INTERACTION OF THE ANTI-APOPTOTIC PROTEIN BAG-1 WITH THE VITAMIN D RECEPTOR

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Interaction Of The Anti-Apoptotic Protein BAG-1 With The Vitamin D Receptor

by

©Michael Witcher

A thesis submitted to the School of Graduate Studies
in partial fulfillment of the requirements for the
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Abstract

BAG-1 (BCL-2 associated anti-death gene) is a multi-isoform, BCL-2 binding protein having anti-apoptotic capabilities. In addition to binding BCL-2, BAG-1 has been found to bind and regulate the function of various steroid hormone receptors. It is postulated that BAG-1's ability to inhibit apoptosis and proliferation induced by hormone receptors could lead to tumor growth if BAG-1 were to become overexpressed. In fact, BAG-1 has been found to be overexpressed in cervical and breast tumors indicating that BAG-1 may be a proto-oncogene. To further investigate BAG-1's role in steroid hormone regulation, I decided to investigate whether or not BAG-1 could bind and regulate another member of the steroid hormone receptor super family - the vitamin D 3 receptor.

Far Western blot analysis and glutathione S-transferase -BAGp50 pull-down assays revealed that the full length 50 KDa isoform of BAG-1 could interact with the vitamin D receptor (VDR). The shorter isoforms however, could not. Gel shift assays using cell extracts from BAGp50 stably transfected U87 glioblastoma cells (U87BAG-1) showed that BAG-1 could inhibit the VDR from binding to its consenus response element, as well as a vitamin D response element (VDRE) from the $p21^{waf1}$ promoter. Furthermore, overexpression of BAGp50 not only resulted in an increased rate of proliferation but rendered the cells resistant to vitamin D-induced growth inhibition. A CAT construct containing a osteocalcin VDRE was employed to demonstrate that BAGp50 could also inhibit vitamin D-mediated gene expression. This was further illustrated by the fact that the presence of BAGp50 in U87 cells blocked the induction of VDR protein levels in response to vitamin D3.

Because BAGp50 could block vitamin D3-mediated transcription of the osteocalcin VDRE it was decided to investigate whether BAGp50 could block vitamin D3-mediated upregulation of $p21^{waf1}$ transcription. Using $p21^{waf1}$ luciferase constructs, it was found that BAGp50 could inhibit vitamin D3 activation of $p21^{waf1}$ transcription. Interestingly, the p46 isoform of BAG-1 decreased the level of $p21^{waf1}$ transcription through an unknown mechanism.

These results demonstrate for the first time that BAGp50 can bind and regulate the function of the VDR. It was also found that BAG-1 may act as a regulator of proliferation. BAGp50 and p46 could also regulate the transcription of $p21^{waf1}$ through two separate mechanisms, suggesting that different isoforms of BAG-1 may work together to achieve the common goal of promoting cell proliferation.

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CHAPTER 1

INTRODUCTION

1.1 Structure of BAG-1

BAG-1 (BCL-2 associated anti-death gene 1) is a multi-isoform protein having antiapoptotic and pro-proliferative activities. In total, there are four known isoforms of BAG-1, having masses of 50, 46, 33 and 29 kilodaltons (kDa), forthwith to be described as BAGp50, BAGp46, BAGp33 and BAGp29 respectively. The full length BAGp50 protein contains a total of 345 amino acids. Each isoform arises from alternate translational start sites (Fig.1.1a), with the p50 isoform of BAG-1 being the product of a rare CTG start site (Yang et al., 1998b). Other genes containing alternate sites of translation include the oncogenes c-*myc* (Spotts et al., 1997) and *cot* (Aoki *et al.*, 1993).

BAG-1 has several structural domains of interest (Fig. 1.1b). The carboxyl (C) terminal of the protein, encompassing approximately the last 112 amino acids (Fig. 1.1b region E), is predicted to assume an α-helical conformation that is highly amphipathic in nature (Takayama *et al.*, 1995). This region has been shown to be important for interactions with other proteins (Takayama *et al.*, 1995 and 1997). Amino acids 169- 200 in the central region of the protein (Fig. 1.1b region C) have 50% identity (66% similarity) with several ubiquitin proteins (Takayama *et al.*, 1995). Although the importance of this region remains unknown, it is possible that this region may confer upon BAG-1 some of the properties of ubiquitin proteins, such as targeting proteins for degradation.

BAG-1 also contains two nuclear localization signals. The first is a simian virus 40-like nuclear localization signal (SV40NLS) (Fig. 1.1b region A) that is only found in the p50

isoform (Packham *et al.*, 1997) and the second is a bipartite signal (Fig. 1.1b region D) located in the central portion of the protein from amino acids 220 to 235 (Zeiner and Gehring 1995). The SV40NLS seems to act as a more dominant signal than its bipartite counterpart, since BAGp50 is found predominately in the cell nucleus while p46 and p33 are found in both the nucleus and cytoplasm and p29 in the cytoplasm alone. What other factors contribute to the compartmentalization of BAG-1 remains to be determined, but may include interaction with other proteins and phosphorylation.

Finally, BAG-1 contains ten repeats of the following amino acid sequence; **TRSEE**. Each repeat being separated by four to five highly variable amino acids. Variation in the consensus occurs primarily within the serine (**S**) residue. These repeats are found within amino acids 79-134 (Fig. 1b, region B). This domain is believed to have an α-helical secondary structure (Packham *et al.*, 1997). Significantly, this region is likely an amphipathic helix with the negatively charged glutamic acid (**E**) residues localizing on one side of the helix's spiral and hydrophobic residues on the opposite side. Proteins with this motif are commonly potent activators of transcription (Latchman 1990) and include the retinoic acid receptor (Durand *et al.*, 1994). The role of the acidic α-helix in activating transcription is to bind and modulate the activity of the TATA box binding proteins TFIID or TFIIB (Transcription Factor II B or D) which are a part of the basal transcriptional machinery. Some proteins having this amphipathic domain do not actually bind DNA. They bind another protein having DNA binding abilities and form a link from this DNA bound transcription factor to TFIID (Latchman 1990). An example of this is the herpes simplex virus VP16 (Stern *et al.*, 1989) which binds Oct-1 to enable itself to consequently bind TFIID.

Fig. 1.1a Positioning of alternate translational start sites and size of each isoform of BAG-1.

Amino acid position of each alternate start site is indicated as is the length of each isoform. Full length BAGp50 is 345 amino acids and the p46, p33 and p29 isoforms are 274, 230 and 207 amino acids in length respectively.

Fig. 1.1b Structural domains of BAG-1

A SV40-like nuclear localization signal
B N-terminal α-helical domain
C Ubiquitin-like domain
D Bipartite nuclear localization signal
E C-terminal α-helical domain

Fig.1 Structure of the four BAG-1 protein isoforms

Fig.1.1a Positioning of alternative translational start start sites

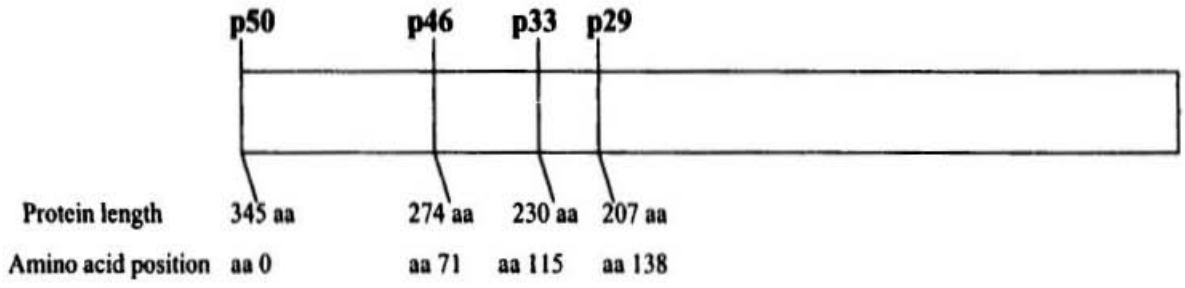
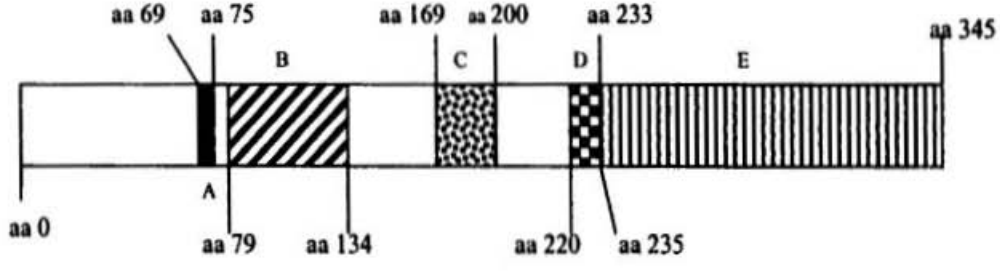


Fig. 1.1b Structural domains of BAG-1



Although it is not known whether or not BAG-1 can bind DNA or TFIID, it has been reported to have transcription regulating properties as will be discussed below.

