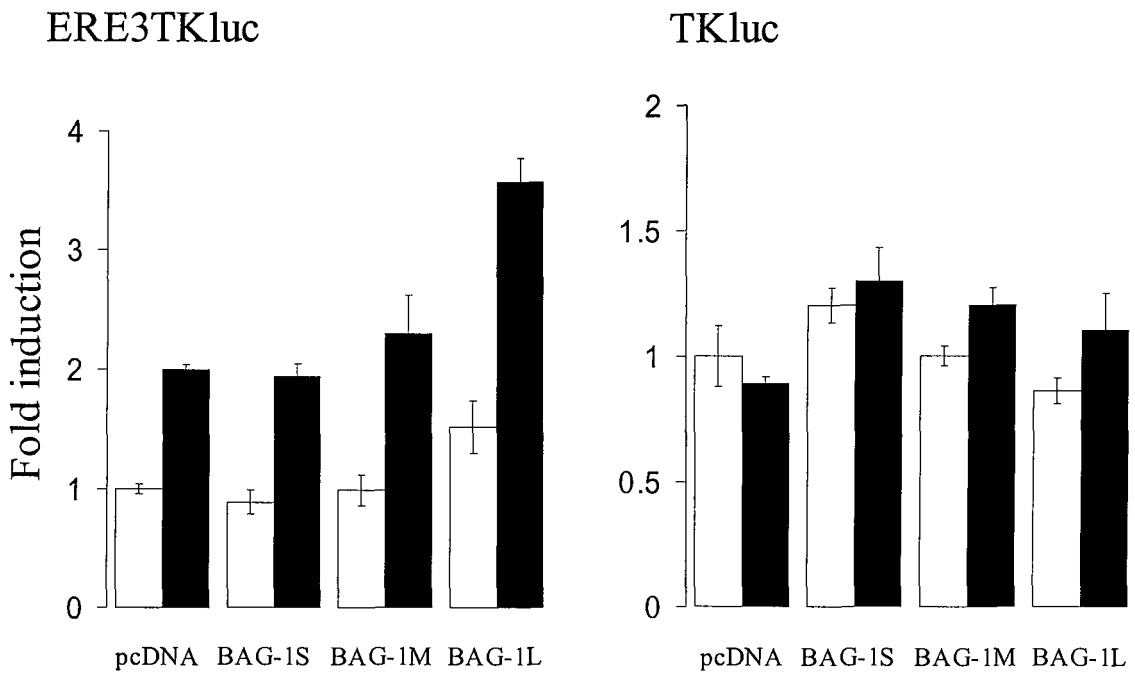


Figure 4.1: Effect of BAG-1 isoform overexpression on ER-dependent transcription MCF7 cells were cotransfected with BAG-1 isoform expression plasmids or pcDNA3 and with ERE3TKluc or control TKluc reporter plasmids. Cells were stimulated with 10 pM 17β-estradiol (closed bars) or left untreated as a control (open bars) for 24 hours and analysed for luciferase activity. The luciferase activity of untreated pcDNA3 transfected cells was normalised to one; the absolute reporter values of untreated pcDNA3 transfected cells were up to 15 fold higher with the ERE3TKluc reporter construct compared to values obtained with the TKluc reporter construct. The levels of transcription in cells transfected with the BAG-1L expression construct and stimulated with 17β-estradiol were significantly higher than levels in cells transfected with pcDNA3 or other BAG-1S or BAG-1M expression constructs ($p=0.005$). The values shown are mean +/- SE of the mean and are derived from 2 separate experiments each with triplicate determinations.

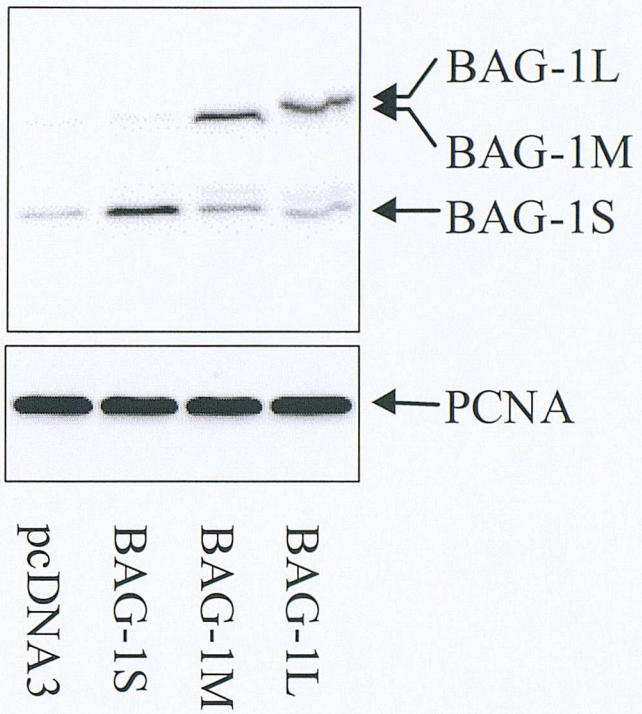


Figure 4.2: Expression levels of BAG-1 constructs in MCF7 cells

MCF7 cells were transfected with the indicated BAG-1 isoform-specific expression constructs, or pcDNA3 as a control, and expression of BAG-1 and PCNA (loading control) analysed by immunoblotting. The position of migration of the BAG-1 isoforms is indicated.

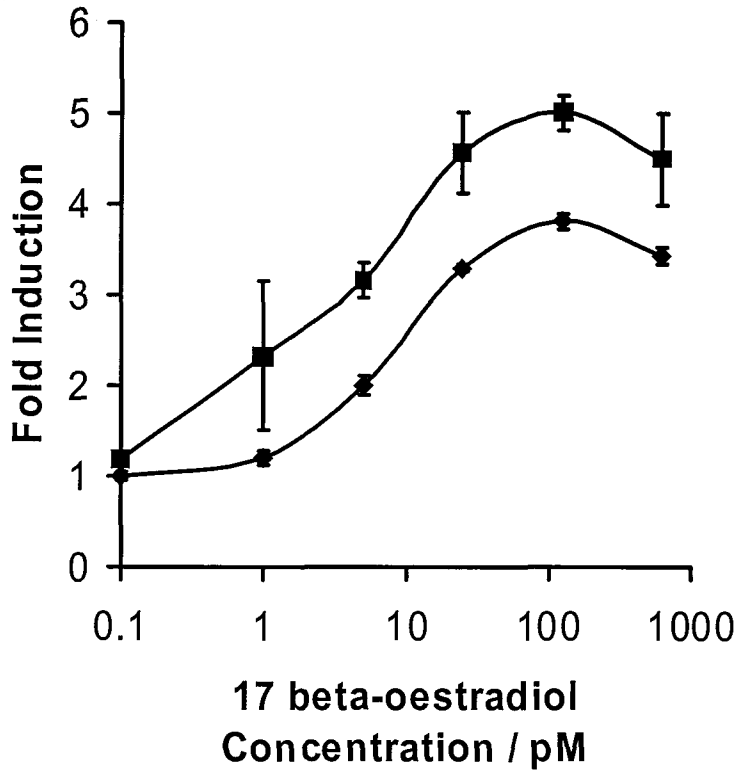


Figure 4.3: Dose response experiments

MCF7 cells were transfected with the BAG-1L expression plasmid (■) or pcDNA3 (◆) and the ERETKluc reporter plasmid. Cells were treated with the indicated concentrations of 17β-estradiol for 24 hours and luciferase activity determined. The values shown are mean +/- SE of the mean and are derived from an experiment with triplicate determinations, representative of 2 such experiments.

The effect of antiestrogens on ER transcriptional activity in MCF-7 cells transfected with BAG-1L or empty pcDNA control plasmid was tested with ICI 182 780 (Faslodex), a pure antiestrogen that unlike Tamoxifen (Nolvadex) has no oestrogenic properties. Cells were treated (one experiment, data points in triplicate) with 100 pM 17β-estradiol to produce maximal transcriptional responses and increasing amounts of ICI 182 780 up to 100 nM was added (data not shown). ICI 182 780 decreased transcription at all concentrations, however at lower concentrations of ICI 182 780, for example 0.1 nM, BAG-1L overexpressing cells were relatively less affected. High levels of ICI 182 780 completely blocked ER dependent transcriptional responses in both control and BAG-1L overexpressing cells. Therefore although transcriptional activity was lower in the presence of antiestrogen both with and without BAG-1L, responses to 17β-oestradiol in BAG-1L transfected cells were still greater until the system was saturated with anti-oestrogen. Presumably in these transient assays the response to oestrogen depends on the competition and ratios between agonist and antagonist. BAG-1L appears to increase the response of the receptor to agonist and so at any given concentration (until saturation) of antagonist BAG-1L attenuates the inhibitory effect by potentiating agonist activity. When the system is saturated with the antagonist, agonist activity is completely blocked and BAG-1L has no effect.

4.3 BAG-1 interacts with both ERα and ERβ

Since BAG-1L potentiates oestrogen dependent transcription and BAG-1 has been reported to bind to the oestrogen receptor in vitro, and in untransfected MCF-7 cells, experiments were performed to determine which BAG-1 isoforms bind to oestrogen receptors in vivo.

4.3.1 BAG-1L interacts with ER alpha and beta

Overexpression studies were performed in HEK-293 cells to determine which BAG-1 isoform(s) interacted with ERα (Figure 4.4). HEK-293 cells express low levels of BAG-1 and do not express oestrogen receptors and therefore were used as a useful model for overexpression studies. Consistent with the low levels of

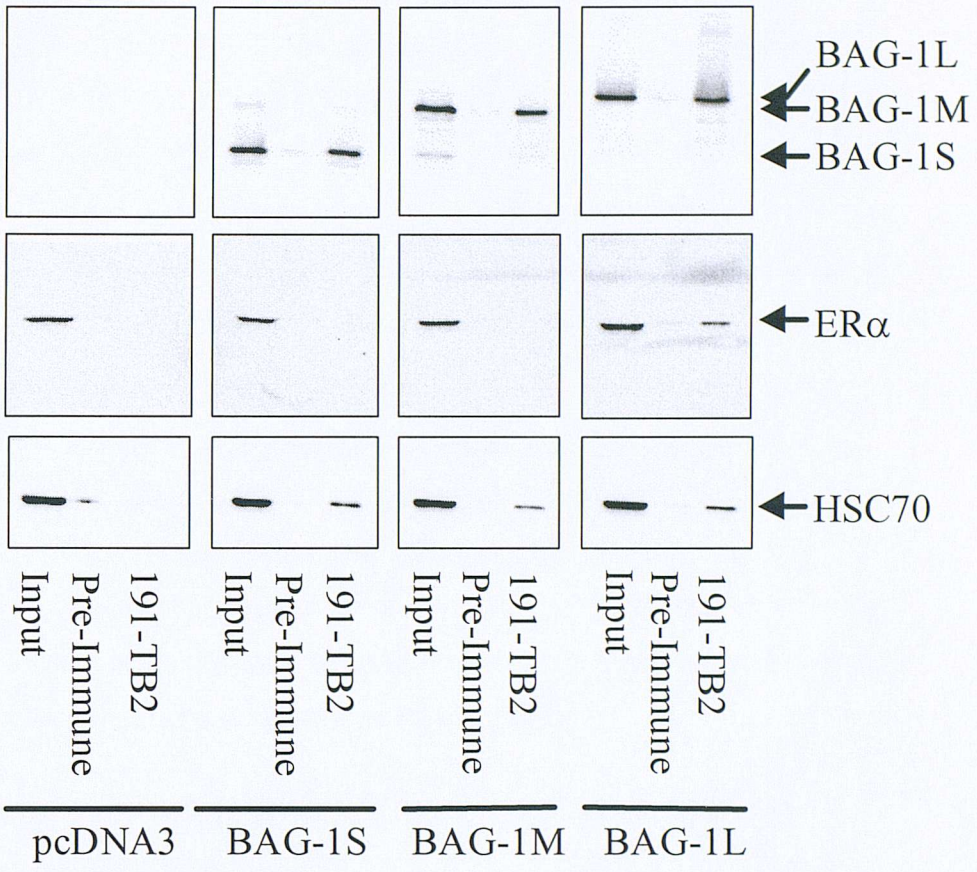


Figure 4.4: Specific interaction of BAG-1L and ERα

HEK-293 cells were cotransfected with an ERα expression construct and BAG-1 isoform expression constructs or pcDNA3 as indicated. BAG-1 proteins were immunoprecipitated using BAG-1 specific polyclonal antibody 191-TB2 (191-TB2) or with preimmune serum (Pre-immune) as a control and immunoprecipitated proteins analysed by immunoblotting using 3.10 G3E2 for BAG-1, 6F11 for ERα and B6 for HSC70. 10% of the lysate (Input) from each transfection was kept to demonstrate expression in each case prior to immunoprecipitation.

endogenous BAG-1 in these cells, significant interaction of endogenous BAG-1 with HSC70 and ERα was not detected. When BAG-1 isoforms were over-expressed however, significant binding to HSC70 was detected with all BAG-1 isoforms. However, when BAG-1L was overexpressed, an interaction between BAG-1L and ERα was detected. Although all BAG-1 isoforms interacted with HSC70, only BAG-1L associated with ERα. It should also be noted from the expression of ERα in the input whole cell lysates (Input) that BAG-1L expression did not alter expression of ERα. This indicates that the BAG-1L mediated increase in transcription (section 4.4.1) was not due to changes in ERα expression mediated by BAG-1L.

To test whether BAG-1L interacted with ERβ, HEK-293 cells were cotransfected with expression constructs encoding BAG-1 isoforms and ERβ in a similar manner. BAG-1 proteins were immunoprecipitated, and ERβ was only detected in BAG-1 precipitates when BAG-1L was co-transfected but not when the other BAG-1 isoforms were cotransfected (data not shown). BAG-1L and not BAG-1M or BAG-1S therefore interacts with both ERα and ERβ.

4.3.2 The interaction between BAG-1L and ERα is not disrupted by DNAse

BAG-1 binds to DNA directly (Niyaz et al. 2001; Zeiner et al. 1999) and it is possible that the interaction observed between BAG-1 and ERα is mediated through or stabilised by DNA. BAG-1 could for example bind to DNA and through HSP70/HSC70 tether the ERα transcriptional complex to the ERE. To test if DNA might be a necessary intermediary in the BAG-1 ERα interaction lysates from HEK-293 cells co-transfected with BAG-1L and ERα were pre-incubated with RQ1 DNAse and then co-immunoprecipitation for BAG-1 was performed. The interaction between BAG-1L and ERα was not disrupted by pre-incubation with DNAse however as ERα was still detectable in BAG-1L but not control immunoprecipitates (Figure 4.5B).

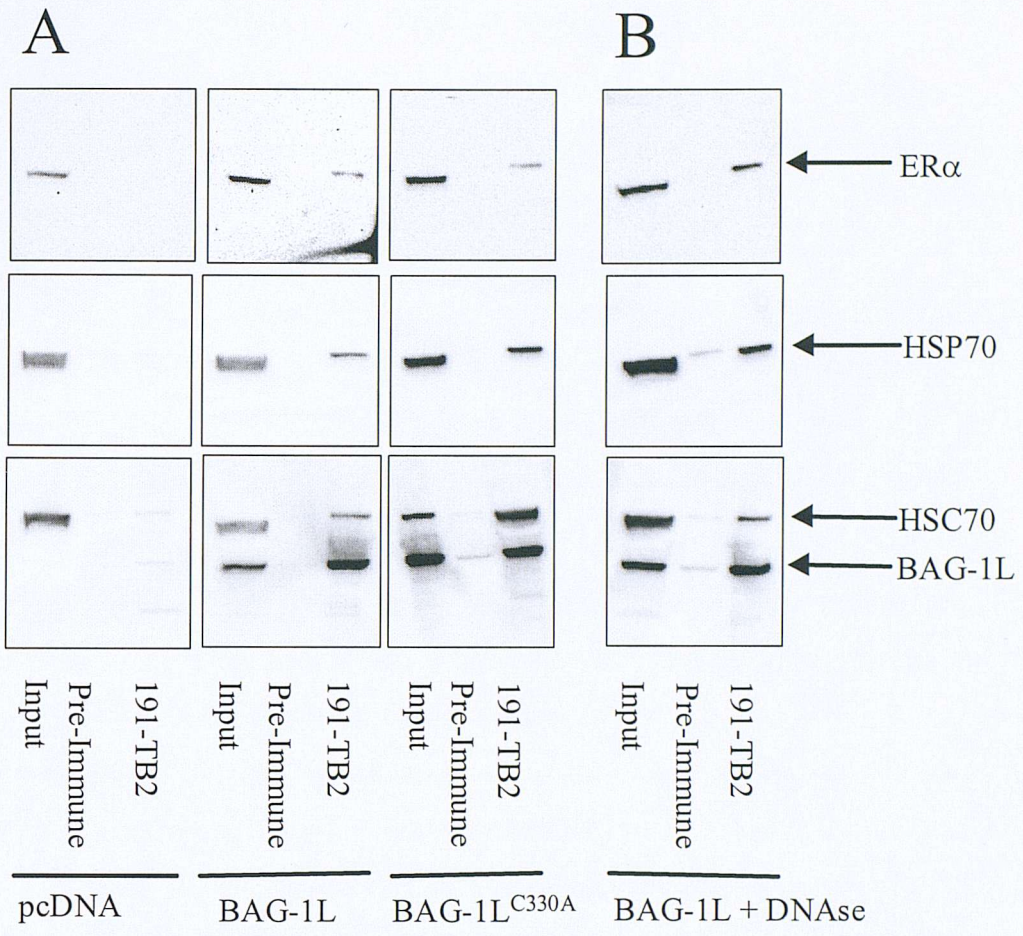


Figure 4.5: Interaction of BAG-1L and BAG-1L^{C330A} with ERα

HEK-293 cells were cotransfected with an ERα expression construct and expression constructs for either pcDNA3 as a control, BAG-1L or the point mutant BAG-1L^{C330A} (A). In (B) cells were co-transfected with ERα expression construct and BAG-1L and incubated with DNAse following cell lysis. BAG-1 proteins were immunoprecipitated using BAG-1 specific polyclonal antibody 191-TB2 (191-TB2) or with preimmune serum (Pre-Immune) as a control and immunoprecipitated proteins analysed by immunoblotting using 3.10 G3E2 for BAG-1, 6F11 for ERα and C92F3A5 for HSP70. The input whole cell lysate (10% of input to immunoprecipitation) is shown to demonstrate expression. Following immunoblotting for BAG-1, filters were reprobed for HSC70 with B6 and therefore demonstrated both BAG-1 and HSC70 immunoreactivity.

4.3.3 The major direct interaction partner of BAG-1 is HSC70/HSP70

It was not clear if BAG-1L bound directly to ERα and ERβ, or if binding was mediated through the chaperones HSC70/HSP70. Far Western blotting was performed to determine whether the interaction between BAG-1L and ERα might be direct. Lysates from a range of cell lines were separated by SDS-PAGE and transferred onto nitrocellulose membrane. The nitrocellulose membrane was then incubated with GST-BAG-1 (Figure 4.6B) or GST (Figure 4.6A), and anti-BAG-1 antibody 191-TB2. GST-BAG-1 was allowed to bind to protein on the filters, and the anti-BAG-1 antibody detected any direct interaction between GST-BAG-1 and proteins on the filter. Bands were detected at 70 kDa consistent with a direct interaction with the chaperones HSC70/HSP70. Bands were not detected in the ERα positive cell lines (MCF-7, ZR-75-1) at the expected size for ERα of 66 kDa. This suggests that the interaction between BAG-1 and ERα is indirect and mediated through heat shock proteins. Some larger molecular weight bands were detected. However co-immunopreciptation assays performed to test if the chaperone Hsp90 bound to BAG-1 did not demonstrate an interaction between BAG-1 and HSP70 (data not shown). BAG-1 proteins were not seen in the presence of GST-BAG-1 because recombinant BAG-1 "blocks" the antigen binding sites of the antibody. Consistent with this BAG-1 isoforms are detected when GST is used in place of GST-BAG-1. Further, this demonstrates that the identification of HSC70/HSP70 as direct binding partners is specific since bands at 70 kDa are not detected in the presence of GST lacking BAG-1.

These results are consistent with other work and implies that at least some of BAG-1's multiple interactions including that with oestrogen receptors, could be mediated indirectly via these heat shock proteins (Zeiner et al. 1997; Zeiner & Gehring 1995). Experiments were also performed using other anti-BAG-1 antibodies including 3.10 G3E2 with essentially the same results (data not shown). Interactions between BAG-1 and HSC70/HSP70 were also consistently and readily detected by multiple other techniques (see for example Figures 4.4, 4.5, 5.3 and 6.1).

A. TB2 + GST

B. TB2 + GST-BAG-1

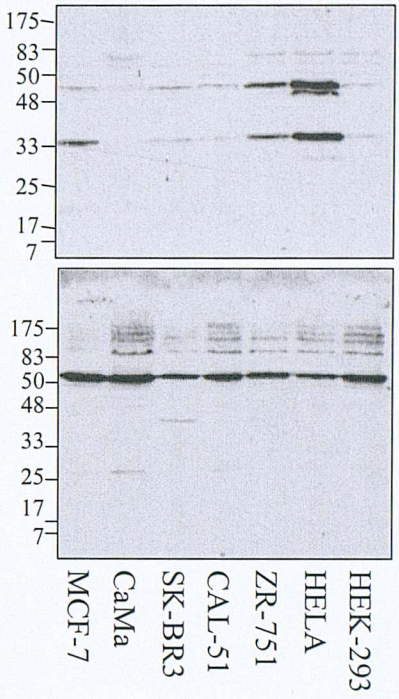


Figure 4.6: Far Western Blot Analysis of BAG-1S direct binding partners
Lysate from a panel of cell lines (40 µg each) was separated by SDS-PAGE, transferred onto nitrocellulose membrane and the filter incubated with TS containing 2% (w/v) non-fat skimmed milk. The filter was then incubated with primary antibody 191-TB2 at 1/1000 dilution in TS with 2µg/ml GST-BAG-1S (B) or 2µg/ml GST (A) added. Bound complexes were probed with an HRP conjugated secondary antibody and detected by chemiluminescence.

4.4 BAG-1 enhances transcription through both ER alpha and beta

4.4.1 BAG-1L enhances ERα transcription in HEK-293 cells

MCF7 cells express both ERα and ERβ and to determine whether the activity of both receptors was regulated by BAG-1L, a model was developed using HEK-293 cells, which lack endogenous oestrogen receptors and contain low levels of endogenous BAG-1. Initial dose response experiments with cells transfected with pcDNA , ERα expression construct and the ERE-TK-Luc and CMV β-galactosidase reporters suggested that the optimal ratios for co-transfection were 4:2:2:1 respectively (data not shown). These ratios produced the maximal BAG-1 expression with the minimal loss in assay sensitivity. Dose response experiments with pcDNA confirmed that, as with MCF-7 cells, half maximal response was obtained with 10 pM 17β-oestradiol (data not shown). This concentration of 17β-oestradiol was therefore used for subsequent experiments with ERα in HEK-293 cells.

BAG-1L overexpression increased transcription approximately fivefold over that obtained in control pcDNA transfected cells in the absence of added 17β-estradiol (Figure 4.7). This was in contrast to MCF-7 cells where BAG-1L did not consistently increase transcriptional activity in the absence of added 17β-estradiol. In control pcDNA transfected cells transcription increased fivefold with the addition of 10 pM 17β-estradiol. Additionally, overexpression of BAG-1L increased transcription approximately threefold over that obtained in control pcDNA transfected cells in the presence of 10 pM added 17β-estradiol. There are several possible explanations for the increase in transcriptional activity obtained with BAG-1L overexpression in the absence of added 17β-estradiol in HEK-293 cells. These include the possibilities that there may be low levels of residual oestrogens in unstimulated cells, or different levels of basal transcriptional activity through the AF1 domain of transfected ERα compared to endogenous ERα, or cell specific differences in co-activators or co-repressors. Enhancement of transcription by BAG-1L was dependent on the presence of ERα, as transfection of empty pSG-5 plasmid lacking

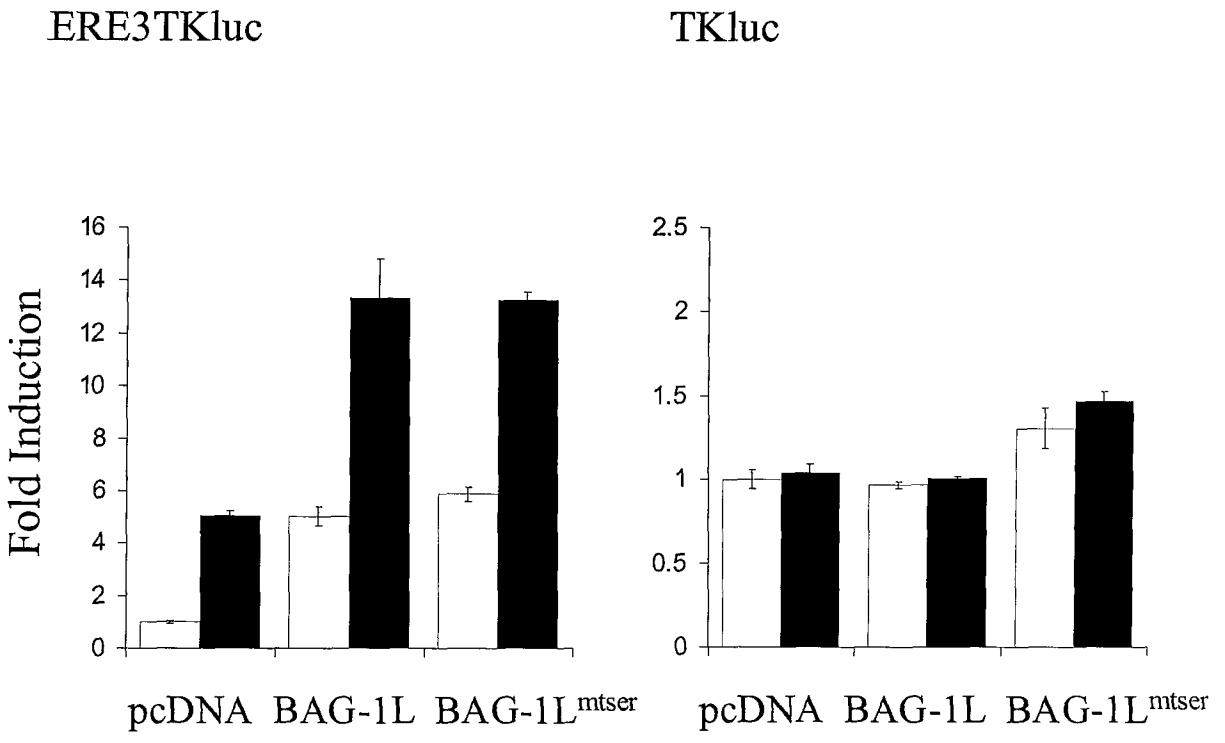


Figure 4.7: Effect of BAG-1L and BAG-1L^{mtser} on ERα mediated transcription in a HEK-293 model system

HEK-293 cells were cotransfected with BAG-1L expression plasmid or pcDNA or BAG-1L^{mtser}, and with ERα expression plasmids and with either the ERETKluc reporter plasmid or with control luciferase reporter lacking the ERE. Cells were stimulated with 10 pM 17β-estradiol (closed bars) or left untreated as a control (open bars) for 24 hours and analysed for luciferase activity. The values shown are mean +/- SE of the mean and are the results of one experiment performed in triplicate.

the ERα sequence did not produce these increases in transcription with 17β-estradiol or BAG-1L overexpression demonstrating that the effect was mediated through the receptor (data not shown). In addition BAG-1L did not increase transcriptional activity in HEK-293 cells transfected with a control reporter construct lacking ERE demonstrating that the effect was specific for the ERE (Figure 4.7).

4.4.2 BAG-1L enhances transcription through both ER alpha and beta

To determine whether the activity of both ERα and ERβ was regulated by BAG-1L HEK-293 cells were transfected with the BAG-1L expression plasmid, the ERE reporter and expression constructs for ERα or ERβ. Initial experiments demonstrated that in HEK-293 cells transfected with pcDNA the maximal response to 17β-estradiol occurred at higher concentrations than with ERα, consistant with previous reports that suggested that ERβ has reduced affinity for the vitellogenin ERE than ERα (Tremblay et al. 1997). With ERβ, half the maximal response was obtained at 100 pM 17β-oestradiol (data not shown) and this concentration was therefore used for further experiments with ERβ. Cells were stimulated with 17β-oestradiol (10pM for ERα, 100pM for ERβ) or left untreated as controls. BAG-1L potentiated ER-dependent transcription in cells transfected with ERα. Although the effects were more modest, BAG-1L also stimulated ERβ mediated transcription (Figure 4.8A). Therefore, BAG-1L stimulates the activity of both ERα and ERβ.

4.4.3 BAG-1L and not the other isoforms increases ER transcription

Since only BAG-1L binds to ERα and ERβ, and only BAG-1L increases oestrogen dependent transcription through endogenous oestrogen receptors in MCF7 cells, experiments were performed to determine which BAG-1 isoforms potentiate transcription in the HEK-293 based model. Similar to MCF-7 cells only the BAG-1L isoform increased transcription through ERα and ERβ (Figure 4.8B and 4.12).

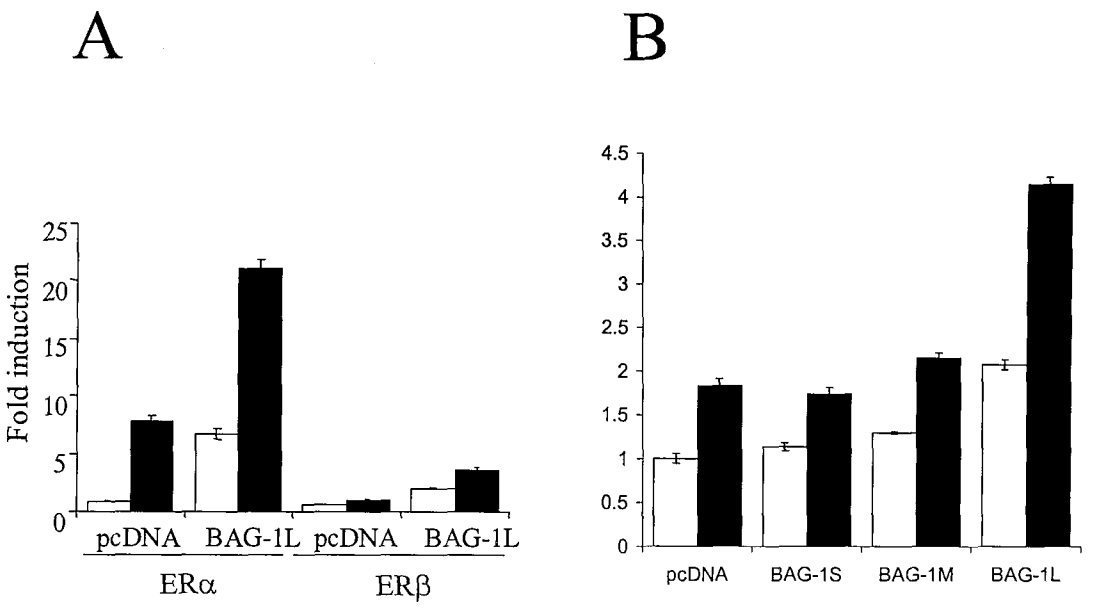


Figure 4.8: Effects of BAG-1 on ERα and ERβ mediated transcription
HEK-293 cells were cotransfected with BAG-1 expression plasmid as indicated or pcDNA3, with ERα or ERβ expression plasmids as indicated (A) or with ERβ (B) and with the ERETKluc reporter plasmid. Cells were stimulated with 10 pM (ERα) or 100pM (ER β) 17β-estradiol (closed bars) or left untreated as a control (open bars) for 24 hours and analysed for luciferase activity. The transcriptional activity of both ERα and ERβ was increased by BAG-1L compared to pcDNA3 ($p<0.01$ for both ERα and ERβ). The values shown are mean +/- SE of the mean and are derived from one experiment with triplicate determinations (A) or an experiment with triplicate determinations, representative of 2 such experiments (B).

4.5 BAG-1L and ERα phosphorylation at serine 118

As BAG-1 activates Raf-1 and the MAP kinase pathway independent of RAS (Song et al. 2001) it was important to exclude the possibility that BAG-1 potentiated ER dependent transcription through activation of this pathway. For example ERα is phosphorylated in-vivo through the MAP kinase pathway at serine 118 and this increases transcriptional activity through AF1 of ERα (Chen et al. 2002a; Kato et al. 1995). This provides a mechanism of cross talk between growth factor receptors and NHR (Kato et al. 2000). In addition overexpression of RAS, which activates the MAP kinase pathway increases transcription through ERα. It was therefore possible that an alternate mechanism by which BAG-1 potentiates ER mediated transcription might be through activation of the MAP kinase pathway, through direct binding to and activation of Raf-1 by BAG-1. To test if BAG-1L increased ERα transcriptional activity through this mechanism, two ERα point mutants were employed (Chen et al. 2002a). HE457 has serine 118 replaced by an alanine and so can not be phosphoylated at this position. HE458 has serine 118 replaced by glutamic acid and so mimics phospho-serine at this position.

Compared to wild type ERα, transcriptional activity was reduced in the absence and presence of added 17β-estradiol when the HE457 construct was co-transfected with pcDNA (Figure 4.9). This is consistent with a role for MAP kinases in ERα function by phosphorylation of ERα at serine 118. The effect of BAG-1L was not altered by co-transfection of these mutants in place of ERα however (Figure 4.9), demonstrating that stimulation of ERα function by BAG-1L is not mediated by phosphorylation at serine 118.

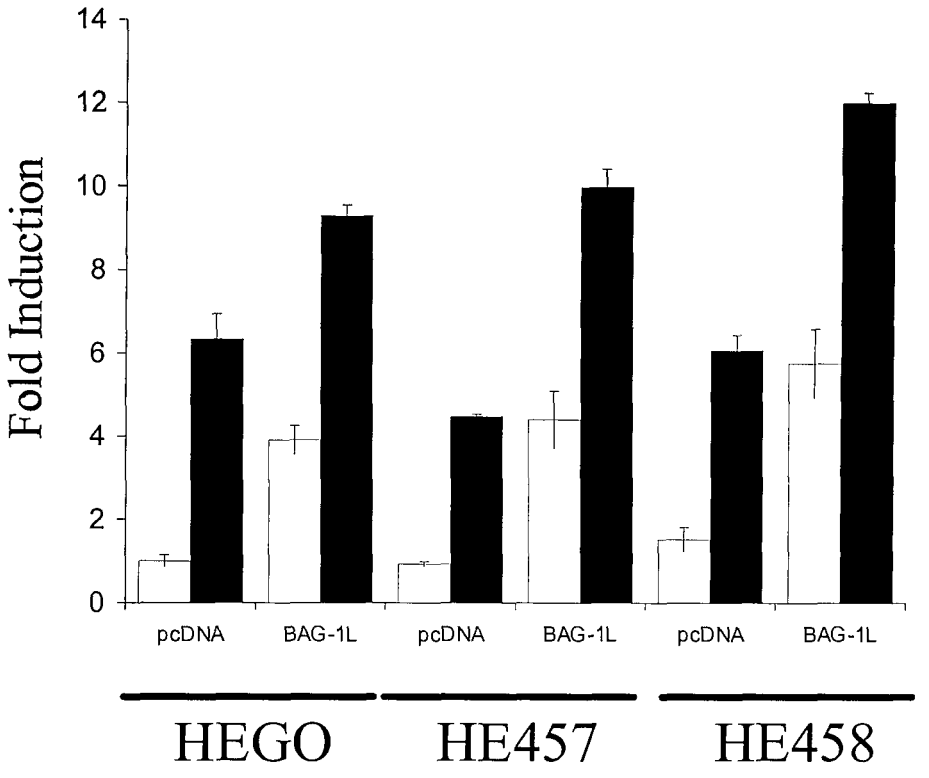


Figure 4.9: Effect of ERα serine 118 mutation BAG-1 mediated potentiation of ERα transcription

HEK-293 cells were cotransfected with BAG-1L expression plasmid or pcDNA3, with ERα or ERα mutants HE457 and HE458 as indicated and with the ERETKluc reporter plasmid. Cells were stimulated with 10 pM 17β-estradiol (closed bars) or left untreated as a control (open bars) for 24 hours and analysed for luciferase activity. The values shown are mean +/- SE of the mean and are derived from one experiment with triplicate determinations.

4.6 BAG-1L structural analysis

To analyse the functional importance of differing regions of BAG-1L various point mutants of BAG-1L were tested in transcription and interaction assays with ERα.

4.6.1 BAG-1L phosphorylation is not required

BAG-1 can be phosphorylated in vivo and the role of this has not been defined. Since kinase pathways and BAG-1 alter ERα function it is important to determine if phosphorylation of BAG-1 plays a role in ERα signalling. BAG-$1L^{mtser}$ is a multiple BAG-1L point mutant that has all 9 serine residues within the multiple repeats substituted with alanine residues. This mutant is not phosphorylated in vivo (Schneikert et al. 2000), and was used to test the requirement of BAG-1L phosphorylation for BAG-1L mediated ERα transcriptional enhancement. This mutant enhanced transcription through ERα similarly to wild type BAG-1L (Figure 4.7) demonstrating that BAG-1L phosphorylation within the repeat region is not required for BAG-1L mediated potentiation of ERα signalling.

4.6.2 BAG-1L BAG domain mutants

Since many of BAG-1's interactions appear to be mediated through HSP70/HSC70 point mutants of BAG-1 that do not bind these chaperones were generated to test in the transcription and interaction assays. In mouse BAG-1S substitution of cysteine to an alanine at amino acid 204 was found to prevent binding of these chaperones (Song et al. 2001). Initially therefore the equivalent point mutant of BAG-1L, BAG-$1L^{C330A}$ was produced. This mutant was tested in the interaction assay and surprisingly it was found to bind to ERα (Figure 4.5). Further analysis demonstrated that unlike the equivalent mouse mutant it did bind to both HSC70 and HSP70 (Figure 4.5). In addition in the functional transcription assays it potentiated oestrogen dependent transcription no differently to wild type BAG-1L (Figure 4.10). This was also surprising since further work demonstrated that although the BAG-

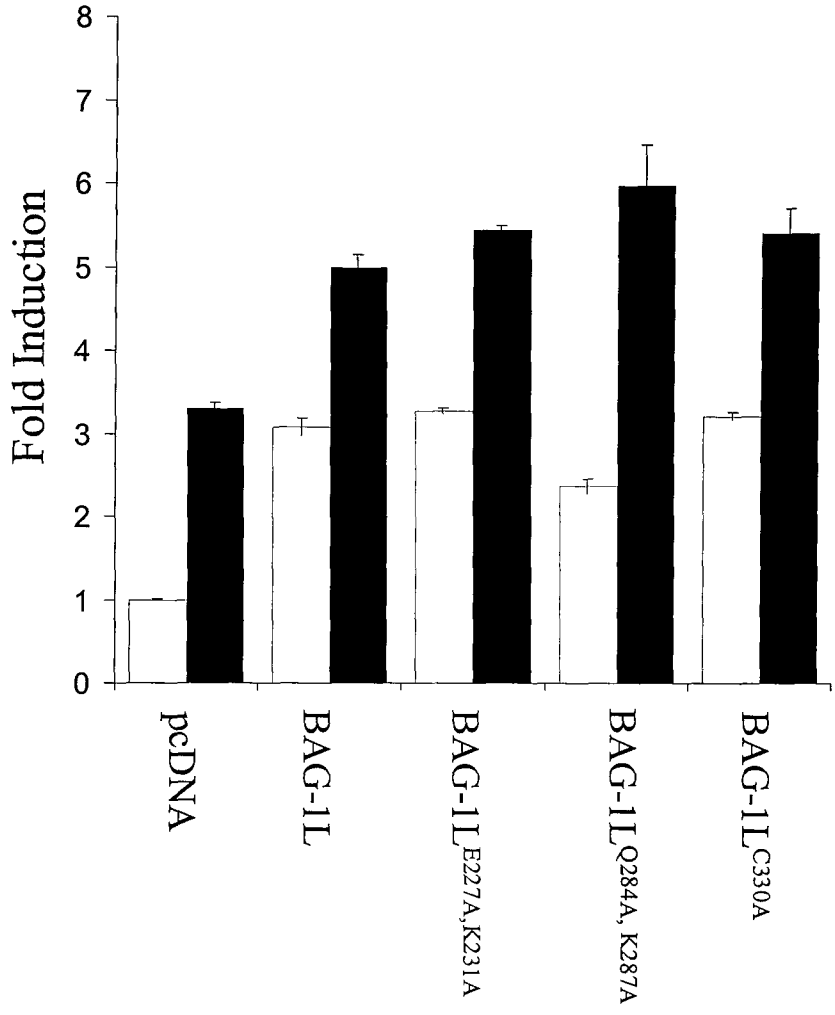


Figure 4.10: Effects of BAG domain mutation on ERα mediated transcription
HEK-293 cells were cotransfected with BAG-1 expression plasmid or BAG-1L mutant as indicated or pcDNA3, and with ERα expression plasmids the ERETKluc reporter plasmid. Cells were stimulated with 10 pM 17β-estradiol (closed bars) or left untreated as a control (open bars) for 24 hours and analysed for luciferase activity. The values shown are mean +/- SE of the mean and are derived from one experiment representative of two such experiments with triplicate determinations.

$1S^{C215A}$ mutation did not affect binding to the chaperones (data not shown) it was unable to rescue MCF7 long term clonogenic potential following heat shock (Dr G Packham and Dr P Townsend; personal communication).

To produce BAG-1L point mutants that could not bind to the chaperones HSP70/HSC70 further mutations were generated within the three helices of the BAG domain of BAG-1L. Since HSC70/HSP70 bind to helices two and three, mutations were made to substitute charged residues in each of the three helices for alanine residues. As expected mutations in helix one (BAG-$1L^{E227A,K331A}$) had no effect on HSC70/HSP70 binding to BAG-1 (data not shown; Figure 5.4 for equivalent data in BAG-1S). In addition they had no effect on transcription (Figure 4.10). Mutations in helix two (BAG-$1L^{Q284A,K287A}$) and helix three (BAG-$1L^{Q316A}$; BAG-$1L^{D323A,Q327A}$) prevented binding to the chaperones HSC70/HSP70 (data not shown; Figure 5.4 for BAG-1S data). These mutations produced a reduction of approximately 50% in BAG-1L mediated potentiation of ERα transcriptional activity in the absence of added 17β-estradiol (Figure 4.10; data not shown), although transcriptional activity was similar to wild type BAG-1L in the presence of 10 pM 17β-estradiol. It is possible that these differences can be accounted for by dual mechanisms of action of BAG-1L on transcription. BAG-1L might for example stimulate ERα mediated transcription both through mechanisms involving chaperone mediated receptor refolding and through chaperone independent actions involving for example BAG-1L DNA binding. It is possible therefore that these two mechanisms affect ERα AF1 ligand independent activity and ERα AF2 ligand dependent activity differently and this may explain the differences in transcriptional potentiation in the presence and absence of 17β-estradiol. Experiments to determine whether the mutants that did not bind chaperones retained oestrogen receptor binding activity were inconclusive (data not shown).