1.2 Function of BAG-1

As is clearly seen from the numerous structural domains and multiple isoforms of BAG-1, this protein has the potential to be involved in a number of cellular functions. In the three years since BAG-1 has been discovered, this potential has come to fruition with BAG-1 being reportedly involved in functions as diverse as inhibiting apoptosis to regulating protein folding. In this section, all these functions will be discussed along with the implications of each function in regard to cancer.

1.2.1 BAG-1 inhibits apoptosis

Apoptosis is defined as a programmed cell death. This process is regulated through a number of mechanisms and occurs in response to a number of intracellular and extracellular stimuli. During embryo development, cells die to shape and sculpt new tissues and dispose of excess neuronal tissue. In adult animals cells die by apoptosis during tissue turnover, in response to cellular damage and white blood cells die at the end of an immune response (Ashkenazi and Dixit 1998). In order for a cell to survive, it must continually receive extracellular survival signals, thus apoptosis is considered to be a cell's default system and a cell must overcome this default mechanism in order to survive. BAG-1 has been found to inhibit apoptosis induced by a number of different stimuli (Takayama *et al.*, 1995; Clevenger *et al.*, 1997). BAG-1 has been found ubiquitously in all tissue types studied

thus far. This indicates that a physiological function of BAG-1 may be to block a cell's default apoptotic program.

Further evidence for this lies in the fact that BAG-1 can inhibit apoptosis induced by the retinoic acid and glucocorticoid steroid hormones. As will be discussed latter, steroid hormone induced apoptosis is important for a number of physiological functions (Kiess and Gallaher 1998). BAG-1 has been found to bind the receptors for these hormones and prevent their normal function (Kullman *et al.*, 1998; Liu *et al.*, 1998). Therefore, one mechanism through which BAG-1 could inhibit apoptosis is through regulation of hormone pathways.

Apoptosis is known to play an important role in tumorigenesis and metastasis. Normally, a cell would die in the event of DNA damage or withdrawal of growth factors. However, if a cell is unable to die, DNA damage can accumulate leading to uncontrolled cell growth. Or, in the event of a cell being able to survive under non-physiological conditions, a cancer cell can migrate to a new body tissue and proliferate under conditions where it normally couldn't survive. Therefore, overexpression of an antiapoptotic protein such as BAG-1 can lead to neoplastic growth and possibly metastasis (Jaattela 1999).

1.2.2 Interaction of BAG-1 with other proteins

Most of the discoveries of potential BAG-1 functions have involved its interaction with other proteins. This section of the thesis will describe in turn the functional significance of each of the BAG-1 binding proteins.

1.2.2.1 BCL-2

BAG-1 was originally cloned due to its ability to bind the BCL-2 protein. Their ability to bind was discovered by screening a mouse embryo library with the BCL-2 protein. BCL-2 is a potent inhibitor of apoptosis and has been directly implicated in the development of malignant lymphoma (Tsujimoto *et al.*, 1985) and indirectly in other cancers such as those of the head and neck (Gallo *et al.*, 1995). The overexpression of BCL-2 in cancer cell lines has been found to make them resistant to many apoptosis-inducing stimuli, including cisplatin (Miyake *et al.*, 1998), oxidative stress (Fabisiak *et al.*, 1997), glucocorticoids and vincristine (Miyashita and Reed 1993). By co-transfecting BAG-1 and BCL-2 into a number of cell lines, researchers have found that BAG-1 can enhance the ability of BCL-2 to block the action of a number of apoptosis-inducing agents such as staurosporine, anti-FAS antibody, (Takayama *et al.*, 1995) and withdrawal of growth factors (Clevenger *et al.*, 1997) in various types of cells. As well, BAG-1 was found to independently confer resistance to staurosporine in NIH3T3 mouse fibroblasts (Takayama *et al.*, 1995).

It is well known that the activation of proteases is an integral part of the apoptotic process. A large family of these proteases are referred to as caspases. The action of caspases is known to be blocked by BCL-2. It has been found in neuronal cells that overexpression of BAG-1 can inhibit caspase activity and can collaborate with BCL-2 when coexpressed to inhibit these proteases to an even greater extent than either protein on its own (Schultz *et al.*, 1997).

BCL-2 is primarily localized in the membranes of the mitochondria and nucleus (Kroemer 1997), while BAG-1 is dispersed throughout the entire cell (Yang *et al.*, 1998b).

Transfection experiments of BAG-1 and BCL-2 have shown that BCL-2 can target BAG-1 to the mitochondrial and nuclear membranes (Takayama *et al.*, 1998), indicating that the interaction of the two proteins has *in vivo* significance.

BCL-2 is thought to prevent cell death through several processes. These include preventing the release of the caspase activator cytochrome c and calcium from the mitochondria (Green and Reed 1998), as well as decreasing the generation of reactive oxygen species (Kane *et al.*, 1993), which are known to be involved in the apoptotic mechanism. BCL-2 seems to be involved in the formation of membrane ion channels, which may be involved in regulating the movement of proteins across these membranes (Schendel *et al.*, 1997). The pore forming ability BCL-2 may allow it to accomplish such diverse antiapoptotic actions. The mechanism by which BAG-1 enhances the anti-apoptotic effect of BCL-2 remains unclear. It can be speculated however, based on the fact that BAG-1 is involved in protein folding (to be discussed latter), that BAG-1 may be involved in regulating the conformation of BCL-2, which could affect ion channel formation. It is also possible that the some isoforms of BAG-1 may induce different conformations of BCL-2, thereby altering its function.

A protein such as BAG-1 that is involved in cell survival on its own and in conjunction with the BCL-2 protein, certainly has potential to be involved in cancer formation. If a suppressor of apoptosis should become overexpressed as the result of some mutation or chromosomal translocation, the cell may be unable to die in the event of DNA damage, thus leading to DNA mutations and ultimately in uncontrolled cell growth and tumor formation. Therefore, the overexpression of BAG-1 may be oncogenic. It has yet to

be shown that BAG-1 is directly involved in tumor formation, but it has recently been demonstrated that BAG-1 can increase pulmonary metastasis in mice (Takaoka *et al.*, 1997) through a yet undiscovered mechanism. Because BCL-2 has been shown to be involved in tumorigenesis (Bonnotte *et al.*, 1998 and Tsujimoto *et al.*, 1985), it is reasonable that the overexpression of BAG-1 in cells could enhance the tumorigenic potential of BCL-2.

1.2.2.2 RAF-1

RAF-1 is a serine/threonine protein kinase involved in the RAS signal transduction pathway (Propst *et al.*, 1993). This pathway is important in cellular growth and proliferation. Constitutive activation or overexpression of RAF-1 can result in uncontrolled cell growth and this situation has been found to occur in lung and renal cancers (Propst *et al.*, 1993). BAG-1 was found to immunoprecipitate with the catalytic domain of RAF-1, similar to the binding of RAF-1 with BCL-2 which results in RAF-1 being targeted to the mitochondria (Wang *et al.*, 1996). Unlike BCL-2, BAG-1 has the ability to activate the kinase activity of RAF-1 *in vitro* and *in vivo*. The exact mechanism by which BAG-1 activates RAF-1 is unknown. It may dislodge negative regulatory proteins from their interaction with RAF-1, alter its conformation, or target it to other proteins. BAG-1 does not have the ability to activate other proteins via phosphorylation (Wang *et al.*, 1996), so this method of activation can probably be ruled out.

The interaction of BAG-1 and RAF-1 would again lead us to believe that BAG-1 has oncogenic potential. If cellular BAG-1 protein levels were to become overexpressed, this might result in a RAF-1 activity much higher than the cellular norm which could lead to

uncontrolled cell growth.