4.6.3 The effect of targeting BAG-1S to the nucleus

The ability of BAG-1L, but not other BAG-1 isoforms, to interact with oestrogen receptors and stimulate oestrogen dependent transcription may stem from the colocalisation of BAG-1L and ER in the nucleus. To determine whether targeting BAG-1S to the nucleus was sufficient to stimulate ER dependent transcription, an

expression construct in which the BAG-1S coding sequence was fused to an heterologous nuclear localization sequence (NLS) was generated. By immunoblot analysis, the BAG-$1S^{NLS}$ protein was expressed at similar levels to other BAG-1 constructs (data not shown). Immunofluorescence analysis demonstrated that when overexpressed, BAG-1S was generally localised diffusely throughout the cell, whereas BAG-1L and BAG-$1S^{NLS}$ were largely present in the nucleus (Figure 4.11). Interestingly whilst BAG-1L localised to the nucleus and produced strong nucleolar staining BAG-$1S^{NLS}$ localised to the nucleus but did not show any punctate staining characteristic of localisation to nucleoli (Hague et al. 2002). Data are shown for MCF7 cells because their morphology more clearly allows discrimination between nuclear and cytoplasmic distribution although similar data were also obtained in HEK-293 cells. Targeting BAG-1S to the nucleus however was not sufficient for BAG-1S to stimulate oestrogen dependent transcription (Figure 4.12). This indicates that the unique amino-terminal sequences of BAG-1L have functions other than and in addition to nuclear localisation necessary for enhancement of oestrogen dependent transcription. It is for example possible that nucleolar localisation is additionally required.

4.7 Discussion

BAG-1L, but not BAG-1S or BAG-1M significantly enhanced transcriptional response to oestrogens in MCF-7 and 293 cell models. This is consistent with the reported effects of BAG-1L on the AR and VDR (Froesch et al.1998; Guzey et al. 2000). In MCF-7 cells total increases in transcriptional activity with BAG-1L were approximately fifty percent, although much greater increases were produced in HEK-293 cells with BAG-1L. BAG-1L overexpression effectively resulted in a shift in the dose response curve of these cells for oestrogens and this would be expected to result in an approximately 5-fold increase in the sensitivity of the cells to oestrogens. High levels of BAG-1L may therefore be expected to have a major impact on the growth and survival of breast cancer cells.

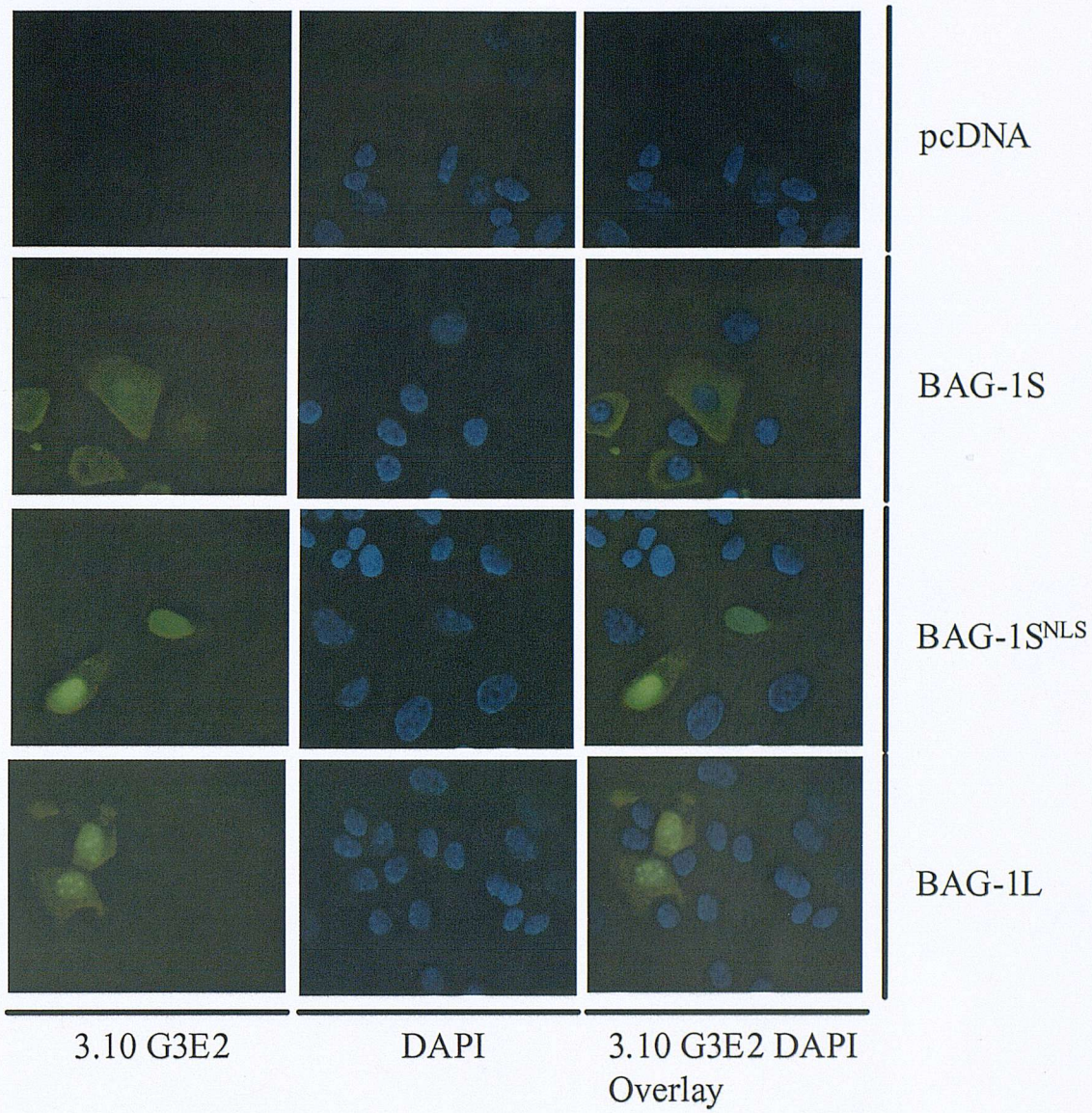


Figure 4.11: Localisation of BAG-1 proteins

MCF7 cells were transfected with expression constructs for BAG-1S, BAG-1S^{NLS} or BAG-1L, or pcDNA3 as a control, and images obtained by flourescence microscopy. Cell nuclei were detected by staining with DAPI, and BAG-1 expression was detected using the 3.10 G3E2 BAG-1 specific antibody.

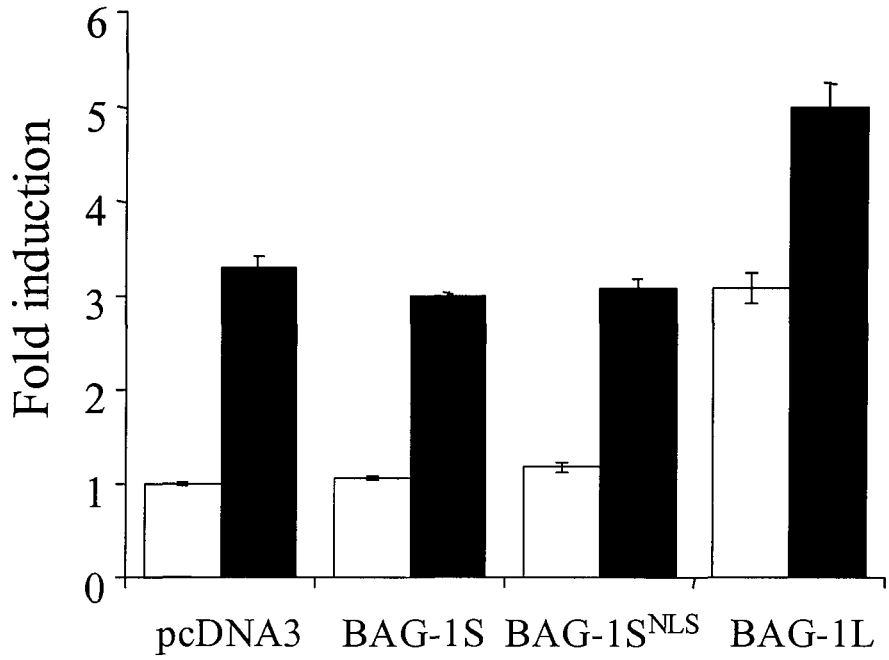


Figure 4.12: Effects of BAG-1S^{NLS} on oestrogen dependent transcription
HEK-293 cells were cotransfected with BAG-1S, BAG-1L and BAG-1S^{NLS} expression plasmids, or pcDNA3 as a control, and with the ERα expression plasmid and ERE3TKluc reporter plasmid. Cells were stimulated with 10 pM 17β-estradiol for 24 hours (closed bars) or left untreated as a control (open bars) and analysed for luciferase activity. The values shown are mean +/- SE of the mean and are derived from one experiment representative of two such experiments with triplicate determinations.

BAG-1L and BAG-1M has been shown to increase general transcription and the activity of the CMV IE promoter in some settings (Niyaz et al. 2001; Zeiner et al. 1999). The activation of ERE dependent transcription reported here was specific however since it was absolutely dependent on the presence of the ERE in both cell systems, and on the presence of cotransfected ER in HEK-293 cells. BAG-1 isoforms did not increase activity of TK-promoter reporter constructs lacking ERE and BAG-1L and did not effect expression of the CMV IE promoter within the β-galactosidase expression construct used to control for transfection efficiency relative to BAG-1S or BAG-1M transfected cells. The reason why BAG-1 overexpression did not increase general transcription in our experiments, unlike experiments described by Niyaz et. al. (Niyaz et al. 2001) is not clear but may relate to the relatively small amounts of DNA used to drive BAG-1 expression in our experiments.

BAG-1L expression had no effect on the steady state levels of the oestrogen receptor and so the increases in transcriptional activity were not due to BAG-1L increasing ER expression. Dose response experiments demonstrated that BAG-1L had no effect on transcriptional activity in MCF-7 cells in the absence of exogenous oestrogen whilst it did however stimulate transcription in the absence of exogenous 17β-estradiol in HEK-293 cells. We can not exclude that this may be due to low levels of residual oestrogens remaining in the charcoal stripped serum, but alternatively BAG-1 may affect background ligand dependent and ligand independent transcription through the AF1 and AF2 domains of the transfected and endogenous receptors differently in the two systems. There may also be cell specific differences in the expression of various co-activators and co-repressors that are involved in transcription through these two ERα domains.

Both ERα and ERβ share close sequence homology of their DNA binding domains (>90%), but other regions are less well conserved (15-20% homology of AF1 domains)(MacGregor & Jordan 1998). Although ERα and ERβ signal in the same way when complexed to oestrogen or Tamoxifen at the classical ERE, they signal in opposite ways in response to these ligands at AP1 sites. At AP1 sites oestrogen activates transcription with ERα whist it inhibits transcription with ERβ (Paech et al. 1997). The prognostic significance of ERβ expression is currently

unclear, although there have been suggestions that treatment with anti-oestrogens may stimulate tumours with high levels of ERβ through AP1 or atypical oestrogen response elements. For example, antioestrogens inhibit the oestrogen stimulated production of cyclin D1 via ERα at the cyclin D1 promoter, whilst they stimulate the production of this cyclin via ERβ (Liu et al. 2002). Given these differences and the observation that BAG-1L stimulates transcription through both ER's at the consensus ERE, it will be interesting to determine if BAG-1 modulates the function of these two receptors differentially at AP1 and other atypical oestrogen response elements.

To determine whether BAG-1L might effect other oestrogen dependent biological endpoints attempts were made to assess the clonogenicity of MCF-7 cells transiently transfected with BAG-1 isoforms in phenol red free media supplemented with charcoal stripped sera, with or without limiting concentrations of oestrogen. These experiments were unsuccessful however largely because of the very poor clonogenicity of MCF-7 cells in phenol red free media supplemented by charcoal stripped sera. Others have also reported clonogenicity as low as 0.1% for MCF-7 cells in the presence of phenol red free media (DeFriend et al. 1994). Furthermore in our hands addition of 17β-estradiol did not fully rescue clonogenic potential from the effects of charcoal stripping of the sera, consistent with the charcoal stripping process removing other important hormones and growth factors from the sera.

Since clonal cell lines that selectively overexpress the various BAG-1 isoforms have now been produced (Figure 5.8), the study of these and other biological endpoints of oestrogenic activity such as induction of endogenous oestrogen target genes and rates of cell proliferation will now be possible. This will compliment the immunohistochemical studies that demonstrate that nuclear BAG-1 expression is associated with ERα and PgR expression in human tumours (Chapter 3). In addition EMSA and chromatin immunoprecipitation studies using these clonal lines will also be possible to investigate the effects of BAG-1L on the binding of ERα to DNA, and complexes with corepressors and coactivators.

To investigate the mechanism of BAG-1 mediated potentiation of ER signalling various BAG-1L and ER mutants were assessed in transcription and binding assays. There is evidence that the repeats present at the amino-terminus of

BAG-1M may be important for BAG-1M's effects on GR function (Schneikert et al. 1999). These repeats can be phosphorylated in vivo, and so the importance of this for BAG-1L and its effect on ER function was studied with the mutant BAG-1L^{mtser}. The ability of this mutant to modulate ER transcription was no different to that of wild type BAG-1L, consistent with results demonstrating that mutation of these repeats in BAG-1M does not affect BAG-1M's ability to modulate GR function (Schneikert et al. 2000). BAG-1 activates the MAP kinase pathway by activating Raf-1 independent of RAS (Song et al. 2001). In addition the ER can be activated by MAP kinase phophorylation at serine 118 (Kato et al. 2000). ERα point mutants at seine 118 were therefore tested in the transcription assays (Chen et al. 2002a). These mutants are modulated by BAG-1L in the same way as wild type ER indicating that phosphorylation of ERα at serine 118 by BAG-1L mediated activation of MAP kinase pathways is not the mechanism by which BAG-1 modulates ER function.

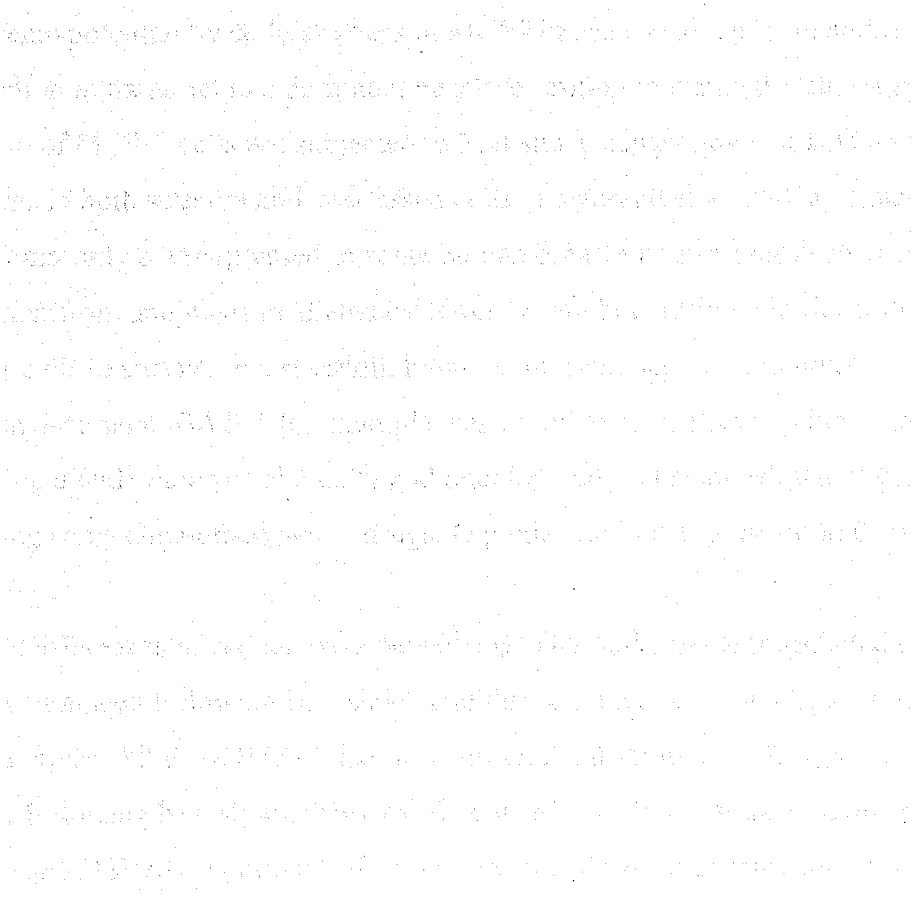
In vitro a carboxy-terminal fragment of BAG-1 containing the BAG domain is sufficient to bind to the AR, and BAG-1M can bind to the ER (Knee et al. 2001; Zeiner & Gehring 1995). Also point mutantions of BAG-1L and BAG-1M that specifically prevent the binding of these proteins to HSC70/HSP70 are deficient in the ability to modulate transcription through the AR and GR respectively (Briknarova et al. 2001; Schmidt et al. 2003). It is assumed but not reported that these mutants do not bind to the receptors. To test if this is the case with the oestrogen receptor point mutants of BAG-1L that do not bind HSC70/HSP70 were made and tested in transcription and binding assays. Co-immunoprecipitations with these mutants and ERα were inconclusive although it was clear that for example the BAG-1L^{Q284A,K287A} did not bind to HSC70/HSP70. Unlike results with the AR and GR where such mutations prevented the effects of BAG-1 on transcription these mutants still potentiated transcription through ERα in the presence of 17β-estradiol, but to a reduced degree in the absence of 17β-estradiol when compared to wild type BAG-1L. The reason for the different effects of the BAG-1L mutants on ERα compared to the AR is unclear and requires further investigation. Further work needs to be performed for example to confirm that the differences detected between BAG-1L and the point mutants are consistent when differing amounts of BAG-1L is transfected. This is

because although an interaction between these mutants and HSC70/HSP70 can not be detected in cells by co-immunoprecipitation experiments, in GST pulldown experiments these mutants can be "forced" to bind to HSC70/HSP70 under conditions with higher concentrations of purified proteins (data not shown).

Similar to the AR (Froesch et al. 1998; Knee et al. 2001), the specific ability of the BAG-1L isoform to activate ERα function correlates with its unique ability to interact with the receptors in cells. Since ER and BAG-1L are nuclear proteins, the specificity of BAG-1L may stem from its colocalisation with the ER. To test this BAG-1S was fused to an heterologous NLS. This protein relocalised into the nucleus as expected but did not potentiate ER transcription demonstrating that as with the AR and GR specific amino-terminal sequences of BAG-1L are required for functions in addition to nuclear relocalisation. One possibility is that nucleolar localisation is important as the BAG-1L unique amino-terminal sequences also direct relocalisation to the nucleolus (Hague et al. 2002). Nucleolar localisation was readily apparent with BAG-1L but not with BAG-$1S^{NLS}$. The exact sequences required for nucleolar localisation are not known and the importance and significance of this phenomena for BAG-1 function has not been tested. Alternatively it is possible that additional functions provided by the amino-terminus, which contains a region rich in basic amino-acid residues that mediates non-specific DNA binding (Zeiner et al. 1999), may be required to stabilise ER DNA interactions or to recruit components of the transcriptional complex. We were however unable to demonstrate this by disrupting DNA with DNAse. A paired cluster of charged residues at the amino-terminus of BAG-1M is important for DNA binding and inhibition of GR function and it will be important to test if mutation of these residues in BAG-1L affects the ability of BAG-1L to potentiate transcription through ERα.

Although it remains to be demonstrated what proportion of nuclear BAG-1 staining in breast cancer cells is due to BAG-1L the results in this chapter may help to explain why patients with high level nuclear BAG-1 expression do relatively well when treated with hormonal therapies. Since life long oestrogen exposure is a risk factor for breast cancer (Clemons & Goss 2001; Colditz 1998; Dickson & Stancel 2000), high levels of BAG-1L effectively increase the effect of this exposure, and

BAG-1L overexpression may therefore play an early role in the malignant process. Consistent with this, changes in BAG-1 expression are seen in premalignant conditions including DCIS (Brimmell et al. 1999). High levels of nuclear BAG-1 may therefore indicate tumours that are highly dependent on ERα-mediated signaling pathways for survival and proliferation. These tumours would be more responsive to the growth inhibitory and pro-apoptotic effects of hormonal therapies. Therefore, nuclear BAG-1 may be a marker of ERα function (via its direct effects on receptor function), similar to PgR (as a downstream measure of receptor activity). Conversely decreased nuclear BAG-1 expression might identify patients that respond poorly to hormone therapy, as seen with loss of ERα expression.



5. BAG-1 and responses to cellular stress

5.1 Introduction

All BAG-1 isoforms protect cells in short term assays from a wide range of apoptotic stimuli (Table 1.2). This is in contrast to the potentiation of ER dependent transcription where only BAG-1L has activity (chapter 4). The exact mechanisms responsible for protection from apoptotic stimuli is incompletely defined and the aim of the experiments presented in this chapter is to better define the molecular mechanisms by which BAG-1 protects cells from apoptosis. Although BAG-1 was initially described as a BCL-2 binding protein, there is no direct evidence that BAG-1 acts through BCL-2. An experimental model developed in Dr Packham's laboratory has demonstrated that BAG-1 overexpression prevents the reduction of long term clonogenic potential by cellular stress in MCF7 breast cancer cells. In addition BAG-1S deletion mutants act in a dominant negative fashion reducing the clonogenic potential of MCF-7 cells not subjected to heat shock suggesting that BAG-1 has functions in both stressed and unstressed cells (Townsend et al. 2003a). Since BAG-1S is commonly overexpressed in some human breast tumours (see chapter 3), protection from apoptosis mediated by BAG-1S might contribute to the ability of tumour cells to survive in a stressful, hypoxic, nutrient deprived, tumour microenvironment. BAG-1 for example was found to rescue clonogenic potential following a wide range of clinically and oncologically relevant cellular stresses including some chemotherapeutic drugs, hypoxia and ionising radiation (Townsend et al. 2003a).

Differences in cell survival between BAG-1 and control transfected cells were most pronounced following heat shock and this was therefore used as a model for cellular stress. All three BAG-1 isoforms protected from loss of clonogenic potential equally following heat shock (Townsend et al. 2003a). Point mutations that prevent HSC70 and HSP70 (but allow Raf-1) binding, and deletion or mutation of the ULD, prevent BAG-1's ability to rescue from heat shock. This confirms that both the ULD

and BAG domain are required for rescue from cellular stress (Townsend et al. 2003a). Furthermore, transfected luciferase was not refolded faster in BAG-1S compared to pcDNA transfected MCF7 cell clones following heat shock (unpublished data, Dr Packham), suggesting that BAG-1 mediated protection from stress could not be explained simply by increases in heat shock protein mediated refolding of cellular substrates.