1.2.2.3 Hepatocyte Growth Factor and Platelet Derived Growth Factor receptors

Hepatocyte growth factor receptor (HGFR) is a transmembrane tyrosine kinase receptor. When stimulated by its ligand (hepatocyte growth factor), it initiates a signal transduction pathway that results in the proliferation of endothelial and epithelial cells (Nakamura *et al.*, 1986) as well as promoting the growth of new blood vessels (Camussi *et al.*, 1997). Bardelli *et al.*, (1996) isolated BAG-1 by screening a mouse embryo cDNA library with the cytoplasmic domain of the HGFR (Bardelli *et al.*, 1996). Using Glutathione-S-Transferase (GST) fusion proteins, the BAG-1 C-terminus was identified as the HGFR binding domain. When the last 83 amino acids of BAG-1 were deleted, binding between the two proteins did not occur. BAG-1 was also found to increase the level of HGF-mediated protection from apoptosis induced by staurosporine and etoposide in mouse liver cells. BAG-1 by itself could not confer resistance to these drugs however. To assess whether BAG-1 could influence the antiapoptotic properties of related receptors, platelet derived growth factor (PDGF) and epidermal growth factor were added to cells in the presence and absence BAG-1. BAG-1 increased the protection by PDGF to staurosporin-induced apoptosis and it was subsequently found that BAG-1 could bind to the PDGFR as well. Interestingly, the whole length of their mouse BAG-1 clone (corresponding approximately to the 33 kDa form of human BAG-1) was needed for cooperative prevention of apoptosis with the HGFR and PDGFR, not just the BAG-1 C-terminus, which as mentioned earlier, is necessary for BAG-1 to bind to BCL-2 or RAF-1. This may point towards BAG-1 acting as a bridge linking the HGFR and PDGFR to other proteins which then transmit the antiapoptotic signal from the

cell membrane to the inner cell, or BAG-1 itself may move from the receptors to transmit the cell survival signal. The fact that BAG-1 is not phosphorylated by these receptors (Bardelli *et al.*, 1996) suggests that it may act as some type of linker molecule, since these survival signals are often conveyed by signalling cascades (Roussel 1998). It is possible that BAG-1 transmits a survival signal from these receptors through some as yet undiscovered mechanism to BCL-2, which then elicits a survival response.

It is reasonable to expect that aberrant expression of a receptor, such as the HGFR, that is tightly linked with proliferation, may result in tumorigenesis. This hypothesis has been proven correct and high levels of HGFR have been linked to several types of cancer including thyroid carcinoma (Di Renzo *et al.*, 1992), several types of sarcoma (Rong *et al.*, 1993), and liver cancer (Boix *et al.*, 1994). These studies have been supported by *in vitro* studies showing that overexpression of HGFR in mesenchymal cells results in increased tumorigenic and metastatic potential of the cells (Taylor et, al 1998). If BAG-1 is an integral member of the HGFR pathway, its overexpression could result in the constitutive activation of the pathway, leading to increased proliferation and potential tumorigenesis.

1.2.2.4 Steroid hormone receptors

Steroid hormone receptors are key elements in the endocrine signal transduction pathway. Members of this superfamily include the glucocorticoid, thyroid, estrogen, androgen, progesterone, retinoic acid, mineralocorticoid and vitamin D receptors. This family of receptors are intracellular transcription factors having similar structures, but eliciting a broad spectrum of cellular responses. They are all modular in structure, having

domains involved in dimerization, protein-protein interactions and a zinc finger DNA binding domain (Lundeen *et al.*, 1996). The receptors elicit a cellular response when activated by hormones by binding to a specific sequence of DNA called a hormone response element in promoter region of a specified gene and thereby regulate the rate of transcription of the gene (Beato 1989). Regulation of transcription by hormone receptors involves chromatin remodeling (Fryer and Archer 1998), cross talk with other transcription factors (Heck *et al.*, 1994) and interaction with the basal transcription machinery through binding to TFIIB or TFIID. Binding of the hormone receptors to these transcription factors can be either direct (Baniahmad *et al.*, 1993, McDonald *et al.*, 1995) or indirect through coactivator/corepressor proteins (Goodrich *et al.*, 1996; Wong and Privalsky 1998).

It was first reported by Zeiner and Gehring (1995) that human BAG-1 could interact with members of the steroid receptor family, including the glucocorticoid, estrogen, androgen and progesterone receptors *in vitro*, but no functional analysis was performed. Since then, a number of studies have investigated the interaction between BAG-1 and these receptors and the ability of BAG-1 to modulate the function of the receptors.

1.2.2.4a Glucocorticoid receptor

Glucocorticoid receptors (GR) are found in almost all human cell types. In accordance with this observation, glucocorticoids have a number of varied normal functions in humans, including, among others, gluconeogenesis (thus regulating blood sugar levels) (Scott *et al.*, 1998), protein catabolism (Louard *et al.*, 1994), anti-inflammatory actions including lowering the number of most leukocytes (lymphocytes, eosinophils, etc.) via

apoptosis (Cato and Wade 1996), and protection against mental stress (Munck and Toth 1994). With all their physiological effects, it is not surprising that the glucocorticoid hormones are necessary to sustain life.

Most of the actions of glucocorticoids are mediated by the binding of the hormone to its receptor, which results in the release of inhibitory heat shock proteins from the receptor, and allows it to bind to a specific glucocorticoid response element (GRE) in a target gene. The GR may up or down regulate gene expression, depending on its interaction with other transcription factors, such as the Jun/Fos AP-1 complex (Mittal *et al.*, 1994; Jonat *et al.*, 1990) and NF-κB (McKay and Cidlowski 1999).

Because of the previously reported ability of BAGp46 to interact with the GR Kullman *et al.*, (1998) investigated whether BAGp46 had any affect on glucocorticoid mediated apoptosis or transcriptional transactivation. The researchers found that BAG-1 could repress 80% of the transactivation by the GR in human JEG-3 and mouse S49.1 cells of CAT reporter constructs containing GREs. Interestingly, it was noted that BAGp46 had no effect on the ability of the androgen receptor (AR) to activate transcription through its response element. Furthermore, it was found that overexpression of BAG-1 could block glucocorticoid-induced apoptosis in S49.1 cells. Finally, the researchers demonstrated that BAG-1 could inhibit the DNA binding activity of the GR. It was found, using electrophoretic mobility shift assays (EMSAs), that the GR DNA binding was absent in cell extracts from simian COS-7 cells transfected with BAGp46. These results show that BAG-1p46 is a direct inhibitor of GR action. This is interesting because even though glucocorticoids can induce apoptosis in almost all lymphocyte types, they inhibit apoptosis in neutrophils (Kato *et al.*,

1995) and in mouse mammary glands (Feng *et al.*, 1995). Therefore, it would stand to reason that BAG-1, by interfering with GR action, may have opposing functions in different cell types. It was not demonstrated that BAG-1 could control GR-mediated transactivation of any of its effector genes.

Glucocorticoids are currently used to treat several types of cancer, including multiple myeloma (Gieseler and Nussler 1998) and leukemia (Kaspers *et al.*, 1994). By inhibiting the binding of the GR to DNA, it is possible that overexpression of BAG-1 may be contributing to inhibition of apoptosis in leukocytes and drug resistance in some cancers. This lends more evidence to BAG-1 being a potential oncogene.

1.2.2.4b Androgen receptor

Androgens are a family of hormones (including testosterone) produced primarily in the testis. As with most hormones, they have a broad range of functions such as aiding in sperm maturation, normal growth of the prostate gland, maintaining secondary male characteristics (deep tone of voice, for example) and increasing muscle mass, etc. Similar to their glucocorticoid cousins, androgens exert their effect by activating the AR, allowing it to bind to a specific androgen response element and thus regulate gene expression. The AR is found in most tissue types and has been found to both inhibit (Zatelli *et al.*, 1998) and promote (Lin *et al.*, 1998) cell proliferation, depending on the cell type. The exact genes involved in androgen-mediated cell proliferation or arrest are still largely unknown. Only gene products seemingly unrelated to cell growth, such as ornithine decarboxylase (Pajunen *et al.*, 1982) have been discovered at present.

Because of BAG-1's interaction with the AR (Zeiner and Gehring 1995), Froesch *et al.*, (1998) decided to further examine the relationship between the two proteins. These researchers showed that the AR can co-immunoprecipitate with BAGp50, but not the shorter isoforms. It was also shown that BAGp50 can significantly heighten the activation of CAT reporter constructs containing AR response elements when cotransfected with the receptor in several cell types, while the p46 isoform had little or no effect. This is consistent with the observation of Kullman *et al.*, (1998), who demonstrated that BAGp46 had no affect on androgen-mediated transcription. In addition, BAGp50 could markedly decrease the ability of antiandrogens to inhibit transactivation of CAT activity by the AR.

Cancer of the prostate is intricately linked to androgens (Culig *et al.*, 1998 and Visakorpi *et al.*, 1995) and both the overexpression of the AR and mutations of the AR have been linked to male breast cancer (Lobaccaro *et al.*, 1993, Hiort *et al.*, 1996). In both types of malignancy, anti-androgens are commonly used in therapy (Newling 1998, Doberauer *et al.*, 1988). However, it is common for hormone-dependent tumors to progress into a largely untreatable hormone-independent state (Crawford *et al.*, 1989). It is possible that, overexpression of BAGp50 in the course of tumor progression could enhance AR-mediated proliferation and impede anti-hormone therapies. It would be interesting to study the expression of BAG-1 in hormone-dependent and independent prostate tumors.

1.2.2.4c Retinoic acid receptor

Retinoic acid is a naturally occurring, active metabolite of vitamin A. It is involved in a plethora of essential life processes (Mangelsdorf *et al.*, 1994) including bone resorption,

cell differentiation and normal embryogenesis. It also affects reproduction, in that it increases the production of testosterone . Retinoic acid is responsible for regulating such genes as p68 kinase (Pelicano *et al.*, 1997), Egr-1, which is involved in cell growth and differentiation (Larsen *et al.*, 1994) and the cell cycle regulator $p21^{waf1}$ (Liu *et al.*, 1996). The retinoic acid receptor (RAR) is functionally the most complicated of the steroid receptors. It has three subtypes (α,β,γ) and its gene activating specificity is partially determined by the heterodimers it forms with the retinoid X, vitamin D and thyroid receptors (Garcia-Villalba *et al.*, 1996). As well, its function is modulated, like other hormone receptors, by a large number of cofactors.

Because of its interaction with other steroid receptors, Liu *et al.*, (1998) thought that RAR-α would be a promising candidate as another BAG-1 binding protein. The researchers found, using GST-fusion proteins, that BAGp33 could interact with the retinoic acid receptor but not with the retinoid X receptor (RXR). This interaction was not negated by deleting the last 47 amino acids from BAG-1's C-terminal, indicating that this region of the carboxyl terminal α-helical domain is not required for their binding. The proteins were also shown to interact using the yeast two-hybrid system. To test the functional significance of the BAG-1-RAR interaction, BAG-1 and RAR were cotransfected into monkey CV-1 cells with CAT constructs containing RAR response elements. It was found that BAG-1 could inhibit RAR-mediated transactivation. In addition, the researchers also noted that BAG-1 could inhibit thyroid hormone receptor transactivation through its response element. Similar to the work performed by Kullman *et al.*, (1998) with the GR, these investigators found that BAG-1 could inhibit the binding of the RAR, RAR/RXR heterodimer and thyroid receptor to their

respective response elements. It could not, however, inhibit the DNA binding ability of the RXR monomer.

RA is known to inhibit the proliferation of breast cancer cells (Ueda *et al.*, 1980). The researchers next explored whether or not BAG-1 could interfere with RA-mediated growth inhibition in MCF-7 and ZR-75 breast cancer cells. They found that BAG-1 could reduce RA-induced growth inhibition in MCF-7 cells by 35% and ZR-75 cells by 25%. Because RA is also known to induce apoptosis in both of these cell lines (Seewaldt *et al.*, 1995) they next investigated whether BAG-1 could inhibit RA-induced apoptosis in these cells. BAG-1 could inhibit RA induced apoptosis by 26% in ZR-75 cells but not at all in MCF-7 cells. In addition, it was found that overexpression of BAG-1 could prevent inhibition of BCL-2 expression by RA in MCF-7 cells. These results indicate that BAG-1 may be an important and novel regulator of the retinoic acid hormone pathway.

RA is currently being used to treat epithelial tumors and promyelocytic leukemia (Alberts and Garcia 1995). Again, if BAG-1 expression were to become upregulated in an uncontrolled fashion, these cancer cells may also become resistant to chemotherapy. There have been no studies at the present which examined the expression of BAG-1 in drug resistant cancers.

1.2.2.5 Heat shock proteins

How can BAG-1 interact with so many proteins? One possible answer is through its interaction with heat shock protein 70 (hsp70) (Hohfeld and Jentsch 1997, Zeiner *et al.*, 1997, Takayama *et al.*, 1997). Heat shock proteins comprise a large family of ubiquitous

proteins that are necessary for the folding of many proteins into their native conformation (referred to as chaperone activity) as well as translocating proteins across intracellular membranes, including nuclear translocation (Bukau and Horwich 1998). Hsp70 is known to bind the GR (Hutchison *et al.*, 1994) and RAF-1 (Hutchison *et al.*, 1993). Therefore, BAG-1 may interact with these proteins indirectly through hsp70. Zeiner *et al.*, (1997) demonstrated that BAGp46 could indeed bind to a number of unidentified proteins indirectly through hsp70. The chaperone activity of hsps involves a heterocomplex of different hsps, including the hsp90 and 70, among others (Czar *et al.*, 1994). This may mean that, even though hsp70 isn't directly involved in binding to theAR or RAR, BAG-1 may bind indirectly to these receptors through hsp70's interaction with the hsp90 which does bind directly to them.

Three very similar papers were published in 1997 describing the interaction between BAG-1 and hsp70 (Takayama *et al.*, 1997, Zeiner *et al.*, 1997, Hohfeld and Jentsch 1997). Hohfeld and Jentsch (1997) documented the binding of mouse BAG-1 to the ATPase domain of hsp70. This binding stimulated the release of ADP from hsp70 and subsequently promoted the formation of the ATP-bound form of hsp70. Zeiner *et al.*, (1997) demonstrated that BAGp46 could bind *in vivo* to the ATPase domain of hsp70 and could inhibit hsp70-mediated protein folding *in vitro.* Similarily, Takayama *et al.*, (1997) showed that BAG-1 could bind to hsp70 *in vivo*, could inhibit hsp70-mediated protein folding *in vitro* and (in at least one human cell line, if overexpressed) could protect cells from heat shock induced apoptosis.

The hydrolysis of ATP is known to be important for the binding of proteins by hsp70 (Szabo *et al.*, 1994). The ADP bound form of hsp70 can form a heterocomplex with binding

Table 1.1 BAG-1 interacting proteins

BAG-1 interacting protein	Effect of interaction	Significance
BCL-2	Enhances ability of BCL-2 to inhibit apoptosis	Overexpression of BAG-1 or BCL-2 may enhance the tumorigenic potential of the other
RAF-1	Enhances kinase potential of Raf-1	Overexpression of BAG-1 could lead to constitutive activation of the RAF-1,pathway resulting in uncontrolled cell growth
HGF, PDGF	BAG-1 enhances anti-apoptotic ability of both proteins	High levels of BAG-1 could increase proliferative or anti-apoptotic potential of pathway leading to cell tumorigenicity
Glucocorticoid receptor	BAG-1 inhibits GR DNA binding and glucocorticoid induced apoptosis	May be involved in resistance to glucocorticoid hormone therapy
Androgen receptor	BAG-1 enhances AR-mediated transcription	May enhance ability of AR to transform prostate cells
Retinoic acid receptor	BAG-1 inhibits RAR DNA binding and retinoic acid-mediated proliferation and apoptosis, as well as blocking the ability of RA to decrease BCL-2 expression	May interfere with ability of RA to treat leukemia
Heat shock protein 70	Involved in hsp70 ATP hydrolysis. Induces hsp70 to release bound proteins. Prevents hsp70 mediated protein folding.	May be involved in hsp70-mediated inhibition of apoptosis. May act on a variety of proteins through interaction with Hsp70

partners, while the ATP-bound form cannot. Taken together, these studies indicate that BAG-1 promotes the release of hsp70-bound proteins by stimulating the ATP bound state of hsp70. This cripples the protein folding ability of hsp70, because it cannot remain bound to target proteins. It is also possible that the function of BAG-1 *in vivo* is to stimulate the release of hsp70-bound proteins when proper folding has been achieved.