The ubiquitin/proteasome system plays a major role in the degradation of proteins damaged or denatured by heat shock and other cellular stresses. Ubiquitin/proteasome inhibitors such as MG132 and ALLN have been reported to induce a heat shock response, and to both increase and reduce cellular resistance to heat shock in different studies (Lee & Goldberg 1998). The ubiquitin gene contains a heat shock responsive element within it's promoter (Bond & Schlesinger 1986). Within 1 to 2 hours following heat shock (42 °C to 44 °C) there is increased ubiquitin production, reduction in ubiquitin bound to H2A histone, and accumulation of high molecular weight ubiquitylated proteins (Fujimuro et al. 1997). In vitro assays demonstrate that this increase is not associated with a reduction of proteasomal activity, but corresponds to increased activity of the ubiquitylation enzyme system (Fujimuro et al. 1997). Mouse ts85 cells which contain a heat labile E1 ubiquitin activating enzyme, and yeast double mutants which lack the E2 ubiquitin transfer enzymes ubc4 and ubc5, exhibit impaired degradation of abnormal proteins following heat shock, and are hypersensitive to increased temperatures (Raboy et al. 1991). In addition enforced overexpression of the E2 ubiquitin transfer enzyme ubc1 restores resistance to heat shock of the yeast double E2 mutant to wild type levels (Raboy et al. 1991). It therefore appears that following exposure to mild heat shock some thermally denatured proteins are degraded by the ubiquitin proteasome system and this is important for cell survival. Those denatured proteins not degraded are presumably renatured by the chaperone system. BAG-1 contains a ubiquitin like domain, is itself ubiquitylated (Sourisseau et al. 2001), interacts with and regulates chaperones, and to binds to the proteasome and the E3 ligases CHIP and Siah (see section 1.6). It is attractive to hypothesise therefore that BAG-1 mediated rescue following heat shock might in part be due to functions where BAG-1 acts as a link

between the chaperone system and the ubiquitin system (Hohfeld et al. 2001). HSC70 for example is essential for degradation of some ubiquitin dependent proteolytic substrates, and BAG-1 co-operates with CHIP to promote ubiquitylation and degradation of substrates such as the glucocorticoid receptor.

The p53 tumour suppresser protein is a key regulator of responses to diverse cellular stresses including ionising radiation and heat shock and other cellular damage. Stabilisation of p53 through a reduction in degradation leads to an increase in p53 protein levels in stressed cells. p53 acts as a transcription factor that binds to specific DNA target sequences, p53 response elements in the promoter of target genes. p53 target genes include those important for cell cycle arrest such as p21 (waf-1) and apoptosis such as BAX. p53 also acts on the Mdm2 promoter as part of a negative feedback loop; p53 increases transcription of Mdm2 which regulates p53, in part through enhanced degradation, and in part through inhibiting p53 dependant transcription. Induction of p53 by various cellular stresses is therefore one mechanism by which various cellular stresses induce apoptosis

Since p53 is induced by heat shock, and BAG-1 overexpression prevents heat shock induced apoptosis and long term growth inhibition in MCF7 cells which contain wild type p53, BAG-1S must presumably disable p53-induced apoptosis pathways. BAG-1S interferes with apoptosis induced by enforced p53 overexpression in 293 cells (Matsuzawa et al. 1998), and in the p53 null osteosarcoma line SAOS-2 (Danen-van Oorschot et al. 1997). BAG-1 for example might act to inhibit stabilisation of p53 following heat shock, or alternatively might act downstream of this, to reduce for example p53 dependent transcription of target genes. BAG-1S does not prevent accumulation of p53 induced by radiation in MCF7 cells (Matsuzawa et al. 1998), but it is not known if BAG-1 attenuates the stabilisation of p53 that occurs following heat shock. In an in-vitro purified protein experimental system, BAG-1 disassociates Hsp90 from a wild type p53 pre-assembled multiple chaperone heterocomplex (King et al. 2001). This therefore provides a potential molecular mechanism by which BAG-1 might modulate p53 localisation and or function. BAG-1S however was found not to effect p53-dependent activation of the BAX promoter in HEK-293 cells. It is important to note however that the maximal amount of BAG-1S

expression construct used in the transcription experiments in this study were significantly lower than those required to suppress p53-induced cell killing (compare Figures 6 and 7 in Ref. Matsuzawa et al., 1998). It was therefore important that these experiments were repeated using transfected levels of BAG-1 that are known to produce a protective effect in functional assays. This chapter describes work undertaken to better understand the molecular mechanisms by which BAG-1 overexpression protects tumour cells from apoptosis. Initial work was directed at BAG-1 itself and its interaction with various binding partners. Subsequently possible BAG-1 target proteins were examined, and the effects of BAG-1 overexpression on p53 dependent transcription were studied.

It has been reported that BAG-1 can prevent the transcriptional repression caused by heat shock (Zeiner et al. 1999), and BAG-1 can regulate both receptor dependent and independent transcription. Microarray analysis was therefore used as a method of identifying novel potential candidate genes or gene expression patterns that may be regulated by BAG-1S. The expression of 5184 genes was compared in control and BAG-1S overexpressing MCF-7 cell lines both before and after heat shock.

5.2 BAG-1 expression and HSC70/HSP70

BAG-1 prevents the loss of long term clonogenic potential that occurs following heat shock. Initial experiments confirmed that the conditions used for experiments presented in this chapter produced the expected rescue of clonogenic potential following heat shock in BAG-1S overexpressing MCF7 cell clones but not vector only pcDNA control clones (Figure 5.1). Cloning efficiencies were similar prior to heat shock but were reduced in the pcDNA clone following heat shock. In contrast cloning efficiencies were largely maintained following heat shock in the BAG-1S clone. To determine how BAG-1S might rescue clonogenic potential following heat shock, BAG-1 and HSC70/HSP70 were initially studied before and after heat shock in BAG-1S overexpressing and pcDNA control clones.

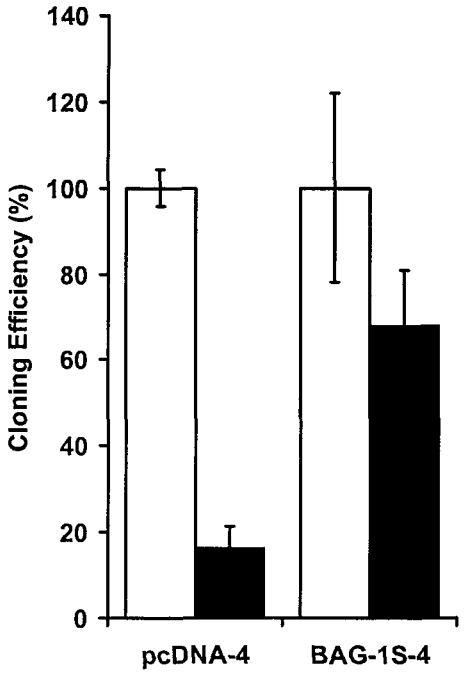


Figure 5.1: Effect of BAG-1S on clonogenic potential following heat shock. MCF7 clones pcDNA4 and BAG-1S4 were plated in duplicate at $2x10^4$ cells per well of a 24 well plate and subjected to heat shock for one hour at 42 °C (closed bars) or remained at 37 °C as controls (open bars). Cells were then cloned out by serial dilution and colonies allowed to grow. Colonies were stained with Giemsa and counted.

5.2.1 BAG-1 and HSC70/HSC70 expression

To determine possible changes in BAG-1 and HSP70/HSC70 expression following heat shock in MCF-7 cells western blotting was performed before and after cells were subjected to heat shock. Experiments were performed in untransfected MCF-7 cells and in four pcDNA and four pcDNA-BAG-1S clones (pcDNA clones 3, 4, 9 and 11 and pcDNA-BAG-1S clones 4, 5, 13, 19). Results in untransfected MCF7 cells were essentially the same as in pcDNA clones and are not described further. Levels of the three BAG-1 isoforms did not change following heat shock although levels of BAG-1S were clearly higher in BAG-1S overexpressing clones than pcDNA control clones as expected (Figure 5.2). The constitutive chaperone HSC70, was not induced by heat shock, but in contrast levels of the inducible HSP70 increased following heat shock in all clones. This demonstrates that both the pcDNA and BAG-1S clones receive and produce an equivalent initial biological response to the cellular stress of the heat shock in terms of HSP70 induction. In addition this demonstrates that BAG-1S acts downstream of initial "interpretation" of the cellular stress and does not prevent the cells from "sensing" the heat shock.

5.2.2 Changes in BAG-1 HSC70/HSP70 interactions following heat shock

Since BAG-1S interacts with heat shock proteins, and deletion or point mutants of BAG-1S that can not bind to heat shock proteins do not rescue from apoptosis following cellular stress, BAG-1 HSC70/HSP70 interactions were characterised by co-immunoprecipitation. Experiments were performed in BAG-1S and pcDNA clones before and after heat shock (Figure 5.3). Experiments were also performed in untransfected MCF7 cells and results were as demonstrated for the pcDNA clone. In the pcDNA clone the interaction between endogenous BAG-1S and HSC70HSP70 was readily detected. This interaction was not detected however following heat shock. This was not due to increased levels of the inducible HSP70 displacing HSC70 from BAG-1S since the BAG-1:HSP70 interaction was also not detected in clone pcDNA4 following heat shock. The BAG-1S clone had higher levels of BAG-1S, and the interaction with the chaperones was maintained following heat shock, although at a reduced levels when compared to levels prior to heat shock.

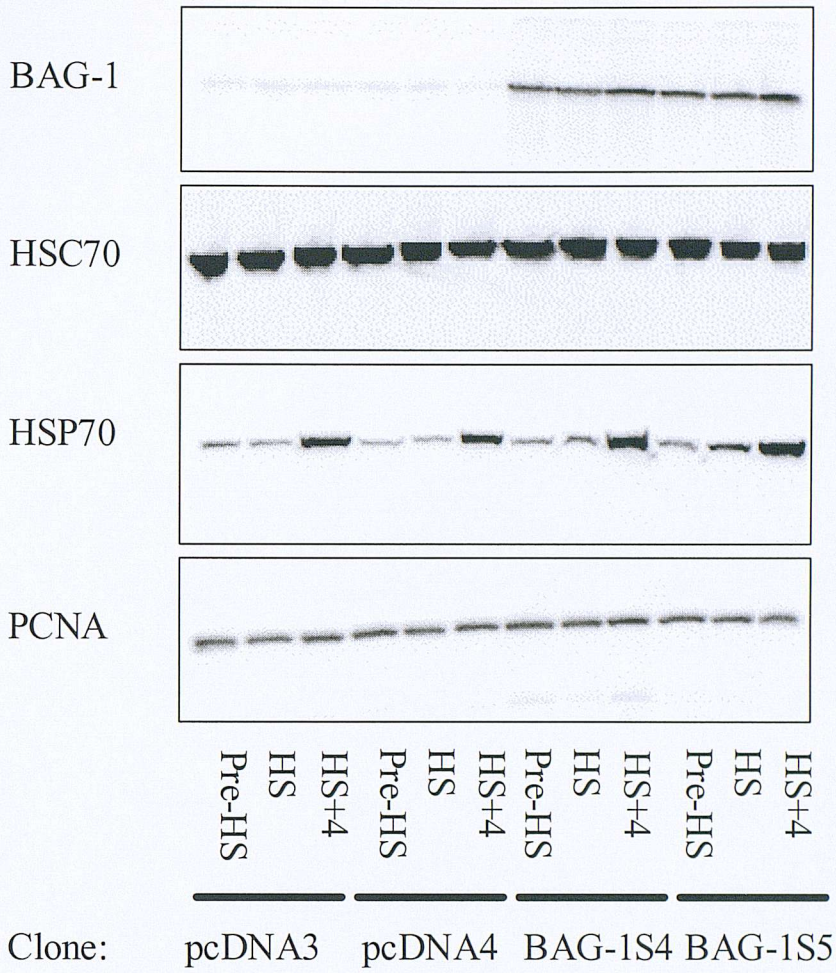


Figure 5.2: BAG-1 and HSC70/HSP70 expression before and after heat shock
Cells were plated in a 90mm dish and subjected to heat shock for 1 hour at 42 ^{o}C in a hybaid oven. Cells were collected for western blotting prior to heat shock (Pre-HS), immediately following heat shock (HS) and 4 hours following heat shock (HS+4). Cells were lysed in RIPA buffer, normalised for protein content and 30 μg of protein loaded per well for SDS-PAGE. Nitocellulose membranes were probed for BAG-1 (3.10 G3E2), HSC70 (B6) and HSP70 (C92F3A5). PCNA (PC10) is shown as a loading control.

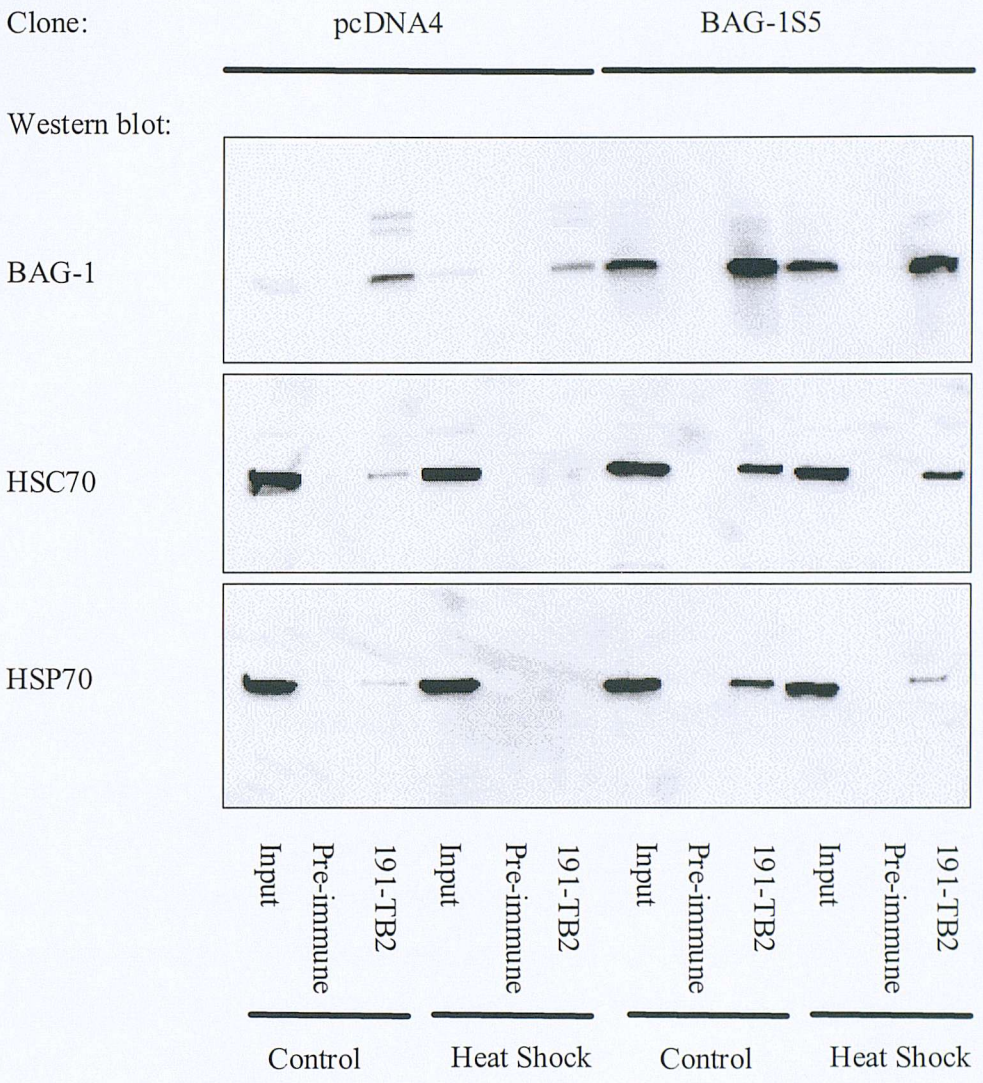


Figure 5.3: BAG-1 HSC70/HSC70 interactions before and after heat shock
MCF7 cell clones pcDNA4 and BAG-1S5 were either subjected to heat shock for one hour, or remained at 37 °C as controls. Cells were harvested after four hours and lysed in HMKEN buffer. Lysate was divided and 10% retained as input whole cell lysate (Input), immunoprcipitated with control pre-immune sera (Pre-immune), or immunoprecipitated with anti-BAG-1 sera 191 TB2 (191-TB2). Western blotting was performed for BAG-1 (3.10 G3E2) and for HSC70 (B6) and HSP70 (C92F3A5).

This supports the notion that the BAG-1:HSC70/HSP70 interaction plays a necessary role in the rescue of cells following heat shock by BAG-1S overexpression.

5.2.3 Mutational analysis and disruption of BAG-1 BAG domain interactions

Mouse BAG-1 constructs that do not bind to HSP70/HSC70 do not rescue clonogenic potential following heat shock (Townsend et al. 2003a). The opportunity was therefore taken to make point mutants in human BAG-1S that also do not bind these chaperones. These would then be available for future functional assays, and would confirm the amino-acid residues important for interaction with HSC70/HSP70. Since HSC70/HSP70 binds to helices 2 and 3 of the BAG domain and Raf-1 to helices 1 and 2 mutations were made in helices 1, 2 and 3 of the BAG domain as described for BAG-1L (see section 4.6.2). In HEK-293 cells the BAG-1 HSP70/HSC70 interaction was readily detected in wild type BAG-1S transfected cells but not in pcDNA transfected cells indicating that there was minimal HSC70 /HSP70 binding to endogenous BAG-1 detected under these conditions (Figure 5.4). This is consistent with the low levels of endogenous BAG-1 in this cell line. HSC70 /HSP70 bound to the BAG-1S^{E112A,K116A} point mutant, but not to the BAG-1S^{Q169A,K172A} or BAG-1S^{Q201A,D208A,Q212A} mutants, although these mutants themselves were immunoprecipitated, demonstrating that they do not bind HSC70 /HSP70. Although these mutants have not yet been used in the heat shock assay they have been used in other studies demonstrating BAG-1 mediated protection from cellular stress. BAG-1S but not for example the BAG-1S^{Q169A,K172A} or BAG-1S^{Q201A,D208A,Q212A} mutants protected cardiac cells from ischemia and reperfusion injury (Townsend et al. 2003c).

5.3 BAG-1 and the Ubiquitin Proteasome machinery

The ubiquitin proteasome system is important for degradation of denatured proteins following heat shock, and there is evidence that BAG-1 links this system to the chaperone system (Hohfeld et al. 2001; Luders et al. 2000a). Western blotting was therefore performed in BAG-1S and pcDNA clones to compare baseline levels of ubiquitylated proteins between clones, and to compare changes in levels following heat shock (data not shown). There were no consistent differences found between the

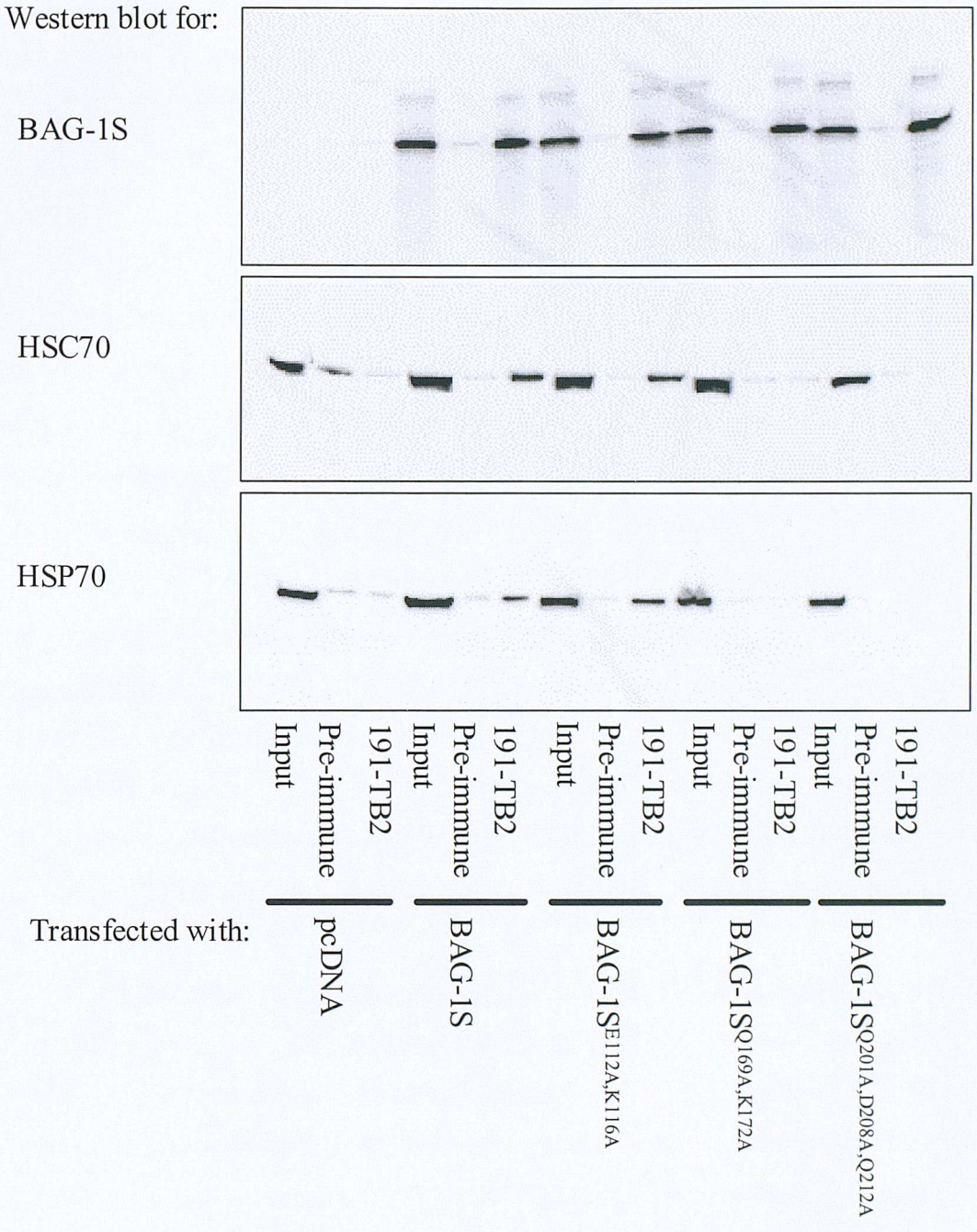


Figure 5.4: Co-immunoprecipitation of HSC70/HSP70 with BAG domain mutants HEK-293 cells were transfected with pcDNA3, BAG-1S or BAG-1S point mutant. Cells were then harvested after twenty-four hours and lysed in HMKEN buffer. Lysate was divided and kept as input whole cell lysate, immunoprcipitated with control pre-immune sera, or immunoprecipitated with anti-BAG-1 sera 191 TB2. Western blotting was performed for BAG-1 (3.10 G3E2) and for HSC70 (B6) and HSP70 (C92F3A5).

clones in baseline levels of total ubiquitylated proteins. A general increase in ubiquitylated proteins was found following heat shock consistent with previous reports on the effects of heat shock on protein turnover (Fujimuro et al. 1997; Raboy et al. 1991), but again no differences were found between the BAG-1S and pcDNA clones.