The two reports showing that BAG-1 can inhibit hsp70-mediated protein folding are somewhat confusing because hsp70 is known to have anti-apoptotic properties (Gabai *et al.*, 1995) and may play a role in tumorigenesis (Kaur and Ralhan 1995; Ralhan *et al.*, 1995 and Jaattela 1995). Therefore, it seems contradictory that BAG-1 should inhibit hsp function, since this would implicate BAG-1 in functions opposite to those previously mentioned (that is, being pro-apoptotic instead of anti-apoptotic). However, it is certainly possible that the interaction between the two proteins is necessary for purposes other than those involved in apoptosis. This hypothesis is supported by the wide range of cellular roles in which the hsps are involved. However, it is still unknown which particular functions are necessary for its ability to inhibit apoptosis. Furthermore, it has recently been shown that hsp70 exerts its antiapoptotic effects downstream of caspase activity (Jaattela *et al.*, 1998), whereas BAG-1 (as mentioned above) acts upstream of caspases (Schulz *et al.*, 1997). Therefore, BAG-1 and hsp70 may act at different steps in the same pathway and their binding may function to connect different steps in the signal transduction pathway. In addition, it may be possible that different isoforms of BAG-1 have different effects on hsp70-mediated folding and may work with hsp70 to release proteins having achieved proper folding or, conversely, inhibit the folding of pro-apoptotic proteins. At this time, the effect of BAGp50 on hsp70-mediated

folded is unknown, as is the precise physiological relevance of BAG-1's interaction with hsp70.

1.3 Further evidence implicating BAG-1 as a tumorigenic protein

Cells that have achieved a growth factor-independent state consistently have enhanced cell survival capabilities. Growth factor independence has also been shown to result in increased tumorigenic cell potential (Piao *et al.*, 1996 and Howard *et al.*, 1996).

Clevenger *et al.*, (1997) demonstrated that overexpression of murine BAG-1 in the IL-3-dependent cell line Ba/F3 could confer IL-3-independence on the cells. It was also found that the addition of IL-3 to Ba/F3 cells increased BAG-1 protein expression, suggesting that BAG-1 is involved in the survival and growth of these cells. It was not shown however, whether BAG-1-induced growth factor-independence could enhance cell tumorigenicity.

There have been several recent studies investigating the status of BAG-1 protein in various tumor types and tumor cell lines. BAG-1 has been shown to be expressed at higher levels in lung, breast and cervical tumor cell lines (Takayama *et al.*, 1998 Yang *et al.*, 1998 b,c Zapata *et al.*, 1998) than their non-tumor counterparts, as well as in 40% of breast carcinomas *in situ* and invasive breast cancers in one study (Zapata *et al.*, 1998). In addition, studies have shown that BAG-1 is expressed at higher levels in human papilloma virus-transformed endocervical cell lines than non-transformed cells (Yang *et al.*, 1998 a,b). One study (Yang *et al.*, 1999a) also demonstrated that BAG-1 is present at much higher levels in cervical tumors than normal cervical tissue. In addition, it was found that overexpression of

BAG-1 correlated with resistance to staurosporine-induced apoptosis in cervical cells.

Zapata *et al.*, (1998) have shown that BAGp33 is the most prevalent isoform in the breast tumors they've studied, while Yang *et al.*, (1998b) have found all isoforms overexpressed in many different types of cancer cell lines. All the data collected thus far supports the hypothesis that BAG-1 is involved in tumorigenesis.

1.4 1,25-dihydroxyvitamin D_3 (1,25-vitamin D3) and the vitamin D3 receptor

As previously mentioned, BAG-1 can interact with a variety of steroid hormone receptors. Another member of the steroid hormone superfamily, with links to cancer, is the 1,25-vitamin D3 receptor (VDR). The primary physiological role of 1,25-vitamin D3 (the active metabolite of vitamin D) is maintenance of serum calcium levels. It regulates the level of calcium in the blood by increasing intestinal absorption of calcium and resorption of calcium from bone. 1,25-vitamin D3 is also involved in bone remodeling (Haussler *et al.*, 1997). 1,25-vitamin D3 maintains calcium homeostasis through the regulation of genes such as osteocalcin, osteopontin and calbindin (Lian and Stein 1992). Like other steroid hormones, 1,25-vitamin D3 elicits a cellular response through activation of its receptor which then regulates gene transcription through a specific vitamin D response element (VDRE). The VDRE consists of a direct hexanucleotide repeat separated by three random nucleotides (Lian and Stein 1992).The consensus VDRE sequence is as follows: AGGTCANNNAGGTCA . Once the VDR is activated by its ligand, it undergoes a conformational change, binds the retinoid X receptor (RXR) and binds VDREs as a heterodimerdimer (Strugnell and Deluca 1997). A number of accessory factors are also

involved in VDR-mediated transcriptional control, such as SRC-1, RIP140 (Masuyama *et al.*, 1997) and GRIP1 (Hong *et al.*, 1997). The VDR is believed to mediate transcription through its direct interaction with TFIIB (MacDonald *et al.*, 1995).

The VDR has also been shown to bind several VDREs as a homodimer (Nishikawa *et al.*, 1994 and Polly *et al.*, 1996). In addition, it has been reported that the VDR can bind DNA as a homodimer independently of ligand activation (Matkovits and Christakos 1995). There has been much discussion on the significance of the VDR homodimer *in vivo*. Some researchers believe that the homodimer has *in vivo* significance based on the fact that CAT constructs containing VDREs that are bound specifically by VDR homodimers can be activated by 1,25-vitamin D3 (Carlberg *et al.*, 1994), while others suggest that the VDR homodimer serves as a negative regulator of transcription (Mackey *et al.*, 1996). Other researchers claim that it is only found in *in vitro* experiments (Haussler *et al.*, 1997). However, the matter remains inconclusive. One theory hypothesizes that the VDR exists as homodimer when not stimulated by 1,25-vitamin D3. This homodimer can bind to a VDRE in the absence of ligand and may actually lower the rate of transcription. But once stimulated with ligand, heterodimerization with the RXR occurs and it subsequently upregulates transcription of the same promoter (Cheskis and Freedman 1994). However, other researchers have found that the sequence of the VDRE is essential in determining which dimerized form of the VDR will bind (Nishikawa *et al.*, 1994; Mackey *et al.*, 1996). In addition it as been found that the binding of VDR as a homodimer may result in an increase in transcription of selected genes (Polly *et al.*, 1996; Kahlen and Carlberg 1994). VDR-mediated transcription is obviously a very complicated process and much more work is

needed before it is completely understood.

The VDR is localized in the cell nucleus. Related to this, is the fact that the VDR is the only steroid hormone receptor not known to bind hsps (Whitfield *et al.*, 1995). Hsps, in addition to being responsible for proper folding of steroid receptors, are known to bind and sequester hormone receptors, such as the GR, in the cytoplasm until they become hormone bound, at which time they are released from hsps and move into the nucleus.

Even though the primary function of 1,25-vitamin D3 appears to be maintaining calcium homeostasis, the VDR has been found in a wide range of cells including leukocytes, neurons, and ovarian cells. This indicates that 1,25-vitamin D3 may have diverse functions. One of theses functions may be the induction of differentiation in various cell types (Abe *et al.*, 1981; Bikle *et al.*, 1988). Another may be the regulation of cell proliferation (Lemire *et al.*, 1984; Clohisy *et al.*, 1987; Casado et et 1998).

In addition to regulating the proliferation of normal cell types, researchers have found that 1,25-vitamin D3 can inhibit the growth of cancer cells both *in vivo* and *in vitro* through inhibition of proliferation and induction of apoptosis (Eisman et et 1987; Dokoh et al; 1984, Simboli-Campbell *et al.*, 1996). 1,25-vitamin D3 may accomplish these functions through the regulation of genes, such as $p21^{waf1}$ (Liu *et al.*, 1996), BCL-2 (Xu *et al.*, 1993) and *c-myc* (Reitsma *et al.*, 1983). However, clinical studies have shown that hypercalcemic effects of 1,25-vitamin D3 make this type of hormonal therapy impractical (Blazek *et al.*, 1992). Recently, new vitamin D analogs have been developed that have potential for cancer therapy because of lowered hypercalcemic effects and greater apoptotic potential (Abe *et al.*, 1991; Van Weelden *et al.*, 1998).

1.5 The Cell Cycle inhibitor $p21^{waf1}$

One of the genes containing a VDRE is $p21^{waf1}$. It is believed that one potential mechanism of 1,25-vitamin D3-mediated inhibition of proliferation is through upregulation of $p21^{waf}$ transcription (Liu *et al.*, 1996c). $p21^{waf1}$ is a cell growth-regulating protein that can block cell proliferation by inducing cell cycle arrest at the G1 phase. It accomplishes this through binding and inhibiting cyclin-dependent kinase-2 (CDK2) which is an activator of several cell cycle promoting proteins called cyclins (Harper *et al.*, 1993). $p21^{waf1}$ can also inhibit cell cycle progression through inhibition of proliferating cell nuclear antigen (PCNA), which is involved in DNA replication (Chuang *et al.*, 1997). In turn, several regulators of proliferation regulate the transcription of $p21^{waf1}$, including 1,25-vitamin D3 (Liu *et al.*, 1996c), RA (Liu *et al.*, 1996b), Smad3 (Moustakas and Kardassis 1998) and p53 (el-Diery *et al.*, 1993).

Even though studies show that $p21^{waf1}$, like p53, can suppress tumor growth in nude mice implants (Chen *et al.*, 1995), there have been few studies which point towards deregulation or mutation of $p21^{waf1}$ being a major factor in human tumor formation (Cox 1997; Nakayama and Nakayama 1998). This is most probably due to overlapping pathways. However, some studies have indicated that the tumor suppressing potential of $p21^{waf1}$ may make it a suitable candidate for gene therapy (Chen et al 1996).

1.6 Objective of this study

Based on the previous findings that BAG-1 can bind and regulate the function of the AR, GR, and RAR, it seems that an important function of BAG-1 is to regulate steroid

hormone pathways. The objective of my study was to further characterize the role of BAG-1 in steroid hormone receptor regulation by investigating if BAG-1 could bind and modulate the function of the VDR. We are particularly interested in the VDR because 1,25-vitamin D3 analogs are currently being developed as potential cancer therapies and the levels of VDR coactivators and corepressors may play an important role in determining whether a tumor is receptive or resistant to these new chemotherapies. In addition, if BAG-1 does exert an effect on the VDR, can BAG-1 effect 1,25-vitamin D3-mediated transcriptional transactivation of VDR target genes?

To achieve these objectives, Far Western blotting and GST-fusion protein pull-down analysis will be utilized to determine if BAG-1 binds the VDR. Cells stably transfected with BAG-1 will also be employed to determine if BAG-1 has any effect on the following; 1) the DNA binding ability of the VDR 2) VDR mediated transcriptional transactivation 3) 1,25-vitamin D3-mediated inhibition of proliferation.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell culture

Human U87 MG glioblastoma and C33A cervical carcinoma cells were grown in Dulbecco's modified Eagles medium (DMEM) (Gibco BRL) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Confluent cells were detached from the plate using trypsin-EDTA (Gibco BRL) and passed 1:10 into new plates. Cell counts were performed using a hemocytometer (Fischer).

2.2 Plasmids

Plasmids were prepared by a cesium chloride gradient method, as described by Sambrook *et al.*, (1989). The p50 and p46 isoforms of BAG-1 (Yang *et al.*, 1998b) were contained in the EcoR1 site of the expression vector pcr3.1 (Invitrogen). The VDR cDNA in plasmid pCMX was a generous gift of Dr. Ronald Evans (Umesono *et al.*, 1991). The $p21^{waf1}$ promoter/enhancer region contained in the luciferase reporter plasmid PGL2 was a gift of Dr. Bert Vogelstein (el-Deiry *et al.*, 1993). The p21 promoter/enhancer in the luciferase reporter plasmid WWP (p21-4) was a generous gift of Dr. Wafik El-Deiry (Zeng *et al.*, 1997). Plasmid VDREtkCAT containing multiple copies of the osteocalcin VDRE was a gift of Dr. Sylvia Christakos (Matkovits and Christakos 1995). The β-galactosidase reporter plasmid PCH110 was purchased from Pharmacia Biotech. The luciferase reporter plasmid PGL3 was purchased from Invitrogen.

2.3 Calcium phosphate-mediated transfections

This protocol was performed with little variation from the method described by Chen and Okayama (1987). Cells were grown to 70% confluence and medium was changed 30 minutes prior to transfection. 2-3 μg DNA/ml of DMEM, 10 μl filter sterilized 0.25 M $CaCl_2$/ml DMEM and distilled H_2O were mixed in a final volume of 100 μl /ml DMEM. Then, an equal volume of filter sterilized 2X BBS pH 6.96 (50 mM N,N-bis[2-hydroxyethyl]-2-amino-ethanesulfonic acid, 280 mM sodium chloride, and 1.5 mM sodium phosphate) was added. This mixture was then left at room temperature for 13 minutes after which it was added dropwise to the plates and the plates gently swirled.

Transfected cells were incubated in a 3% CO_2 incubator for 16-24 hours. The cells were washed with PBS, fresh medium added and the cells incubated in 5% CO_2 for a further 48 hours.

2.4 Stable transfections

U87 cells were seeded in 24-well plates at a density of 0.5 X 10^4 cells/well. Cells were then grown in the presence of varying amounts of G418 (Gibco-BRL) to determine the concentration of the drug necessary to kill all of the cells in 4-5 days. For this particular cell line, 750 μg/ml of media was determined to be the lowest concentration of drug necessary to kill all the cells in a 5 day period.

U87 cells were next subcultured 1:12 from a 10 cm plate to a single well of a 6 well plate. The cells were next transfected by calcium phosphate precipitation with 5 μg BAGp50 or its parent vector pcr3.1. At 72 hours post-transfection, the cells were subcultured into three

10 cm plates and grown in the presence of 750 μg/ml G418 for 14 days. On day 14, well separated colonies were transferred to 96-well plates. The cells were continued to be grown in the presence of G418 and transferred to 24-well and then 6-well plates as they became confluent until enough cells were present for DNA or protein preparation.

2.5 RNA extraction

RNA was extracted from cells using a cesium chloride gradient method, as described previously by Sambrook *et al.*, (1989). Generally, RNA was collected from four plates of 80% confluent cells. Cells were washed with phosphate buffered saline (PBS) (154 mM sodium chloride, 1.54 mM potassium dihydrogen orthophosphate and 2.71 mM sodium phosphate) and 1.8 ml of lysis solution (4 M guanidine isothiocyanate, 17 mM sodium N-lauroylsarcosine, 8.5 mM sodium citrate pH 7.0 and 53.5 μl mercaptoethanol/1.8 ml total lysis volume) in diethyl pyrocarbonate (DEPC)-treated water (add 4 ml of DEPC to 4 liters water, cover top with foil, place in 45°C water bath overnight and autoclave) was added to each plate. Plates were scraped with a rubber policeman, the lysate transferred to another plate using a 10 ml syringe with a 20 gauge needle and the process repeated using cells from all other plates. For the final plate, the combined lysates were passed 10 times through the needle and carefully overlain onto 3.5 ml of a cesium chloride solution (5.7 M cesium chloride and 100 mM ethylenediamine-tetraacetic acid (EDTA) made up in DEPC treated water) in a Beckman SW41 tube (also DEPC treated). The tubes were then balanced using lysis buffer and centrifuged at 30,000 rpm for 20 hours at 22°C. After centrifugation, the supernatants were decanted and the pellets were air-dried for 10-30 minutes. The pellets were

subsequently dissolved in 200 µl DEPC treated water, transferred to DEPC treated 1.5 ml Eppendorf tubes, the SW41 tubes were rinsed with an additional 200 µl water which was also added to the Eppendorf tube. Next, 40 µl 3M sodium acetate and 880 µl 100% ethanol were added to the tubes to precipitate the RNA. Tubes were placed in -70°C for at least two hours (can be left overnight). Next, the tubes were spun in a microcentrifuge at 4°C for 15 minutes, the supernatants aspirated after which the pellets were washed in ice cold 70% ethanol and respun. The supernatants were again aspirated and the pellets allowed to air-dry for 5 minutes followed by 5 minutes in a vacuum. The RNA pellets were reconstituted in DEPC-treated water (usually 25-50 µl) and stored at -70°C.

2.6 Northern blots

Northern blots were performed as described by Sambrook *et al.*, (1989), with several modifications. Gel electrophoresis equipment was soaked in 0.1 M sodium hydroxide for 15 minutes to prevent RNase contamination and then washed with DEPC-treated water. RNA was resolved on a 1% agarose/formaldehyde denaturing gel (4 g agarose, 40 ml 10X 3-[N-Morpholino]propanesulfonic acid (MOPS) buffer (0.2M MOPS, 0.05 M sodium acetate pH 7.0, 0.1 M EDTA in DEPC treated water) 21 ml formaldehyde made up to a final volume of 400 ml with DEPC treated water). 20 µg of total RNA was adjusted to a final volume of 11.2 µl with DEPC-treated water. 20µl formamide, 4 µl 10X MOPS and 4.8 µl formaldehyde were added to the RNA, the solution mixed and subsequently denatured at 65°C for 15 minutes and chilled on ice for 5 minutes. 4 µl of gel loading buffer (50% glycerol, 1mM EDTA pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol) was added to each sample.