BAG-1 contains a ubiquitin like domain and interacts with E3 ligases including Siah and CHIP (Demand et al. 2001; Matsuzawa et al. 1998), and in addition interacts with the proteasome (Luders et al. 2000a). Siah functions as an E3 ligase for DCC and β-catenin. These proteins were therefore examined as possible target proteins whose expression might be regulated by BAG-1S. No consistent differences were found however between pcDNA clones and BAG-1S clones before or after heat shock in the expression of DCC (Figure 5.5). There were also no large quantitative changes in the levels of β-catenin expression, although levels were perhaps higher in the BAG-1S clones compared to the pcDNA clones prior to heat shock, and there was perhaps a tendency towards increased expression following heat shock. These results need to be confirmed in further studies but suggest that BAG-1 may therefore be involved in the control of expression of certain specific growth control molecules by regulating their turnover through E3 ligase mediated ubiquitylation.

Co-immunoprecipitation experiments in HEK-293 cells transfected with BAG-1S, but not pcDNA, demonstrated a band of higher molecular weight than BAG-1S that ran just below BAG-1M (Figure 5.6A). It was suggested that this might represent ubiquitylated BAG-1S. Interestingly this band was not clearly seen in the ubiquitin domain point mutant BAG-1S^{K80A} that does not rescue from heat shock. When western blotting was performed on these lysates for ubiquitylated proteins it was not possible to demonstrate any evidence of a ubiquitylated protein migrating at the molecular weight of the band that might represent modified BAG-1S (Figure 5.6B). It has since been confirmed by transfection with HA tagged ubiquitin that this form is indeed ubiquitylated BAG-1S (Alberti et al. 2002). It is still not clear however why this band was not recognised by the monoclonal antibody to ubiquitin used in these experiments. Posssible explanations include the possibility that the epitope is

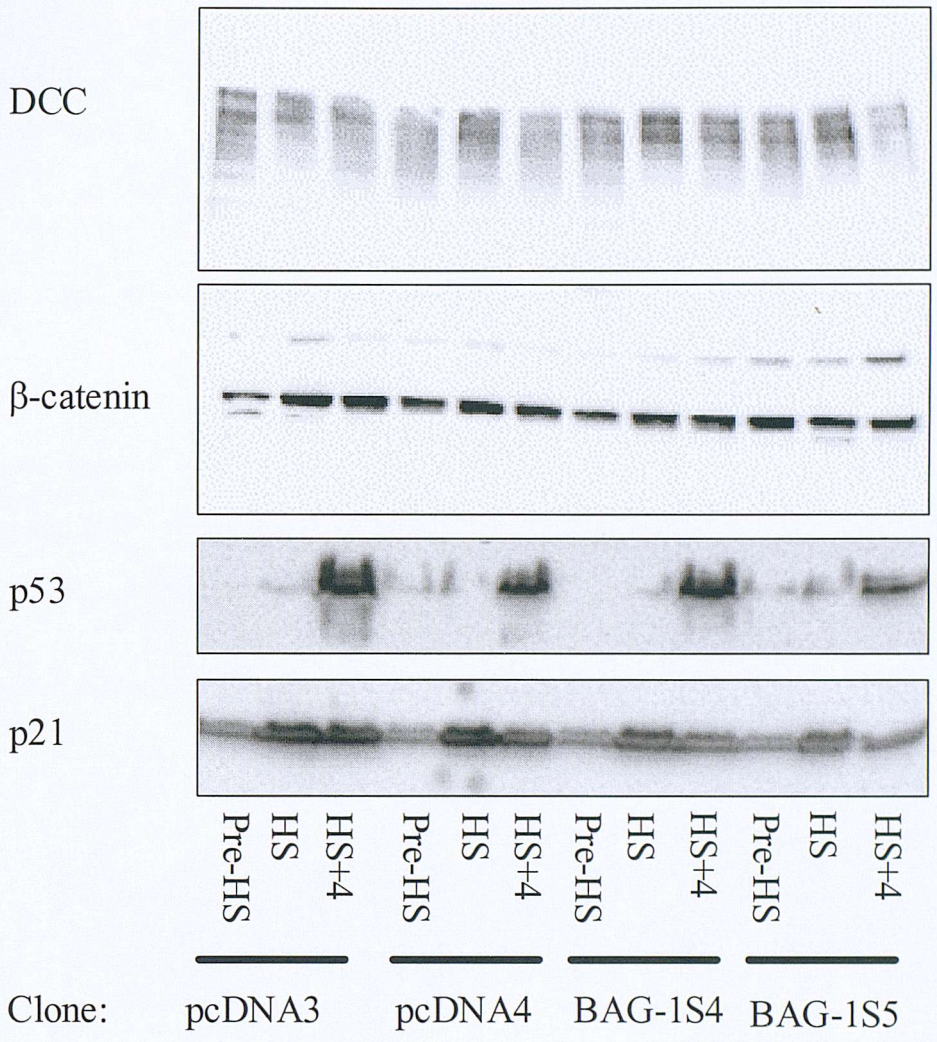


Figure 5.5: Expression of potential BAG-1 target proteins in pcDNA and BAG-1S clones before and after heat shock

Cells were plated in a 90mm dish and subjected to heat shock for 1 hour at 42 °C in a hybaid oven. Cells were collected for western blotting prior to heat shock (Pre-HS), immediately following heat shock (HS) and 4 hours following heat shock (HS+4). Cells were lysed in RIPA buffer, normalised for protein content and 40 μg of protein loaded per well for DCC (A20) and β-catenin (C18), 60 μg was loaded for p53 (DO1) and p21 (SX118). Western blotting for p53 and p21 in this experiment was kindly performed by Dr J Blaydes.

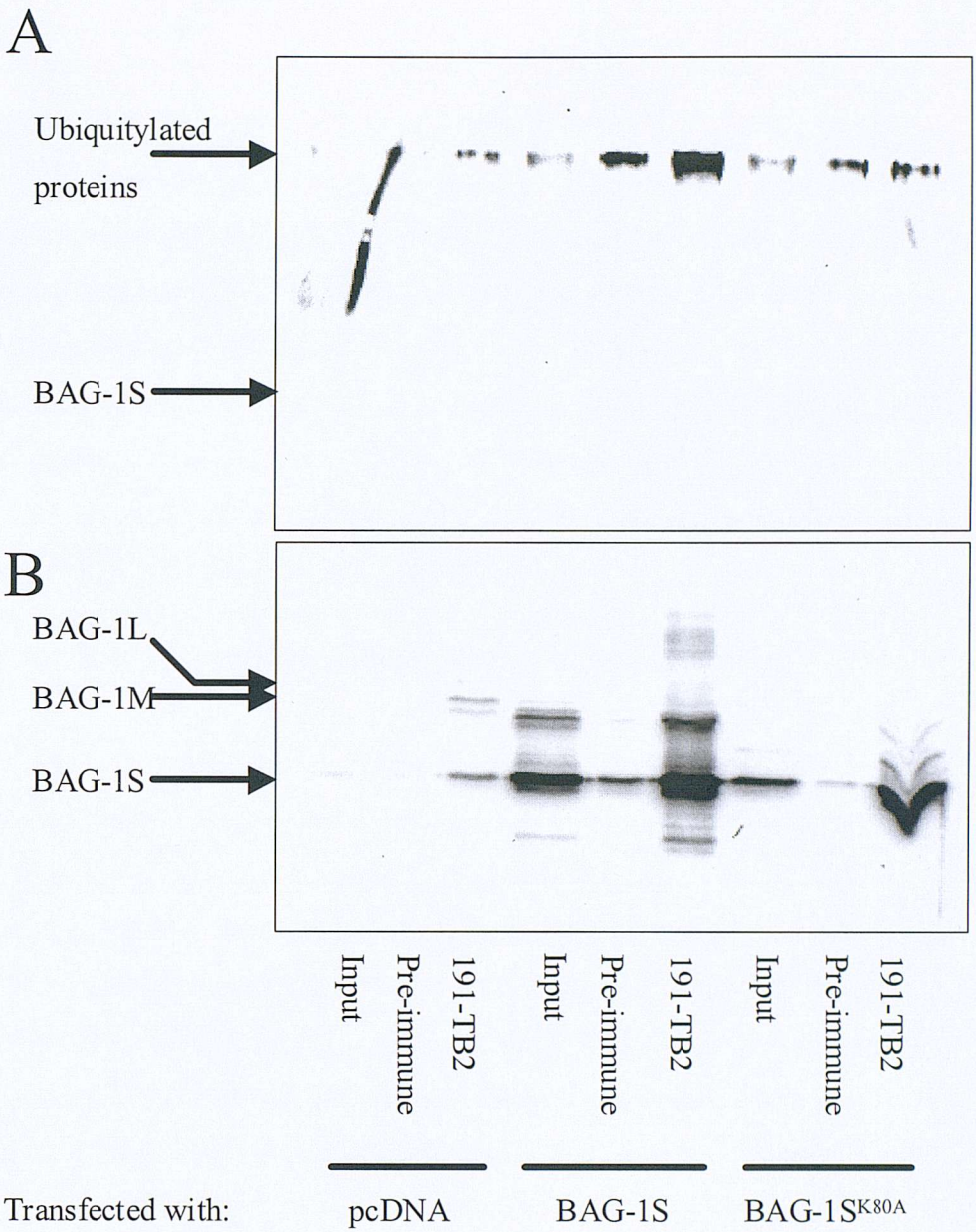


Figure 5.6: Co-immunoprecipitation with BAG-1S and BAG-1S^{K80A}

HEK-293 cells were transfected with pcDNA3, BAG-1S or BAG-1S^{K80A} point mutant. Cells were harvested after twenty-four hours and lysed in HMKEN buffer. Lysate was divided and kept as input whole cell lysate (Input), immunoprecipitated with control pre-immune sera (Pre-immune), or immunoprecipitated with anti-BAG-1 sera 191-TB2 (191-TB2). Western blotting was performed for BAG-1 (B; 3.10 G3E2) and for ubiquitylated proteins (A; FK2).

not recognised by the monoclonal antibody used since the poly-ubiquitin chain is conjugated atypically through lyseine 11 (Alberti et al. 2002).

Western blotting for ubiquitin with this antibody did however demonstrate high molecular weight ubiquitylated proteins bound to transfected BAG-1S but not to transfected BAG-$1S^{K80A}$, or in control pcDNA transfected cells. The immunoprecipitations do demonstrate non-specific interaction with high molecular weight ubiquitylated proteins in all the control immmunorecipitations. However, following immunorecipitation with 191-TB2 immunoreactivity for high molecular weight ubiquitylated proteins is increased in lysates from BAG-1S transfected, but not BAG-$1S^{K80A}$ or control pcDNA transfected cells.

5.4 BAG-1, Raf-1 and the MAP kinase pathway

BAG-1 binds to Raf-1 and activates Raf-1 independent of RAS (Wang et al. 1996). In addition, UO126, a MAP kinase inhibitor partially reduces the ability of BAG-1S to rescue clonogenic potential following heat shock (Dr P Townsend and Dr G Packham, personal communication). It is therefore possible that BAG-1 mediated modulation of MAP kinase signalling pathways via Raf-1 might play a part in BAG-1 mediated rescue of clonogenic potential following heat shock. To determine if there were changes in the MAP kinase pathway following heat shock western blotting was performed on BAG-1S and pcDNA clones (pcDNA clones 3, 4, 9 and 11 and pcDNA-BAG-1S clones 4, 5, 11, 19), before and up to eight hours following heat shock. Western blotting was performed for total and phosphorylated MAP kinase (p44/42), total and phosphorylated p38, and total and phosphorylated SAP/Jun kinase. General increases in the activity of these stress kinases were observed following heat shock however no consistent differences were found however between BAG-1S and pcDNA clones (data not shown), suggesting that this may not be the major pathway by which BAG-1 produces its survival effects following heat shock.

Since BAG-1 binding to HSC70/HSP70 is altered following heat shock and HSP70 and Raf-1 compete for binding to BAG-1 (Song et al. 2001), it is possible that changes in BAG-1 HSC70/HSC70 binding are due to changes in other binding partners such as Raf-1. To determine the significance of the BAG-1 Raf-1 interaction

in BAG-1 mediated protection from heat shock attempts were made to demonstrate the BAG-1:Raf-1 interaction by co-immunoprecipitation and GST pull down assays. Overall, although there were suggestions of an interaction in some experiments, results were not compelling, even when BAG-1S and Raf-1 were co-transfected (data not shown). Using the same buffers and techniques Dr Townsend has clearly demonstrated the interaction in cardiac cells (Townsend et al. 2003c), indicating that our negative results were not necessarily simply due to the choice of the buffers or techniques employed. The absence of a clearly demonstrable interaction between BAG-1S and Raf-1 in these cells is consistent with the absence of clearly demonstrable differences in MAP kinase signalling pathways between BAG-1S and control pcDNA transfected clones following heat shock. The implication is that at least in this model, BAG-1S does not produce its survival effects through modulation of the MAP kinase pathway.

5.5 BAG-1 and the p53 response

The p53 protein is a major effector of cell death following stress, and therefore to rescue long-term clonogenic potential following heat shock BAG-1 must presumably either prevent, or act in some way to reduce the downstream effects of the stabilisation of p53 in BAG-1S clones. Expression of p53 was initially studied by western blotting (Figure 5.5). p53 was stabilised following heat shock consistent with previous descriptions of the response of p53 to cellular stress (Cadwell & Zambetti 2001). Similar to the increase in HSP70 expression that occurred following heat shock, p53 stabilisation occurred in both control pcDNA clones and in BAG-1S clones suggesting that BAG-1 might act downstream of the initial stabilisation of p53.

One possible mechanism by which BAG-1 might act downstream of p53 stabilisation to attenuate p53 induced apoptosis following heat shock is through modulation of p53 dependent transcription. There is evidence that BAG-1S does not modulate p53 dependent transcription (Matsuzawa et al. 1998), but since levels of BAG-1S used in the transcriptional experiments were lower than those required to produce a survival effect it was felt that the conclusions of these experiments were

unreliable. Reporter assays were therefore performed to assess the impact of BAG-1 on p53 dependent transcription (Figure 5.7). BAX reporter activity increased 20 fold when p53 was transfected into HEK-293 cells but was unchanged when BAG-1S was transfected in the absence of p53. When BAG-1S and p53 were co-transfected BAG-1S attenuated p53 dependent transcription through the BAX promoter by 34%.

Experiments were also performed with BAG-1L. In this experiment BAG-1L did not produce a reduction in p53 induced reporter activity through the BAX promoter, although there was a more pronounced effect on the MDM-2. Significant reductions in the reporter activity of the p21 promoter were not seen (data not shown). BAG-1 isoforms therefore reduce p53 dependent transcription at some but not all p53 regulated promoters.

Western blot analysis was performed to identify candidate p53 target proteins whose endogenous levels might be regulated by BAG-1 mediated modulation of p53 transcriptional activity (Figure 5.5 and data not shown). p21 is a cyclin dependant kinase inhibitor involved not only in regulating entry into the cell cycle, but also in control of differentiation and apoptosis (Dotto 2000). p21 induction occurred immediately following heat shock, whilst p53 stabilisation occurred at four hours following heat shock. The p21 induction observed was therefore not due to p53 stabilisation as the p21 induction occurred prior to the p53 stabilisation. Interestingly, an abrogation of p21 induction was seen in BAG-1S but not pcDNA clones. It is possible therefore that the differences in p21 levels between BAG-1S and pcDNA clones after heat shock are due to changes in rates of degradation since this is also recognised to be an important mechanism by which the levels of this protein are controlled (Gorospe et al. 1999). It is possible therefore that p21 mediated cell cycle arrest occurs in pcDNA clones but not BAG-1S clones. Western blotting was also performed for other p53 target genes including BAX (data not shown). There were no overall consistent differences between pcDNA and BAG-1S clones before and after heat shock in levels of BAX. Since induction of p21 was abrogated by BAG-1S, expression of p27, another cell cycle kinase inhibitor regulated by protein turnover was studied. No differences were found in levels of p27 after heat shock between BAG-1S and pcDNA clones (data not shown).

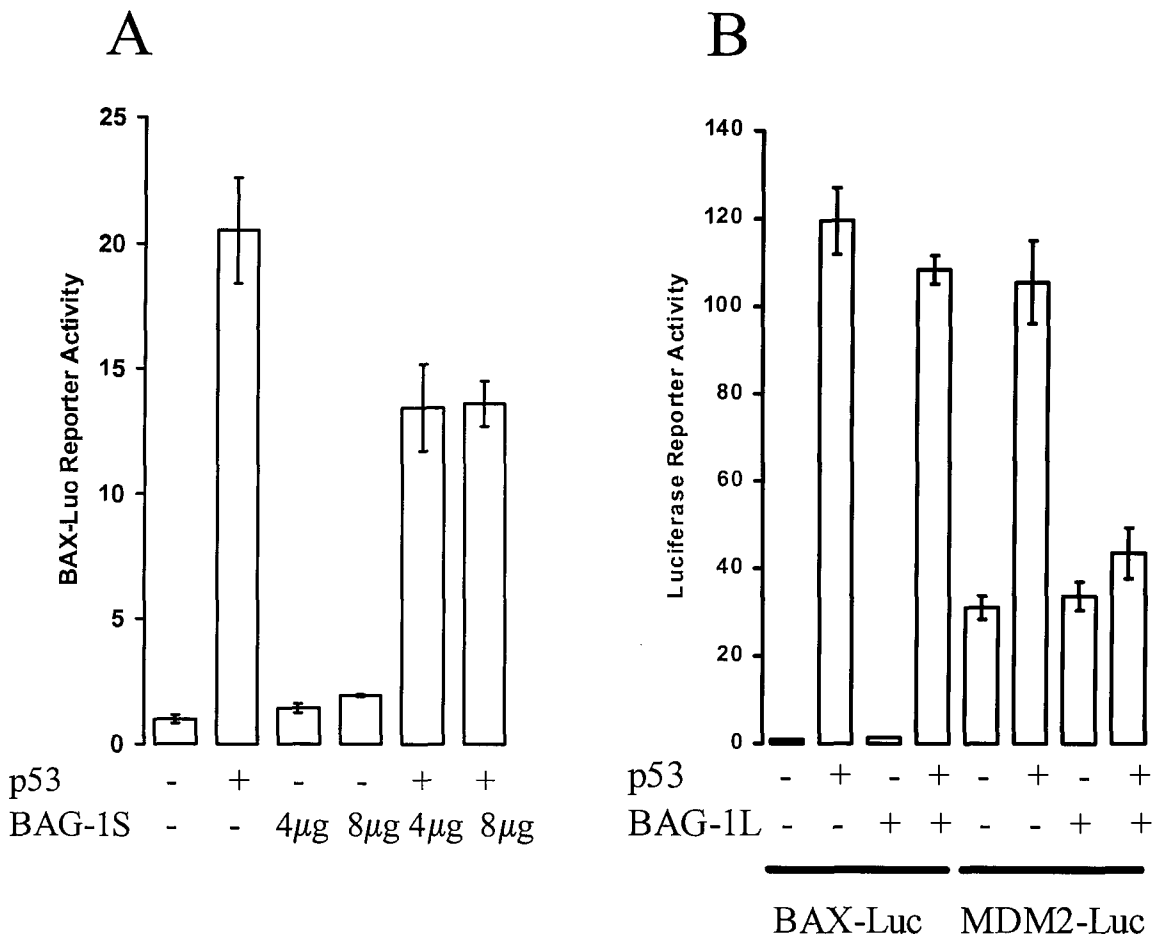


Figure 5.7: The effect of BAG-1S overexpression on p53 dependent transcription HEK-293 cells were plated in duplicate wells and co-transfected with a BAX promoter reporter construct (A, BAX-luc in B) or an MDM-2 (MDM2-luc in B) promoter reporter construct. Cells were co-transfected with empy pcDNA control vector or p53 expression construct SN3 and either control pcDNA constuct or BAG-1S expression construct (A) or BAG-1L expression construct (B). Cells were cultured for 24 hours and then harvested and assayed for luciferase and β-galactosidase activity.

5.6 Identification of candidate BAG-1 target proteins by microarray analysis

Since BAG-1 modulates transcriptional activity, including p53 dependent transcription, general transcriptional activity (Niyaz et al.2001; Zeiner et al. 1999), and transcription through nuclear hormone receptors (see section 1.7.1 and chapter 4) microarray analysis was performed to identify candidate genes whose transcription might be affected by BAG-1S. Early passage stable transfectant MCF-7 clonal cell lines were produced for these experiments (Figure 5.8). Individual colonies were selected and grown in G418 following transfection with pcDNA or BAG-1S, BAG-1M or BAG-1L expression construct. Approximately half the colonies selected overexpressed BAG-1 isoforms and examples are shown (Figure 5.8). In total four pcDNA control colonies (A, C, H and J), two BAG-1S colonies (D and J), three BAG-1M colonies (D, F and G) and three BAG-1L colonies (B, J and I) were identified and cryopreserved for further study.

For the microarray experiments BAG-1S clone D and pcDNA clone A were subjected to heat shock at 42 ^{o}C for one hour or kept at 37 ^{o}C as controls. Experiments performed under these conditions of heat shock had demonstrated that BAG-1S clones survive the shock whilst pcDNA clones undergo apoptosis. 12 hours later cells were harvested and RNA prepared and ^{33}P labelled cDNA probes generated. These probes were hybrised to breast specific gene filters, and bound probe detected by autoradiography. The most striking feature following visualisation of these four arrays was the overall similarity of the expression patterns demonstrated, although some differences were clearly discernible (Figure 5.9). This indicates firstly that the fundamental cellular processes in the two clonal cell lines are comparable and that they provide a "clean" system for studying the effects of BAG-1S overexpression. Secondly the similarity demonstrates that the heat shock the cells were subjected to was reasonably mild and therefore analogous to a "physiological cellular stress" rather than a catastrophic event. Thirdly it demonstrates that the timing at which the cells were harvested was appropriate as the aim was to identify primary changes following heat shock rather than multiple downstream effects.

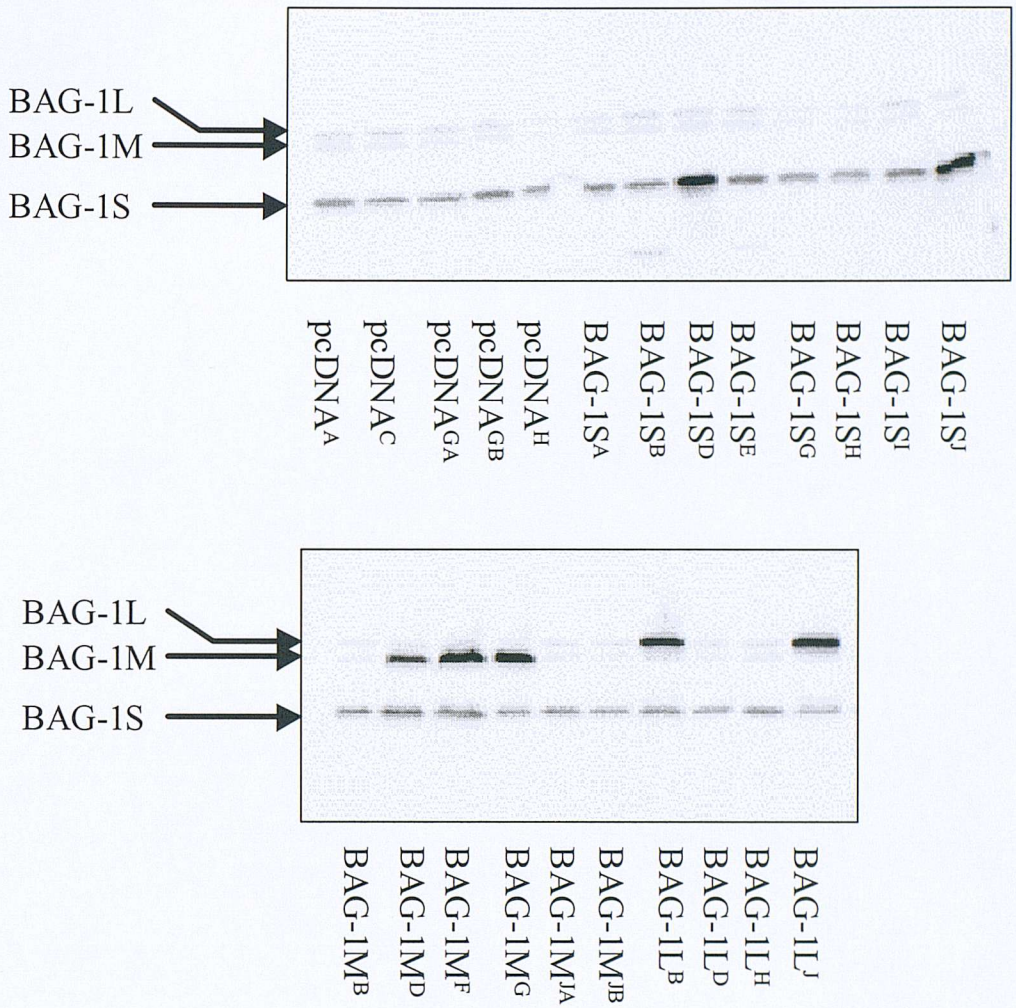


Figure 5.8: Examples of MCF-7 stable transfectant clonal cell lines

MCF-7 cells were transfected with control pcDNA plasmid or BAG-1 expression construct and grown in the presence of G418 to select for transfected cells. Individual colonies (originating from single cells) were selected by trypsinisation in cloning rings and plated and grown separately. Cells from surviving colonies were harvested, lysed in RIPA buffer and subjected to SDS-PAGE. Protein was transferred to nitrocellulose and blotted for BAG-1 expression with 3.10 G3E2.

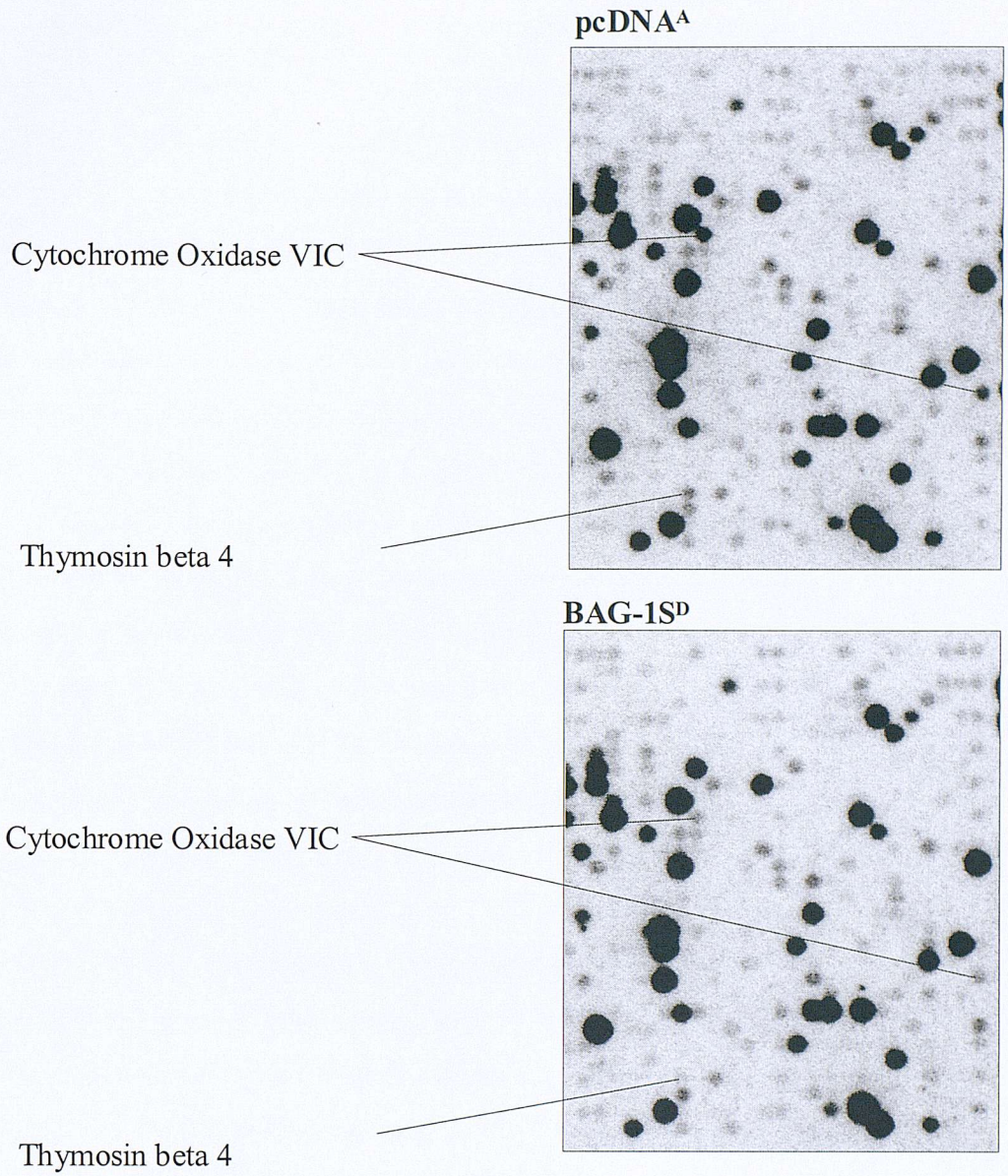


Figure 5.9: Examples of the microarray filters

RNA was extracted from MCF-7 clonal cell lines pcDNA^A and BAG-1S^D and a ^{33}P-labelled cDNA probe produced by reverse transcription in the presence of ^{33}P-dCTP. The probes were allowed to hybridise in a hybridisation oven at 42°C for 18 hours following which unbound probe was removed by washing and bound probe detected by autoradiography using a phosphor-imager. A magnified portion of each filter is shown.

The "Pathways" software allows for pairwise comparisons between gene filters and it was decided that for consistency all the filters should be compared to a single control filter. Pairwise analyses were therefore performed to compare the filter of the $pcDNA^A$ clone kept at 37 °C with each of the three other filters. Initial data processing involved performing a statistical test (Chen test) to identify all genes in which the difference in signal was likely to be due to differential regulation with a greater than 90% probability. Such statistical tests are important as they take not only the distribution of the data into account, but also the fact that microarray techniques involves multiple hypothesis testing across thousands of genes and so tests have to be more stringent than when testing single hypotheses.

Traditionally genes with a greater than two fold variation are considered differentially regulated. This is not necessarily the optimal cut off for all filters however since the experimental methodology and technique may affect the full distribution of the expression patterns across filters (Quackenbush 2002). With the experiments described here the full range of variation of expression between genes was not always apparent as those genes with very high expression produced saturated pixels. To compensate for this a cut-off of 1.8 was chosen to define differentially regulated genes in these experiments. Further data processing involved one further criteria of an absolute difference in expression. Only genes with an absolute difference in signal of greater than or equal to 0.9 were considered significant. This is because at very low levels of gene expression the effects of experimental noise become much more pronounced and so it becomes impossible to separate biologically important differences from experimental artefact.

The results of the three pairwise comparisons that meet these criteria are shown (Tables 5.1-5.3). Although it should be stressed that changes observed in all these candidate genes will require confirmation, several patterns emerge. Comparison of the BAG-1S clone with the pcDNA clone under control conditions revealed changes in expression in proteins involved in signal transduction, particularly through tyrosine kinase receptor / MAP kinase pathways. There was increased transcription of a tyrosine kinase substrate, reduced transcription of MADD, a protein implicated in TNF-α signalling through activation of the ERK pathway, and reduced transcription

Table 5.1: Microarray analysis of control BAG-1S^{D} compared to control pcDNA^{A} cells.

Accession No.	Ratio	Difference	Protein and comments
			Increased in control BAG-1S^{D} relative to control pcDNA^{A} cells
AA481540	5.43	2.24	Uncharacterised protein. Contains three consecutive armadillo/β-catenin like repeats believed to be involved in protein:protein interactions and a karyopherin domain implicated in intracellular trafficking.
AI004316	3.38	1.60	EST. Protein contains one domain of unknown function (DUF143).
AI369331	2.49	1.05	Signal regulatory protein α-1. A substrate of activated receptor tyrosine kinases.
R44202	2.34	2.87	Catechol-O-methyltransferase. Methyl conjugation is an important pathway in the metabolism of many drugs, neurotransmitters and hormones. COMT inactivates catechol oestrogens that are believed to contribute to the development of oestrogen induced cancers (Lavigne et al. 1997;Lavigne et al. 2001;Weinshilboum et al. 1999).
AA035310	2.16	1.08	Hypothetical protein. Contains an Sm domain present in small nuclear ribonucleoprotein particles (snRNPs) involved in pre-mRNA splicing.
R61332	1.82	1.74	The ubiquitin activating enzyme E1. Catalyses the first step of ubiquitylation. Temperature sensitive mouse ts85 cells contain a defective E1 enzyme that is heat labile.
			Decreased in control BAG-1S^{D} relative to control pcDNA^{A} cells
AA669532	0.2	1.67	EST.
AA281945	0.27	2.69	MADD (MAP kinase activating death domain). Interacts with tumour necrosis factor (TNF) receptor 1 and activates ERK and nuclear factor κB. Alternatively spliced isoforms can either increase or reduce TNF-α mediated apoptosis through downstream modulation of caspase-8 and 3 (Al Zoubi et al. 2001;Schievella et al. 1997).
AA976544	0.29	2.11	Melanophilin. Involved in melanosome transport by linking melanosomes to the actin transport machinery (Fukuda et al. 2002;Strom et al. 2002).

Continued			
Accession	**Ratio**	**Difference**	**Protein and comments**
AA456931	0.40	3.02	Cytochrome c oxidase subunit (COX) VIc. A subunit of cytochrome c oxidase, the terminal complex of the mitochondrial respiratory chain. A nuclear gene encodes this subunit. COX deficiency is the most frequent cause of human respiratory chain defects including Leigh syndrome. In conjunction with nitric oxide COX may be involved in mediating apoptotic and survival responses to hypoxia (Barrientos et al. 2002;Moncada & Erusalimsky 2002;Rahman et al. 2000)
AA634103	0.43	1.47	Thymosin-beta 4. Beta thymosins are present at high concentrations in all cells and bind monomeric actin preventing polymerisation into filaments, but supplying a pool of monomers when the cell requires filaments (Huff et al. 2001;Otto et al. 2002).
R59927	0.48	2.31	COX VIc
R59927	0.49	2.09	COX VIc
AA598508	0.50	1.31	Cellular retinoic acid binding protein. Binds to retinoic acid and may be involved in the regulation of skin growth and differentiation (Cornic et al. 1994;Napoli et al. 1991;Wolf 2000).
AA486746	0.54	1.04	Ribosomal subunit L28. Ubiquitylation of this ribosomal subunit is associated with the S phase of the cell cycle. Abnormal expression of RNA transcripts of this subunit occur in colorectal carcinomas (Spence et al. 2000).
AA996131	0.56	.93	SH3 binding glutamic acid rich (SH3BGR) protein. Contains a PXXP motif like BAG-3 responsible for binding to SH3 domains of proteins involved in signal transduction. Structural homology to the Thioredoxin superfamily and suggesting that this protein may be involved in the control of redox dependent processes (Egeo et al. 1998;Egeo et al. 2000;Mazzocco et al. 2001;Mazzocco et al. 2002).

Table 5.1: Microarray analysis of control BAG-1S^{D} compared to control pcDNA^{A} cells.

Genes included are upregulated or downregulated more than 1.8 fold with an absolute difference in intensity score of more than 0.9 units.

Accession No.	Ratio	Difference	Protein and comments
			Increased in pcDNA^A following heat shock relative to control pcDNA^A
AA452140	2.36	0.96	EST. A putative kinase with structural similarity to doublecortin, a brain specific transmembrian protein. Contains a putative tyrosine kinase catalytic domain.
			Decreased in pcDNA^A following heat shock relative to control pcDNA^A
AA976544	0.24	2.27	Melanophilin.
AA281945	0.25	2.77	MADD
AA669532	0.27	1.53	EST
W48713	0.48	2.64	The epidermal growth factor receptor (EGFR).
AA488631	0.50	1.22	Image clone. No conserved domains within putative protein encoded.
AA634008	0.53	2.08	Ribosomal protein S13. A component of the small 40S ribosomal subunit[b].
AA666180	0.53	1.05	Ear-2. A nuclear orphan receptor with close homology to other steroid hormone receptors.
AA670315	0.53	2.31	Hypothetical protein with no conserved domains.
AA991507	0.55	2.04	Selectin P ligand. The P selectin ligand is expressed on various leukocytes and mediates binding of leukocytes to P selectin expressed on activated platelets and endothelium and thus accelerates fibrin formation and deposition during thrombogenesis (Shebuski & Kilgore 2002).

Table 5.2: Microarray analysis of gene expression in the pcDNA^A clone before and after heat shock.

Genes included are upregulated or downregulated more than 1.8 fold with an absolute difference in intensity score of more than 0.9 units.

Table 5.3: Microarray analysis of BAG-1S^{D} (following heat shock) compared to pcDNA^{A} (unheat shocked).

Accession No.	Ratio	Difference	Protein and comments
			Increased in BAG-1S^{D} following heat shock relative to control pcDNA^{A}
AA454618	4.84	2.37	Associated molecule with the SH3 domain of STAM (AMSH). AMSH binds to STAM and Smad6 which are components of the TGF-β superfamily signalling pathway. Overexpression of AMSH increases growth inhibitory signalling through these pathways. AMSH deficient mice show extensive hippocampal neuron apoptosis (Ishii et al. 2001;Lohi & Lehto 2001;Tanaka et al. 1999)
R71093	2.71	1.23	Hsp47. Hsp47 is a heat inducible chaperone specific for collagen and is required for normal collagen synthesis and for maintaining the structure of collagen during thermal stress. Hsp47 deficient mice die in utero and exhibit multiple defects consistent with a severe deficiency in the mature processed form of collagen (Hendershot & Bulleid 2000;Nagai et al. 1999;Williams 2000).
R44202	2.51	3.23	COMT
AA485959	2.13	1.42	Keratin 7. Keratin 7 is one of approximately 20 keratin subtypes, which together form essential structural components of the intracytoplasmic cytoskeleton. Carcinomas of epithelial origin, particularly glandular including breast carcinomas express high levels of keratin 7. Immunocytochemical analysis of ketatin subtype is used to identify the origins of metastases from unknown primary sites (Chu & Weiss 2002;Tot 2002).
H16958	1.99	2.60	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Classically involved in glycolysis catalysing the conversion of Glyceraldehyde 3-phospate to 1,3-Bisphosphoglycerate yielding NADH. Increases in GAPDH expression occur in prostate cancer and during oxidative stress. Induction of apoptosis in cerebellar granule cells is dependent induction and translocation of GAPDH to the nucleus, and is independent of glycolytic activity (Berry & Boulton 2000;Sirover 1999).
H79353	1.98	1.39	The Fc fragment of the high affinity IgE receptor 1. The binding of antigen to IgE triggers the release of histamine and other substances from mast cells.

continued

Accession	Ratio	Difference	Protein and comments
AA425435	1.96	1.77	Protein of unknown function. Contains a GTPase domain.
N21338	1.94	1.52	Protein of unknown function. No conserved domains.
AA429483	1.86	2.18	Mitochondrial ribosomal protein S21. Mammalian mitocondrial ribosomal proteins are encoded by nuclear genes and catalyse protein synthesis within mitochondria.
AA481464	1.81	1.29	Cyclophilin B. Cyclophilins are heat inducible chaperones involved in protein synthesis, refolding misfolded proteins following cellular stress and steroid receptor maturation. Cyclophilin B complexes with prolactin and binds to Stat5 enhancing Stat5 mediated DNA binding and transcriptional activity resulting in increases in cell viability (Andreeva et al. 1999;Andreeva et al. 1997;Fluckiger et al. 2002;Rycyzyn et al. 2000;Rycyzyn & Clevenger 2002).
AA088749	1.81	1.16	Hypothetical protein. Putatively contains a CIDE-N domain present in CAD nuclease and I-CAD the inhibitor of CAD nuclease, two proteins involved in apoptotic DNA fragmentation.
			Decreased in BAG-1S^{D} following heat shock relative to control pcDNA^{A}
AA976544	0.21	2.36	Melanophilin
AA669532	0.25	1.56	EST
AA281945	0.26	2.73	MADD
AA488631	0.32	1.66	Image clone
R59927	0.38	2.77	COX VIc
AA634103	0.43	1.46	Thymosin beta 4
R59927	0.47	2.19	COX VIc
AA996131	0.55	0.97	SH3BGR protein

Table 5.3: Microarray analysis of BAG-1S^{D} (following heat shock) compared to pcDNA^{A} (unheat shocked).

Genes included are upregulated or downregulated more than 1.8 fold with an absolute difference in intensity score of more than 0.9 units.

of the SH3GBR protein. As BAG-1 itself can activate MAP kinase pathways such changes are consistent with our understanding of BAG-1 function. There is an increase in the transcription of E1, the ubiquitin activating enzyme, and a reduction in the transcription of ribosomal subunit L28, ubiquitylation of which is associated with S phase of the cell cycle. The E1 cDNA was present twice on the filter, but only one of these spots met the stringent criteria outlined for the definition of differential regulation of candidate genes. On reviewing the second spot E1 transcription was also increased in this duplicate spot in the BAG-1S clone but not to the same degree. Given the close association of BAG-1 and the ubiquitin/proteasome system and the importance of the E1 enzyme this candidate should be considered for further testing, despite the discrepancy in the degree of difference between duplicate spots. In contrast to the E1 enzyme cytochrome c oxidase (COX) VIc showed a reduction in transcription in the BAG-1S clone at three distinct positions. Although cytochrome c oxidase is involved in the terminal stages of the mitochondrial electron transport chain, it has also been implicated in mediating responses to hypoxia. As subunit specific antibodies have been produced, primarily to investigate respiratory chain disorders, it will be relatively easy to test for changes in COX VIc protein expression.

Following heat shock in the pcDNA^{A} clone only one gene met our criteria for upregulation, and this for a hypothetical protein. This perhaps is consistent with the general reduction in transcription that occurs following such cellular stresses. Also associated with this there was a reduction in the transcription of the S13 ribosomal protein and also of the S23 ribosomal protein (ratio 0.47, absolute difference 0.85). There was also reduction in transcription of the epidermal growth factor receptor, which may be expected to make the cells more dependent on growth factor signalling, and reduction in transcription of an orphan NHR.