After loading, the gel was run at 35 volts overnight in 1X MOPS buffer.

The RNA was next transferred from the gel to a nylon membrane by capillary transfer. A glass tray was filled with 20X SSC (3 M NaCl, 0.3 M trisodium citrate). A glass plate was placed across the tray to use as a platform. A wick, composed of two pieces of Whatman 3MM filter paper saturated with 20 X SSC, was placed across the platform and into the 20X SSC. The gel was placed on the wick, being careful to avoid trapping any air bubbles under the gel. A piece of nylon membrane (Hybond-N, Amersham) cut to the size of the gel, was placed on the gel again being careful to avoid trapping any bubbles. If bubbles appeared, they were removed by rolling with a pipette. The edge of the gel was surrounded with Saran wrap to prevent dispersion of the buffer. Three sheets of Whatman 3MM filter paper wetted with 20X SSC were placed on top of the membrane. Finally, a stack of paper towels (approximately 5 cm high) was placed on top of the membrane with a 0.75 - 1 kg weight in turn being positioned on top of the paper towels. Transfer was allowed to proceed overnight.

The RNA was next fixed on the membrane by baking the membrane at 80°C for 1.5 hours followed by exposure to UV light (254 nm) for 3 minutes.

Blots were prehybridized at 65°C in QuickHyb buffer (Amersham) for a minimum of 10 minutes in a sealed plastic bag, at which time a cDNA probe was added. Probes were prepared using the Rediprime labeling kit (Amersham). Briefly, 25-50 μg cDNA in 40 μl water was boiled for 5 minutes after which it was added, along with 5 μl [α-P^{32}]-dCTP to a tube of reaction mix. The reaction is allowed to proceed at 37°C for 20-30 minutes after which the probe was purified using a Nick column (Pharmacia) as recommended by the

manufacturer.

Hybridization buffer (2 X 10^6 cpm probe/ml) was mixed with 1 mg salmon sperm DNA per 10 ml hybridization buffer and boiled for 5 minutes, after which it was immediately chilled on ice for 5 minutes before being added to the membrane. The probe was incubated with rocking at 65°C with the membrane for two hours. Membranes were then washed twice for 15 minutes at room temperature in 2X SSC, 0.1% sodium dodecyl sulfate (SDS) and twice for 15 minutes at 50- 65°C (depending on the specificity of the probe) in 0.1X SSC, 0.1% SDS. After washing, the membrane was wrapped in plastic wrap and exposed to Kodak Biomax film at -70°C.

2.7 Cell extracts for gene expression assays

Cell extracts were prepared according to the method of Gorman *et al.*, (1982). The cells were scraped from the plates with a rubber policeman, spun for 1 minute in a microcentrifuge and resuspended in 0.25 M Tris buffer (tris[hydroxymethyl]-aminomethane) pH 7.8. Suspensions were stored at -70°C or were used immediately to prepare cell extracts. Cell extracts were obtained via three freeze-thaw cycles of 5 minutes each in liquid nitrogen and a 37°C water bath, respectively. The lysed cells were chilled on ice for 10 minutes and spun for 5 minutes at 4°C in a microcentrifuge. Supernatants was collected and stored at -70°C until it was to be used for chloramphenicol acetyl transferase (CAT), β-galactosidase or luciferase assays.

2.8 CAT assays

CAT assays were performed as originally described by Gorman *et al.*, (1982). 10 µl cell extract was mixed with 20 µl CAT reaction buffer (4 µl of 4 mg/ml acetyl coenzyme A, 1 µl [^{14}C]-labeled chloramphenicol (1 µci), 1 µl water and 14 µl of 1 M Tris buffer pH 7.8) and incubated for 1 hour. To stop the reactions and isolate the chloramphenicol reaction products, 500 µl ethyl acetate was added to the reaction, tubes were vortexed and microcentrifuged for 1 minute. The supernatant (organic layer) was collected, put into another eppendorf tube and placed into a speed vacuum (Savant) until the ethyl acetate was completely evaporated. Pellets were dissolved in 15 µl acetyl acetate and spotted on a thin layer chromatography (TLC) sheet (Kodak). The acetylated and non-acetylated chloramphenicol products were separated by ascending chromatography in chloroform:methanol (95:5). The TLC plates were then exposed to Kodak film overnight at room temperature. The percentage of acetylated chloramphenicol was subsequently quantitated by densitometry using the Cyclone phospho-imaging system (Canberra Packard). All CAT and luciferase assays were performed at least three times.

2.9 β-Galactosidase assay

β-galatosidase expression assays were performed as a control for CAT and luciferase assays, as recommended by Pharmacia Biotech. 10 µl cell extract were mixed with 200 µl buffer Z (60 mM sodium dihydrogen orthophosphate dihydrate, 40 mM sodium phosphate, 10 mM potassium chloride, 1 mM magnesium sulfate, 50 mM β-mercaptoethanol) pH 7.0 after which 40µl 4mg/ml ortho-nitrophenyl-β- D-galacto-pyranoside was added and the

solution immediately incubated at 37°C. After a yellow colour developed, the reaction was stopped with 100 µl 1 M sodium carbonate and the absorbence measured at 420 nm.

2.10 Luciferase assays

20 µl cell extract was mixed with 100 µl luciferase reagent (Biorad) and the resulting luminescence was immediately measured for 60 seconds in a luminometer (Monolight 2010, Analytical Luminescence Laboratory).

2.11 Polymerase chain reaction (PCR)

DNA for PCR was extracted from cells using the QiAmp blood kit (Qiagen). PCR was performed as recommended by the Strategene company, with minor modifications. 50 ng DNA (1 µl) and 10 pmol of each primer (1µl each), were mixed with 2 µl 10X PCR buffer (Stratagene), 0.4 µl 10 mM deoxynucleoside triphosphates (Gibco BRL), 0.2µl pfu polymerase (Stratagene) and 14.4 µl water. Reactions were carried out in the Perkin Elmer Gene Amp 2400 PCR thermocycler. Reactions were carried out under the following conditions: 94°C for 3 minutes; 35 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute; followed by 94°C for 1 minute, 55°C for 1 minute and 72°C for 8 minutes.

2.12 Protein extraction

Proteins were extracted for western blotting as described by Yang *et al.*, (1997). Cells were washed twice with ice-cold PBS. 500 µl lysis buffer (50 mM Tris pH8.0, 150 mM sodium chloride, 0.02% sodium azide, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate,

10 µg of 10 mg/ml phenyl methyl sulfanyl fluoride (PMSF) and 5 µl aprotinin (2mg/ml)) was added to each 10 cm plate and the cells were harvested using a rubber policeman. Cells were chilled on ice for 30 minutes to allow for their lysis. Lysates were then microcentrifuged at 4°C for 10 minutes, after which the supernatants were transferred to another tube and stored at -70°C. Protein concentration was determined using the BioRad DC Lowry protein assay kit as instructed by the manufacturers.

2.13.1 Western blots

Western blots were performed according to the method of Yang et al (1997). Proteins were boiled in 2X SDS gel loading buffer (200 mM Tris pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol) for 3 minutes and loaded onto a SDS-polyacrylamide gel. The resolving portion of the gel was prepared with 8-10% acrylamide, 375 mM Tris pH 8.8, 0.1% SDS, 0.1% ammonium persulphate, and 6 µl tetramethylethylenediamine (TEMED). The stacking gel was composed of 5% acrylamide, 125 mM Tris pH 6.8, 0.1% SDS, 0.1% ammonium persulphate and 5 µl TEMED. Gels were electrophoresed at 150 mV in SDS-PAGE running buffer (25 mM Tris, 250 mM glycine) using a Protean II minigel apparatus (BioRad).

Following electrophoresis, gels, Hybond nitrocellulose membranes (Amersham) and extra thick filter paper (BioRad) were equilibrated in several changes of Towbin transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) for 30 minutes.

Proteins were transferred to nitrocellulose membranes using the Trans-Blot SD transfer cell (BioRad). Soaked filter paper was put onto the stainless steel cathode and rolled

with a pipette to remove air bubbles. Next, the membrane was placed on the filter, carefully avoiding trapping any bubbles. The gel was then placed onto the membrane followed by the second piece of filter paper, which is also rolled with a pipette. The platinum anode was put in place on top of the stack and the proteins were allowed to transfer for 45 minutes at 20 volts.