In contrast to the pcDNA clone the pattern in the BAG-1S clone following heat shock was quite different, and significantly more genes were upregulated. There was only one gene that met the criteria that was downregulated in the pcDNA clone following heat shock and was also downregulated in the BAG-1S clone (Table 5.4). All other genes that were downregulated in the BAG-1S clone after heat shock were also downregulated prior to heat shock. This is consistent with reports that BAG-1

Accession	Gene	pcDNA^{A} control	BAG-1S^{D} control	pcDNA^{A} heat shock	BAG-1S^{D} heat shock
AA669532	EST	1	0.2	0.27	0.25
AA281945	MADD	1	0.27	0.25	0.26
AA976544	Melanophilin	1	0.29	0.24	0.21
AA488631	Image clone	1	ns	0.50	0.32
R44202	COMT	1	2.34	ns	2.51
R59927	COX VIc	1	0.48	ns	0.38
AA634103	Thymosin beta 4	1	0.43	ns	0.43
AA996131	SH3BGR	1	0.56	ns	0.55

Table 5.4: Relative expression of selected genes

Genes included are those from tables 5.1 to 5.3 that exhibit significant changes in expression control pcDNA^{A} cells on more than one filter. ns: Not significantly different to control pcDNA^{A} cells.

can overcome the general transcriptional repression that can occur following heat shock (Zeiner et al. 1999). Consistent with this there was an increase in the transcription of ribosomal proteins including the mitochondrial ribosomal protein S21 and the ribosomal protein S13 (which was reduced in the pcDNA clone following heat shock) which may allow increased protein production following heat shock. Following heat shock there were also increases in various structural and stress inducible proteins that did not occur in the pcDNA clone following heat shock. These include increases in cyclophilin B, keratin 7 and Hsp47, which is important for the production of collagen. The expression of AMSH, a protein associated with survival signalling from the TGF-β receptor superfamily is notable as the gene most upregulated in the BAG-1S clone following heat shock, and is therefore an important potential novel BAG-1 target.

5.7 Discussion

Cellular stress and heat shock classically induce heat shock proteins which perform critical functions necessary for cell survival following these stresses (Kiang & Tsokos 1998). These include chaperoning and refolding of proteins misfolded by thermal stress, but also include a more direct role in the regulation of apoptosis (Mosser et al. 1997). As BAG-1 regulates the function of HSP70 and HSC70 initial experiments to determine the molecular mechanisms by which BAG-1 can regulate apoptosis focused on these proteins. Expression of the inducible HSP70, but not of the constitutive HSC70, increased within four hours, in both BAG-1 and control pcDNA clones following heat shock. This demonstrates that BAG-1 does not modulate the immmediate response of cells to produce HSP70 for example by modulating transcription of HSP70 by heat shock factors. Increases in transcription of HSP70 RNA were not seen at 12 hours in the microarray experiments however, possibly because the induction of HSP70 had already occurred by this time.

Previous reports have suggested that the increased levels of HSP70 following heat shock lead to increased in the amounts of HSP70 bound to BAG-1 (Song et al. 2001). The opposite was seen here, and it was found that heat shock lead to the

complete dissociation of BAG-1 from both HSC70 and HSP70 in pcDNA clones, but only a partial dissociation in BAG-1S clones. It may well be that this difference is simply a result of the equilibrium kinetics and the interaction in the BAG-1S clones is maintained because there is much more BAG-1S present. This does not mean that the differences are not biologically significant however, and these results suggest that the maintenance of the BAG-1:HSC70/HSP70 interaction is important for cell survival following heat shock consistent with previous work using BAG-1S point mutants (Townsend et al. 2003a). Importantly the levels of heat shock used in the experiments described here were carefully titrated so that they led to biologically significant outcomes in particular survival in the BAG-1S clones and death in the pcDNA clones. It is possible for example to envisage that lesser or greater levels of heat shock, as used in other studies, may produce differing results.

Raf-1 is activated by BAG-1 by binding to a site on BAG-1 that overlaps with the binding site of HSC70 and HSP70. The possibility that BAG-1 mediated its effect following heat shock by modulating signalling through the MAP kinase pathway was therefore explored. Raf-1 was initially identified as the cellular homologue of v-raf the transforming gene of the murine sarcoma virus 3611 (Rapp et al. 1988). Observations that the catalytic domain of Raf-1, without the regulatory functions of amino-terminal sequences, are sufficient to transform murine cells (Schultz et al. 1988) clearly demonstrate that Raf-1 signalling is inherently pro-survival and therefore makes it an attractive possible BAG-1 target. The situation is more complex however when the whole network of MAP kinase signalling is considered. Although MAP kinases are generally activated after cellular stress the cellular effects are dependent on a wide range of interacting factors. Therefore prolonged activation of ERK1/ERK2 generally is generally required for transcription of cyclin D1, whilst activation of p38 inhibits transcription of cyclin D1 and has a negative role in cell proliferation (Robinson & Cobb 1997). It was therefore important that all the main MAP kinase pathways were investigated. Despite an intensive and thorough search however no consistent differences were found by western blotting in MAP kinase signalling between BAG-1S and pcDNA clones following heat shock. A clear interaction between BAG-1 and Raf-1 was also not demonstrated in these cells. It is

therefore possible that the importance of the BAG-1:Raf-1 interaction and signal modulation is cell type specific, and is not an important target of BAG-1 in this system. At the time these experiments were initiated the effects of point mutations within the BAG domain of BAG-1S on long term survival were not known. It was subsequently demonstrated that BAG-1S helix 3 point mutants that retain the ability to bind Raf-1, but lose the ability to bind to the chaperones, also lose the ability to rescue MCF-7 cells from heat shock (Townsend et al. 2003a). This provides further evidence that in at least in this system, the BAG-1: HSC70/HSP70 interaction is of greater importance than the BAG-1:Raf-1 interaction in protecting from heat shock induced apoptosis.

In contrast to Raf-1, both BAG-1 amino-terminus deletion mutants and a ULD point mutant suggest that the BAG-1 ULD is important for BAG-1 mediated survival in this system (Townsend et al. 2003a). The ULD mediates interactions between BAG-1 and the proteasome (Luders et al. 2000a) and so clonogenic assays were performed to asses the effect of proteasome inhibitors, for example MG132, on the protective effect of BAG-1 following heat shock. Unfortunately these inhibitors significantly reduced clonogenic potential, even in the absence of heat shock, and so this approach did not produce meaningful results and was not pursued (data not shown). The BAG-1 ULD does not however appear to stimulate increases in total ubiquitylation of cellular proteins either before or after heat shock (Section 5.3), but may effect the expression of specific targets of E3 ligases to which BAG-1 binds such as β-catenin which is ubiquitylated by Siah (Section 5.3). This is supported by evidence presented demonstrating that BAG-1S but not BAG-1S^{K80A} is associated in complexes with high molecular weight ubiquitylated proteins (Figure 5.6). It is possible to envisage ternary complexes between BAG-1, E3 ligases and specific E3 ligase targets such as β-catenin that may be important growth regulatory or survival proteins. BAG-1 may be involved in the regulation of E3 ligase activity whilst the E3 ligases themselves confer substrate specificity. Since BAG-1 itself is ubiquitylated at lysine 80, this may explain in part the importance of this domain. Ubiquitylation of BAG-1 may act as a signal or for E3 ligase targeting or activation, or may act as a cofacotor in the ubiquitylation of specific substrates by E3 ligases.

The p53 protein is inactivated by mutation in approximately 50% of human cancers and therefore is an important tumour suppressor. Since wild type p53 is pro-apoptotic and growth inhibitory and is induced by types of damage that may produce DNA mutations, induction of p53 prevents the proliferation of cells that may harbour cancer-causing mutations by deleting such damaged cells. Many tumours however are able retain wild type p53 and to survive and proliferate despite having such DNA changes. In particular the MCF-7 cells that overexpress BAG-1 proliferate despite p53 induction after heat shock, whilst controls transfected do not survive. In the BAG-1 transfected cells there must presumably be mechanisms of inactivating the function of wild type p53. Reporter assays demonstrated that BAG-1S and BAG-1L were both able to inhibit p53 dependent transcription in HEK-293 cells. These results have now also been confirmed by others in Dr Packham's laboratory in MCF-7 cells (Dr D O'Connor and Dr G Packham; personal communication). These effects of BAG-1 on p53 dependent transcription, although preliminary, are potentially a novel mechanism by which p53 function and cell survival is regulated by BAG-1. For example BAG-1 overexpression may provide a mechanism by which tumour cells can tolerate wild type p53. Consistent with this in breast cancer BAG-1 overexpression commonly occurs in well differentiated tumours, and these tumours commonly retain wild type p53. Further work must be performed to characterise the effect fully. This will include comparison of the various BAG-1 isoforms for the ability to inhibit p53 dependent transcription in other cellular backgrounds for example in a p53 null background such as Saos-2 oseosarcoma cells, and with other p53 responsive promoters, and control promoters.

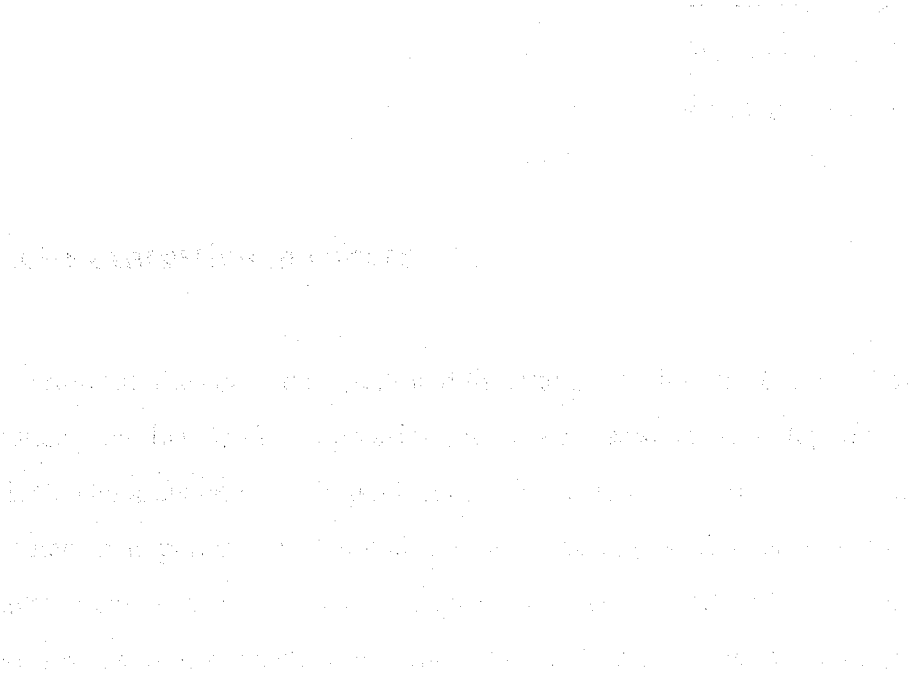
The stabilisation of p53 that occurs in MCF-7 cells following heat shock is not altered by BAG-1S and so BAG-1S does not significantly enhance p53 ubiquitylation and degradation. It is possible however that BAG-1 may regulate p53 dependent transcription through regulation of the degradation of other specific target proteins involved in the control of p53 function. For example the ASPP proteins enhance DNA binding and activation by p53, and are frequently down regulated in human breast carcinomas expressing wild-type p53 (Samuels-Lev et al. 2001), which will also tend to express high levels of BAG-1.

To determine if BAG-1 expression altered the expression of p53 target genes, p21 expression was studied before and after heat shock. Although p21 levels did appear to be regulated by BAG-1S following heat shock induction of p21 occurred too early to be due to changes in p53 transcriptional activity since it preceded induction of p53. The literature on the induction of p21 by heat shock demonstrates that p21 can be induced both by p53 dependent and p53 independent mechanisms, and that p21 degradation is often regulated by ubiquitylation. For example in primary human fibroblasts, cell cycle arrest after heat shock is p53 dependent and due to p21 induction which occurs after 6 hours (Nitta et al. 1997). In contrast in a immortalised fibroblast cell line (MDA HO41; p53 null), p21 induction following heat shock occurred within 1 hour, and in the presence of cycloheximide suggesting that it was due to changes in the rate of p21 degradation (Fuse et al. 1996). Thus p21 may be an important target of BAG-1S, but may be more associated with BAG-1 effects on protein turnover then on p53 function per se.

In addition to the directed analysis of potential BAG-1 target proteins a microarray analysis was performed as a systematic technique to identify novel BAG-1 potential targets. It is important when assessing the microarray data to bear in mind the preliminary nature of the results. Three genes (MADD, Melanophilin and an EST) appear to be differentially regulated both by BAG-1S and by heat shock (Table 5.4). It is conceivable that this result is not real and that the levels of RNA expression of these genes were artefactually high in the pcDNA sample (cells maintained at 37 ^{o}C) that was used as a control for all the pairwise comparisons. It is possible for example that the signal produced for these genes was due to non-specific adherence of the probe to dust or a slightly damaged point on the filter. The changes found in these stably transfected clonal cell lines will also need to be confirmed in other BAG-1S and pcDNA clones to confirm that they are representative of the effects of BAG-1 as a whole and not an artefact of the particular clonal cell line used. Also PCR techniques will be required to confirm the changes in RNA expression since the cDNA clones can occasionally be "spotted" inaccurately during the production of the filters or libraries may be cross-contaminated. In addition the changes will ultimately have to be confirmed at a protein level, as changes in RNA levels may not always be

translated into changes in protein levels. Indeed increases in RNA production could be a response to reduced protein levels for example as part of a negative feedback loop responding to increased protein degradation.

Despite these cautions the technique was successful in achieving the aims of identifying several candidate BAG-1 targets. Interestingly many of these fall into functional categories that BAG-1 might be expected to impinge on, such as proteins involved in cell signalling through tyrosine kinase pathways. It is reassuring that in all instances examined where there was more than one spot for a particular gene then both spots demonstrated the same trend, if not always to the same degree. Where differences occurred it was usually apparent from studying the filter that they were due to saturation of one of the spots. There are therefore consistent changes in the expression of multiple candidate genes, and although many of the changes are consistent with what is understood of the biology of BAG-1 and heat shock, all the candidates will require further testing and evaluation before any can be confirmed as novel targets important for BAG-1 action.



6. Discussion

It is likely that BAG-1 plays an important role in breast cancer. Expression of BAG-1 is frequently altered in human cancers and may have prognostic value (Cutress et al. 2002; Takayama & Reed 2001; Tang 2002). Work described here demonstrates that nuclear BAG-1 expression is associated with expression of ERα and PgR receptors, and in patients treated with hormone therapy, with patient outcome (Chapter 3). BAG-1 is known to regulate nuclear hormone receptors and thus the sensitivity of cells to a variety of nuclear hormones, and hence modulate signaling involved in controlling a myriad of important cellular processes (Cato & Mink 2001; Cheung & Smith 2000). Work described in this thesis demonstrates that BAG-1L interacts with and potentiates signalling through both ERα and ERβ and the mechanisms underlying this have been examined by mutagenic analysis (Chapter 4). Multiple studies using a wide variety of techniques have previously demonstrated that BAG-1 protects cells from diverse inducers of apoptosis and activates key signaling molecules such as Raf-1, important for cell proliferation and survival (Takayama & Reed 2001; Tang 2002; Townsend et al 2003b). It is now known that BAG-1 attenuates p53 dependant transcription and preserves long term clonogenic potential following heat shock in MCF-7 cells, and microarray analysis has provided further clues of potential BAG-1 targets (Chapter 5). These new findings are likely to increase the understanding of role and function of BAG-1.

6.1 BAG-1 expression in cancer

Various studies have demonstrated that the expression BAG-1 is altered in breast cancer and that BAG-1 expression patterns are associated with patient outcome (Table 1.3). The association of BAG-1 expression with hormone receptor status and patient outcome in patients treated with hormone therapy is addressed in chapter 3. The results obtained here, that BAG-1 expression is associated with improved patient outcome appears to be consistent with the results of several other studies of patients

with this disease. Both Townsend et al (Townsend et al. 2002), and Turner et al (Turner et al. 2001) and work described in chapter 3 suggested that BAG-1 expression was associated with improved outcome. Whilst work described in chapter 3, and by Townsend et al suggested that nuclear BAG-1 was associated with improved outcome, Turner suggested that cytoplasmic BAG-1 expression was associated with improved outcome. A close association between nuclear and cytoplasmic BAG-1 expression was described in chapter 3 and this may partly explain this difference. In contrast the study of Tang (Tang et al. 1999) demonstrated an association between BAG-1 expression and shorter overall and disease free survival. Sjostrom et al (Sjostrom et al. 2002) however found no association between BAG-1 expression and outcome, but they did not separate nuclear from cytoplasmic BAG-1 expression unlike other studies that demonstrate differences in patient outcome. Although BAG-1 expression therefore appears to be predictive of outcome from breast cancer there are differences in detail between the studies, and differences in cohort characteristics and treatment regimens may partly account for this (Cutress et al. 2001).

The association found in chapter 3 between nuclear BAG-1 expression and improved outcome in patients treated with hormone therapy is intriguing given the effects of BAG-1 expression on oestrogen dependent transcription (chapter 4). It is postulated that increases in BAG-1 expression help drive oestrogenic signalling in ERα positive tumours that are dependent on these pathways. This is also found with AIB1, which like BAG-1 enhances oestrogen dependent transcription and commonly undergoes gene amplification in breast cancer which correlates with ERα and PgR positivity (Anzick et al. 1997; Bautista et al. 1998; Planas-Silva et al. 2001). This hypothesis also explains the observed association found between nuclear BAG-1 expression and expression of the ERα target gene PgR. In contrast tumours that are less dependent these pathways can afford to lose expression of ERα and nuclear BAG-1, and thus lose PgR expression. Hence as with loss of ERα expression, loss of nuclear BAG-1 expression is associated with poorer patient outcome. In contrast to other downstream markers of ERα function such as PgR and pS2 it is hypothesised

nuclear BAG-1 expression may be partly responsible for the increased oestrogenic signalling, and may therefore serve as a potential target for therapy in these tumours.

Despite the successful production of a BAG-1L specific antibody however it has still not been formally proven if nuclear BAG-1 expression in these tumours is due to increased expression of BAG-1L. Given that the multiple BAG-1 isoforms can be functionally distinct, and in particular given the fact that only BAG-1L potentiates oestrogenic signalling, the consequences of of relocalisation of the cytoplasmic isoforms to the nucleus might be very different from those of high level BAG-1L expression. As discussed these proteins were not discriminated by the antibodies previously used in immunohistochemical analyses of cancer samples. Since the same mRNA encodes all BAG-1 isoforms it is also not possible to use RNA-based approaches to measure their relative levels of expression. The BAG-1L specific antibody produced is therefore an important reagent. It is unfortunate that it does not produce staining on formalin fixed paraffin embedded material, and that BAG-1 epitopes in the nucleus appear to be hidden to both this antibody and to the pan-isoform antibody 3.10 G3E2 in frozen material. The BAG-1L specific antibody demonstrates that BAG-1L may be present in the cytoplasm in breast cancer cells. As with colon cancer where it is known the relocalisation of BAG-1L to the cytoplasm does occur under certain situations (Arhel et al. 2003), it is possible that BAG-1L can relocalise to the cytoplasm in breast cancer. The further development and application of isoform specific antibodies will enable further characterisation of the significance and expression patterns of specific BAG-1 isoforms. This should enable improved dissection of the relationship between differing BAG-1 isoforms and clinical outcome.

One limitation of immunohistochemical studies that simply assess protein expression is that functional changes may pass undetected. It is not known for example if mutations occur within BAG-1, and if so what the functional consequences might be. BAG-1 may be phosphorylated under some conditions (Cato & Mink 2001; Schneikert et al. 2000). The functional significance of this and other potential post-translational modifications (such as BAG-1 ubiquitylation (chapter 5) (Sourisseau et al. 2001)) are poorly understood and presumably are not discriminated

by immunohistochemistry with current antibodies. It is also likely that the key targets of BAG-1 will differ between cell types. For example, ERα is a key regulator of breast epithelial cells and BAG-1L may therefore be a key determinant of survival in breast cancer. By contrast, in oral cancer, suppression of retinoid-induced differentiation may be crucial. Since the BAG-1S isoform suppresses RAR function, overexpression of BAG-1S might also be of importance. Thus, we should not expect a simple pattern of changes in BAG-1 expression in all cancer types due to differences in "key" BAG-1 targets in different cell types. Improved understanding of these factors may further clarify the possible role of BAG-1 as a novel prognostic marker and therapeutic target in a wide range of malignancies.

Overall there is increasing evidence that BAG-1 expression is frequently altered in the most common human cancers. Studies have focused on breast cancer, non-small cell lung cancer and colon cancer. As the second most common cancer in men and the fourth most common cancer in the United Kingdom overall it is very important that a study of the expression of BAG-1 in prostate cancer is undertaken. This is particularly important given the known functional consequences of BAG-1 overexpression on androgen receptor signalling, and responsiveness to anti-androgens, and since like breast cancer prostate cancer is often dependent on hormonal signalling and hormone therapy is an important form of treatment. BAG-1 expression has also been examined in squamous cell carcinoma of the head and neck, and there are indications that BAG-1 expression may also be altered in glioblastoma, cervical cancer and haemopoietic malignancies (Table 1.4). Although some inconsistencies have been reported, there appears to be broad agreement that BAG-1 is overexpressed in breast and non-small cell lung cancer and that this can correlate with clinical parameters and improved patient outcome. Conversely BAG-1 overexpression is associated with poorer prognosis in colo-rectal and oral squamous cell carcinomas. Differences between tumour types may be due to differencies in the relative importance of the many BAG-1 functions in the different tumours. Further work, including prospective trials, is required to confirm these findings. Larger prospective studies should be more representative of the spectrum of breast cancer as a whole and have increased power to detect independent prognostic predictors in

multivariate analysis, in particular in the presence of possible confounding associations such as tumour grade and ERα status. In addition given the ability of BAG-1 to target multiple biological pathways, these should be sufficiently large to allow pre-defined subgroup analysis to determine the effect of BAG-1 on survival in patients treated with specific therapeutic regimens (e.g., hormonal therapy versus chemotherapy). The TACT study is a randomised-controlled trial that has recruited over three thousand patients to investigate the effect of the addition of docetaxol, a drug that inhibits the production of tubulin, to standard adjuvant chemotherapy regimes. Patients within this study have consented to have various "biological predictors of therapeutic response" measured, and an application has been co-written by the author to consider inclusion of BAG-1 as one of these markers.

Since the deregulation of BAG-1 expression has functional consequences for a wide range of cancers an increased understanding of the mechanism by which BAG-1 expression is regulated and can be deregulated will be important. The BAG-1 promoter has only been studied to a limited degree and the importance of transcription factors, signalling pathways and external stimuli in controlling the expression of BAG-1 will need further study. Cell type specific differences in BAG-1 isoform expression patterns and reporter analysis with the BAG-1 promoter and various deletion constructs may provide a starting point for such studies. For example BT-474 cells express high levels of BAG-1L compared to other breast cancer cell lines and CAL-51 cells express low levels of BAG-1S compared to other breast cancer cell lines (Figure 3.11) (Brimmell et al. 1999). It is likely that increases in BAG-1S expression are due to increased IRES activity since increased promoter activity may be expected to increase BAG-1L expression preferentially. As BAG-1 expression is also regulated by post-transcriptional mechanisms (Townsend et al. 2002), it will be important to understand better exactly how this occurs and under what circumstances. Mutations in the c-myc IRES have been described in multiple myeloma that lead to increased c-myc translation for example (Chappell et al. 2000), and it is conceivable that such mutations may occur in the BAG-1 IRES and lead to the high levels of BAG-1S found in some tumours.

6.2 BAG-1 and NHR transcription

The predominant expression of BAG-1L in many hormone dependent cancer cell lines (Takayama et al. 1998) suggests that BAG-1 may play an important role in modulating responses to nuclear hormones and hormonal therapies in cancer cells. The list of hormone receptors affected by BAG-1 now includes the oestrogen receptors ERα and ERβ. BAG-1L potentiates transcription through both these receptors at the consensus ERE but it will be interesting to determine if BAG-1 can modulate their function differentially under some conditions, or at certain atypical oestrogen response elements such as at AP1 sites where the responses of these two receptors to oestrogens differ. Further studies are also underway to determine the relationship between BAG-1 and ERβ expression in cohorts of breast cancer patients. It is hoped that these studies will increase the understanding of the relationship between BAG-1 expression and the differing nuclear hormone receptors in breast cancer.

There are notable differences in the effects of BAG-1 isoforms on the various nuclear hormone receptors. In some cases such as with the AR and as shown here with ERα and ERβ BAG-1 potentiates receptor activity, whereas in others such as with the GR it inhibits activity. The spectrum of isoforms that regulate activity is also distinct for each receptor. BAG-1L, but not BAG-1S or BAG-1M interacts with and modulates AR and ERα and ERβ signalling whilst both BAG-1L and BAG-1M inhibit GR signalling. In addition interaction with receptors is frequently dependent on the BAG-1 carboxy-terminus, suggesting it is mediated via the chaperones HSC70/HSP70, but this is not the case with the RAR where carboxy-terminal deletions of BAG-1 do not affect function (Liu et al. 1998). Interestingly BAG-1L function in the ER transcription assays reported here did not appear as dependent on interaction with heat shock proteins, as evidenced by the experiments with the BAG-1L point mutants that fail to bind HSC70/HSP70, as had been previously described for the AR or GR (Briknarova et al. 2001; Schmidt et al. 2003).

For receptors other than the RAR and TR the ability of BAG-1 to bind to HSC70/HSP70 is not sufficient for regulating receptor function and the acidic repeats

or residues at the extreme amino-terminus of BAG-1M may also be required. A carboxy-terminal truncation of BAG-1L that removes the HSP70 binding site acts as a trans-dominant repressor of AR function suggesting that the unique amino-terminus has important interactions with the AR transcriptional complexes unrelated to HSP70 (Froesch et al. 1998). Moreover, since all BAG-1 proteins bind the chaperones this can not explain why only the larger isoforms are active in most cases. Indeed the nuclear localisation of BAG-1L is also not sufficient to account for its activity and relocalisation of BAG-1S with a heterologous fused NLS did not potentiate ERα dependent transcription in studies described here. Such results were also reported with the AR where fusion of an NLS to BAG-1M but not BAG-1S produced activity (Knee et al. 2001).

A cluster of charged residues at the extreme amino-terminus of BAG-1M is required for binding to DNA and their deletion prevents in-vitro receptor independent transcriptional activity (Zeiner et al. 1999). Substitution of these residues with alanine also abrogates BAG-1M mediated attenation of GR activity in cells (Schmidt et al. 2003). Some of these residues however form part of the BAG-1L SV-40 large T-like NLS, and the effects of these mutations on BAG-1 cellular localisation (BAG-1L) and relocalisation with ligand associated receptor (BAG-1M and the glucocorticoid receptor) has not been studied. It will for example be important however to determine if these mutations are associated with changes in DNA binding ability secondary to changes in cellular localisation. It will also be important to determine if such mutations in the context of BAG-1L affect the ability of this protein to modulate ERα transcriptional activity.

As a result of these differences and findings it has been proposed that there are two potential mechanisms of action that account for the effects of BAG-1 proteins on NHR (Cato & Mink 2001; Cheung & Smith 2000). BAG-1 appears to be able to act both pre- and post-hormone binding. For example GR complex assembly and affinity for hormone is influenced by BAG-1 (Kanelakis et al. 1999; Kanelakis et al. 2000), and BAG-1 also inhibits binding of the GR to DNA (Schneikert et al. 1999). Possibly BAG-1 may act both to influence receptor refolding by chaperones, and additionally act to serve as a scaffold to recruit chaperones or transcriptional coactivators or

corepressors to NHR complexes. BAG-1 can therefore appears to modulate hormone receptor function through regulation of chaperone refolding function, and possibly through affecting the stability of hormone receptor DNA complexes. It is therefore conceivable that the potentiation of ERα signalling compared to AR signalling by BAG-1L is more dependent on stabilisation of receptor/cofactor interactions with DNA than on receptor refolding. In this respect the effect of BAG-1L on ERα may be more similar to its effect on the RAR than its effect on the AR. This would provide a possible functional explanation for the lesser effects observed of BAG-1L point mutations that prevent chaperone binding on BAG-1L mediated potentiation of ERα transcription. Key to this is the question of whether hormone receptor binding to BAG-1 is always through the chaperones. There have been no reports in the literature as to the effects of these point mutations that prevent chaperone binding on the binding of the receptors on BAG-1, although there are now two reports describing the effects on transcriptional activity. With ERα no clear-cut answer as to whether these mutations completely prevent binding has been obtained, and it is possible that the interaction is stabilised by, rather than mediated through the chaperones.

6.3 BAG-1 and cell survival

BAG-1 appears to suppress apoptosis induced by a broad range of agents in different cell types upstream of caspase activation (Schulz et al. 1997; Takaoka et al. 1997), but few studies have determined the domains and important residues required for suppression of apoptosis. Both the amino- and carboxy-terminus of BAG-1S appear to be required (Bardelli et al. 1996; Yang et al. 2000), and BAG-1M and BAG-1L also possess anti-apoptotic activity but the poorly expressed p29 isoform does not (Chen et al. 2002b). Overexpression of BAG-1 for example suppresses apoptosis induced by chemotherapeutic agents, radiation and growth factor withdrawal (Table 1.2). Therefore, in addition to contributing to reduced cell death in cancer development, BAG-1 may also contribute to resistance to important therapeutic modalities. Suppression of apoptosis may also be responsible for the

ability of BAG-1 to promote metastatic spread (Takaoka et al. 1997; Yawata et al. 1998).

BAG-1 proteins interact with and modulate the activity of the 70 kDa heat shock proteins, HSC70 and HSP70, via helices two and three of the carboxy-terminal BAG domain. The pro-survival effects of BAG-1 are often ascribed to this interaction since HSP70 and HSC70 play important roles in regulating cell survival (Hohfeld 1998;Jolly & Morimoto 2000). In addition deletion of the BAG-1 carboxy-terminus abrogates its pro-survival activity, as well as many other BAG-1 functions (Bardelli et al. 1996; Froesch et al. 1998; Kudoh et al. 2002; Yang et al. 2000). Consistent with this it has been demonstrated that BAG-1 and HSC70/HSP70 interactions are maintained in BAG-1S MCF-7 clones following heat shock. These clones survive and maintain long term clonogenic potential. In contrast untransfected MCF-7 cells and control pcDNA transfected MCF-7 clones do not maintain the BAG-1:HSC70/HSP70 interaction following heat shock and do not survive (Chapter 5).

Refolding of denatured proteins through modulation of HSC70/HSP70 refolding activity alone is unlikely however to explain the pro-survival effects of BAG-1, in particular since BAG-1 generally inhibits chaperone-dependent protein refolding. Also the BAG-1 carboxy-terminus is generally sufficient for inhibiting refolding but amino-terminal regions of BAG-1 are required for the pro-survival activity. It is therefore possible that BAG-1 is involved in the regulation of specific target molecules either through heat shock proteins or possibly acting as a molecular "bridge" recruiting chaperones to target proteins.

The BAG-1 carboxy-terminus is required for interaction and activation of Raf-1 (Wang et al. 1996), and this provides an alternative mechanism by which BAG-1 might promote cell survival (Morrison & Cutler 1997). Since both Raf-1 and the 70KDa heat shock proteins interact with the BAG domain of BAG-1 simple deletion of this domain will not distinguish between these possibilities and so the relative importance of these two pathways is unclear. In addition HSP70 and Raf-1 compete for binding to the BAG domain of BAG-1 and high levels of HSP70 prevent activation of Raf-1 by BAG-1. It has therefore been suggested that the pro-survival effects of BAG-1 may be mediated by activation of Raf-1 dependent MAP kinase

pathways, and that these are negatively regulated by chaperone binding (Song et al. 2001). Therefore the suggestion has been made that BAG-1 isoforms may act as a "molecular switch" in signalling pathways, and direct cells towards different states depending on whether environmental conditions are hospitable or stressful (Song et al. 2001; Takayama & Reed 2001). Although these hypotheses are compelling and elegant little evidence has been found from work described in chapter 5 that these pathways play a major role in BAG-1mediated survival from heat shock in MCF-7 cells. Extensive analysis of MAP kinase pathways by western blotting before and after heat shock in pcDNA control and BAG-1S MCF-7 clones failed to demonstrate any consistent differences between the BAG-1S clones that survived the heat shock and the pcDNA clones that did not. In addition no interaction was observed by co-immunoprecipitation between BAG-1 and Raf-1 in MCF-7 cells.

Since deletion of the BAG-1 amino-terminus prevents rescue from apoptosis, protein regions in addition to the BAG domain that binds HSC70/HSP70 are required for biological effects. BAG-1 possibly acts as a scaffold molecule, to functionally link chaperone function with specific target molecules (Hohfeld et al. 2001; Takayama & Reed 2001). For example, BAG-1 may protect cells from the apoptotic effects of cell stress induced by radiation or heat shock by enhancing the delivery of chaperone-bound denatured proteins to the proteasomal degradation system. The BAG-1 amino-terminus contains a ULD, similar to ubiquitin and the ULD found in other proteins such as Rad23, involved in DNA repair (Takayama et al. 1995). Like Rad 23, BAG-1 interacts with the proteasome via its amino-terminus and has been suggested that BAG-1 acts as bridge linking chaperone molecules with the proteasome (Demand et al. 2001; Luders 2000a). HSC70/HSP70 plays an important role in refolding cellular proteins following cellular stress and models have been proposed whereby BAG-1 acts to direct HSC70/HSP70 bound to a whole range of denatured proteins, that can not be refolded, to the proteasome for degradation. Alternatively BAG-1 may act in a specific manner in conjunction with HSC70/HSP70 to target specific substrates that might be important for regulation of cellular growth, division or survival for degradation and hence play a part in the control of the levels of certain key regulatory proteins.

Although BAG-1 amino-terminal deletions inactivate BAG-1's survival functions (Bardelli et al. 1996; Yang et al. 2000a) specific residues within the ULD important for survival had not been previously determined. It is now clear however that a conserved lysine (K80 within BAG-1S) within the ubiquitin like domain of BAG-1S is necessary for BAG-1 mediated survival from heat shock (Townsend et al. 2003a), and it has been demonstrated here that this is a site of BAG-1 ubiquitylation. The exact role of this, and how this may be involved with targeting substrates to the proteosome however is unclear. BAG-1 interacts with two E3 ligases, Siah and CHIP, and it is possible that BAG-1 acts as a cofactor or intermediate in the ubiquitylation of certain substrates, in addition to linking such complexes to the proteasome. Alternatively the atypical poly-ubiquitin chain formed on BAG-1 through this lysine may serve as a cellular signal to target and bind the complexes to the proteasome.

The transcriptional activity of BAG-1 is likely to be of importance in the ability of BAG-1 to protect cells from apoptosis. In addition to effects on NHR, where in general BAG-1 inhibits those receptors that are growth inhibitory and stimulates those that are growth and survival promoting, BAG-1 can inhibit apoptosis associated with p53 stabilisation. It is possible that the observations described here that BAG-1 can inhibit the transcriptional activity of p53 provide an explanation for this. Mechanistically the function of p53 is highly confirmation dependent and it is possible that BAG-1 regulates chaperone mediated folding of p53 and thus regulates p53 transcriptional function in this way. Microarray analysis described in chapter 5 has identified various novel potential targets of BAG-1 action. Reassuringly many of these candidate proteins are involved in processes that BAG-1 is known to play a role. BAG-1 therefore potentially affects the transcription of various candidate proteins involved in responses to cellular stress, cellular signalling, the ubiquitin activating enzyme E1, and various ribosomal proteins.

Although work in this thesis has focused on overexpression recent antisense and dominant negative experiments have demonstrated that endogenous BAG-1 plays an important role in maintaining cancer cell survival (Kudoh et al. 2002; Sawitzki et al. 2002a; Townsend et al. 2003a). Small molecules targeting other components of the chaperone machinery, such as geldanamycin derivatives, are producing encouraging

results in early clinical trials (Workman & Maloney 2002). Criteria have been suggested to select biological targets for rational therapies (Alberti et al. 2002), and BAG-1 fulfils many of these. In addition as BAG-1 is multifunctional it is possible that blocking BAG-1 function may affect multiple pathways important for the development and progression of cancer. As many of the functions of BAG-1 appear to be dependent on the BAG-1 HSC70/HSP70 interaction it is felt that targeting this interaction with competitor peptides may lead to novel therapeutic approaches, and may prove to be an important experimental tool. Such approaches have been used successfully to block the mdm-2:p53 interaction to activate p53 (Bottger et al. 1997), to mimic p21 and thus inhibit cyclin D1:Cdk4 complexes and induce cell cycle arrest (Ball et al. 1997), and to block complexes formed with the eIF4E translation initiation factor leading to apoptosis (Herbert et al. 2000). In preliminary pilot experiments a peptide of 21 amino acids was designed with sequence homology with part of helix 3 of the BAG domain (Figure 6.1A). This peptide successfully inhibited the BAG-1:HSC70 interaction in GST pulldown assays (Figure 6.1B) suggesting that at least in principle it is possible to block these interactions using relatively small peptides. Further work is now in progress characterising and optimising various peptides for the ability to inhibit the BAG-1:HSC70 interaction. Such approaches are however dependent on the importance of such interactions and processes in tumour development and survival. It remains possible, despite evidence to the contrary that the altered expression of BAG-1 in cancer is a consequence of malignant conversion, rather than a causal or contributory factor. The results of gene ablation and transgene experiments in mice will help elucidate the normal function of BAG-1 and to determine whether it plays a causal role in cancer. Ultimately the success of any agent will also depend not only on the importance of these interactions for tumour cells, but also on the importance of these interactions for normal processes and therefore the resultant toxicity and therapeutic ratios will prove crucial. In any case such approaches should prove to be important tools in the elucidation of BAG-1 function, even if the ultimate goal of successful anti-cancer therapy is not realised.

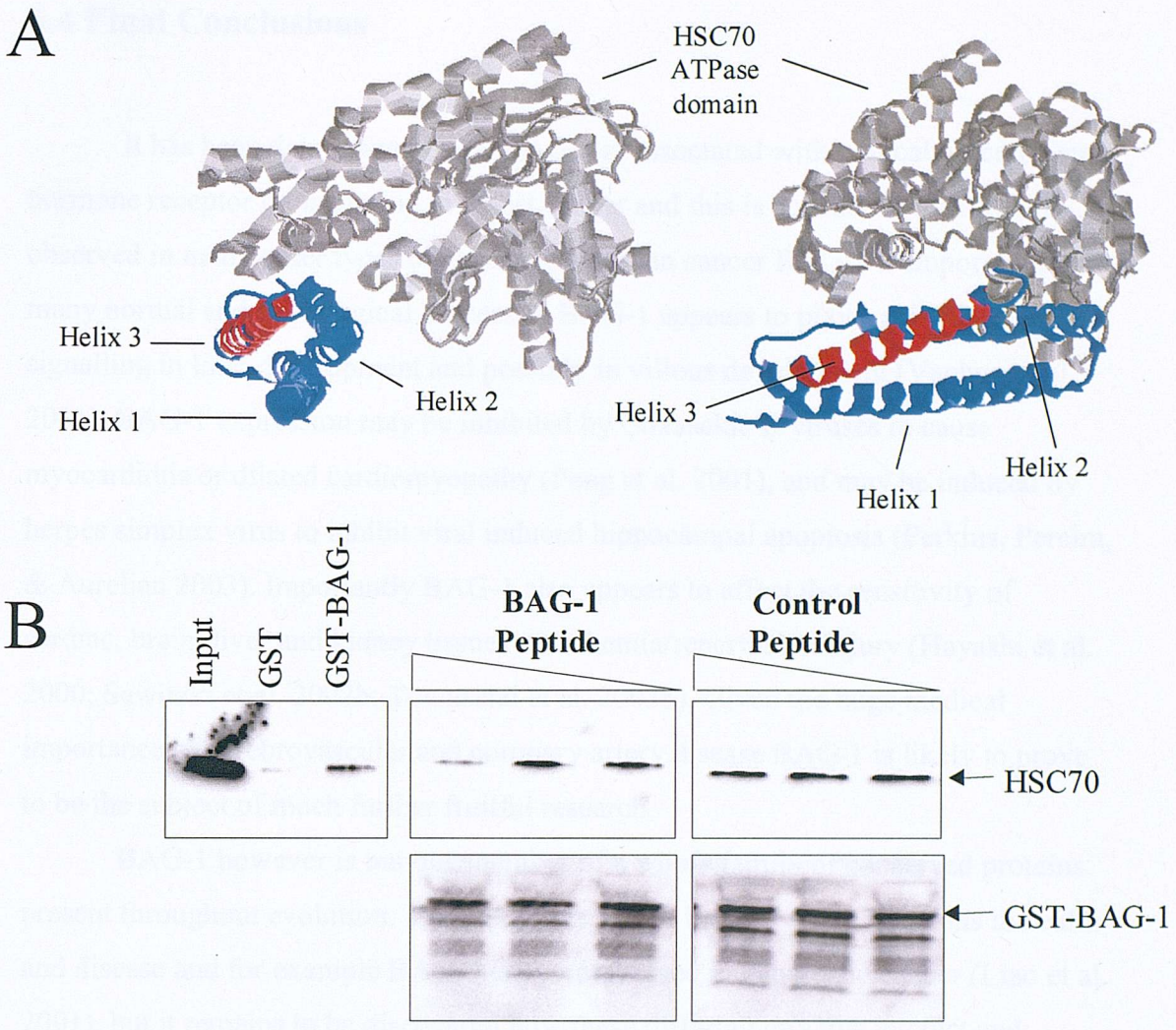


Figure 6.1: The effect of competitor peptides on the BAG-1 HSC70 interaction
Synthetic peptides with sequences corresponding to part of helix 3 of the BAG-1 BAG domain (A; red, and BAG-1 peptide in B) and a control peptide with sequences corresponding to part of the BCL-2 protein (control peptide in B) were used in GST pulldown assays (B). GST pulldown assays were performed in the absence of peptide to demonstrate HSC70 expression in the input rabbit reticulocyte lysate (Input) and following pulldown with GST-BAG-1S (GST-BAG-1) or control pulldown with GST (GST) alone. BAG-1 or control peptide (10 μM, 1 μM and 100nM), was also incubated with lysates prior to GST pulldowns with GST-BAG-1S. Following GST pulldown bound complexes were washed, separated by SDS-PAGE, transferred onto nitrocellulose membrane and analysed by immunoblotting for HSC70 (B6) and BAG-1 (191-TB2).

6.4 Final Conclusions

It has been demonstrated that BAG-1 is associated with clinical outcome and hormone receptor transcription in breast cancer and this is consistent with what is observed in many other types of cancer. Other than cancer BAG-1 is important in many normal and pathological processes. BAG-1 appears to play a role in retinoid signalling in limb development and possibly in villous development (Vachon et al. 2001). BAG-1 expression may be inhibited by Coxsackie B viruses to cause myocardidtis or dilated cardiomyopathy (Peng et al. 2001), and may be induced by herpes simplex virus to inhibit viral induced hippocampal apoptosis (Perkins, Pereira, & Aurelian 2003). Importantly BAG-1 also appears to affect the sensitivity of cardiac, brain, liver and kidney tissues to ischemia/reperfusion injury (Hayashi et al. 2000; Sawitzki et al. 2002b; Townsend et al. 2003b). Given the huge medical importance of cerebrovascular and coronary artery disease BAG-1 is likely to prove to be the subject of much further fruitful research.

BAG-1 however is but one member of a whole family of conserved proteins present throughout evolution. These proteins are all likely to have functions in health and disease and for example BAG-3 is overexpressed in pancreatic cancer (Liao et al. 2001), but it remains to be discovered how these differing proteins interact and function with each other. Work described here and elsewhere demonstrates that BAG-1 and probably this whole family of proteins appears to function by linking chaperone proteins to other effector proteins. These proteins thus play a pivotal role linking together protein production, refolding and repair with other fundamental processes such as degradation cell death and cell survival.

7. References

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