Blots were blocked for 1 hour with gentle shaking in TBS-T (20 mM Tris, 137 mM sodium chloride pH 7.6 with 0.1% Tween-20) and 5% skim milk powder. Primary antibody was diluted with TBS-T containing 5% skim milk powder. Membranes were incubated with the antibody overnight at 4°C. Next, membranes were rinsed briefly in TBS-T and then washed in TBS-T once for 15 minutes and twice for 5 minutes. Membranes were incubated with the secondary antibody, which was also diluted in TBS-T and 5% skim milk powder, for 1 hour at room temperature. Membranes were washed in the same manner as described for the primary antibody. Protein detection was performed using the ECL system (Amersham) and subsequently exposed to ECL film as instructed by the manufacturers. After exposure membranes could be reprobed, if desired.

2.13.2 Reprobing of Western membranes

This protocol is an adaptation of the technique first described by Krajewski *et al.*, (1996). The membrane was incubated in 5 ml TBS containing 50 µl diaminobenzedine (DAB) and 2 µl 30% hydrogen peroxide with gentle agitation for 30 minutes. The reaction of the proteins with the oxidized chromogenic substrate DAB, renders the protein-antibody complexes unreactive during future reprobings. Blots were rinsed with tap water to stop the

reaction and washed for two 15 minute periods with TBS at which time the blot could be blocked and reprobed, as described above.

2.14 Far Western blots

This method of detecting protein-protein interaction was taken from Inostroza *et al.*, (1992), but with many modifications. 15 µg of proteins were resolved on a 10% polyacryamide gel and fixed on a nitrocellulose membrane, as described previously. The membranes were blocked with buffer A containing 20mM N-[2-Hydroxyethyl]piperazine-N-[2ethanesulfonic acid] (HEPES) pH 7.9, 50 mM sodium chloride, 1mM EDTA and 10 mM β-mercaptoethanol and 5% skim milk powder at room temperature for 2 hours with gentle shaking. Membranes were next incubated with 50 µl [^{35}S]-radiolabelled *in vitro* translated VDR in 2 ml buffer B (20 mM Tris pH 7.5, 140 mM sodium chloride, 2 mM EDTA, 0.15% NP-40, 2 mM dithiothreitol, 0.05% bovine serum albumin and 5% glycerol) overnight at 4°C. Next, the membranes were washed 3 times for 15 minutes with TENNS buffer (10 mM Tris pH 7.4, 2.5 mM EDTA, 140 mM sodium chloride, 1% NP-40 and 2.5% sucrose). Membranes were subsequently subjected to autoradiography.

2.15 Preparation of whole cell extracts for Electrophoretic Mobility Shift Assays (EMSAs)

Preparation of whole cell extracts was performed as described by Tasset *et al.*, (1990), with some minor alterations. Cultured cells were grown to 80% confluence in 10 cm plates, washed twice with PBS and harvested with 500 µl high salt extraction buffer (0.4 M

potassium chloride, 20 mM Tris pH 8.0, 2 mM dithiothreitol, 20% glycerol, 50 µg PMSF and 5 µg Aprotinin). The cells were lysed with three freeze-thaw cycles in liquid nitrogen and ice, respectively. The homogenate was microcentrifuged at 4°C for 15 minutes at 12,000 rpm and stored at -70°C.

2.16 Oligonucleotide probes for EMSA

The sequence of the sense strand of the 27-mer double stranded oligonucleotides used for protein-DNA interaction assays is shown below. The VDRE contained in each strand is underlined. The oligonucleotide containing a consensus sequence VDRE (Santa Cruz) is a DR3 type VDRE. It consists of a direct repeat of the sequence AGGTCA separated by three nonspecific bases. This element is known to be bound by a VDR-retinoid X receptor (RXR) heterodimer. A mutated consensus was also used having two of the GT bases changed to AA (shown below in italics). The second VDRE is found in the $p21^{waf1}$ promoter region (Liu *et al.*, 1996). It is a non-consensus VDRE, which has been found to be bound by both a VDR-RXR heterodimer and a VDR homodimer.

		A A *A A*
Consensus VDRE	5'	AGCTTCAGGTCAAGGAGGTCAGAGAGC 3'
$p21^{waf1}$ VDRE	5'	TGTAGGGAGATTGGTTCAATGTCCAAT 3'

2.17 End labeling probes for EMSA

10 picomoles of sense oligonucleotide was incubated in a 500 µl PCR tube with 2 µl of 5X forward reaction buffer (Gibco BRL), 10 units T4 polynucleotide kinase (Gibco

BRL), and 5 µl γ-[^{32}P]-ATP (Amersham) and water to a final volume of 10 µl. The reaction was carried out in a PCR thermocycler at 37°C for 45 minutes and stopped by heating at 70°C for 10 minutes. Then, 10 pmol of antisense oligonucleotide, 7.5 µl water and 1 µl 20x oligonucleotide binding buffer (200 mM Tris pH 7.8, 40 mM magnesium chloride, 1 M sodium chloride and 20 mM EDTA) are added to the same tube and incubated at 65°C for 5 minutes and 25°C for 45 minutes to allow the oligonucleotides to anneal. The double stranded oligonucleotides were purified using the nucleotide removal kit from Qiagen.

2.18 EMSAs

EMSAs were performed according to the instructions set forth by Pharmacia Biotech in their EMSA technical manual. 10 or 15 µg of protein from whole cell extract was incubated with 1 X 10^{-7} M 1,25-vitamin D3 in EMSA binding buffer (10 mM Tris pH 7.5, 10% glycerol, 80 mM sodium chloride, 1 mM EDTA, 5 mM dithiothreitol, 0.05% NP-40 and 1.25 µg of the non-specific competitor poly dI-dC) for 10 minutes to activate the VDR. Next, 50,000 cpm of [^{32}P] end-labeled DNA probe was added for a final volume of 20 µl and the reactions were allowed to proceed for 30 minutes at room temperature. The reaction was terminated with 2 µl of 10X gel loading buffer (250 mM Tris pH 7.5, 0.2% bromophenol blue, 0.2% xylene cyanol and 40% glycerol) and loaded onto a 5% polyacrylamide gel. The gel was made with a final concentration of 0.5x TBE (45 mM Tris pH 8.0, 45 mM boric acid and 1 mM EDTA. The gel was run in the same 0.5 X TBE low ionic strength buffer at 15v/cm. After electrophoresis, the gel was dried and exposed to x-ray film (Kodak) for autoradiography.

2.19 Glutathione S-transferase fusion protein purification

Glutathione S-transferase (GST) fusion proteins were purified according to the manufacturer's (Pharamacia Biotech) instructions. Briefly, the BL21 strain of *E. coli* containing either an empty pGEX-4T-3 plasmid (Pharmacia Biotech) or with an inserted gene were grown at 30°C until the optical density at 600 nm reached 0.7. At this time isopropyl-β-D-thiogalactoside was added to a final concentration of 0.1 mM and the culture allowed to incubate for a further 5 hours. The bacteria were centrifuged, resuspended in PBS and sonicated for eight 15 second bursts on ice. Triton X-100 was added to a final concentration of 1% and the lysates mixed gently for 30 minutes. Lysates were centrifuged at 12,000 X g and the supernatants collected. 400 µl of glutathione Sepharose beads (Pharmacia Biotech) were then added to the supernatant containing the desired proteins and the mixture was incubated at room temperature for 30 minutes with gentle agitation. After this, unbound proteins were removed with three PBS washings. GST proteins were eluted with 10 mM reduced glutathione (Sigma) in 50 mM Tris pH 8.0. Protein concentrations were determined by SDS-PAGE comparison of 1 µl of protein with bovine serum albumin standards. Protein bands were observed by subsequent Coomassie blue staining.

2.20 GST protein binding assays

GST protein interactions were performed as originally described elsewhere (Hanada *et al.*, 1995). 10 µg of GST protein was incubated with 11µl of Glutathione-Sepharose beads

for one hour in HKM buffer (10 mM Hepes pH 7.2, 140 mM sodium chloride, 5 mM magnesium chloride, 1 mM EDTA and 0.15% Nonidet P-40) to attach the proteins to the beads. Next, 5 µl of [^{35}S]-methionine *in vitro* translated VDR prepared using the TNT T7 Quick coupled transcription/translation system (Promega) was incubated with the GST proteins for 2.5 hours at 4°C. The mixture was washed six times with HKM buffer, after which the beads were boiled in 20 µl 2X SDS gel loading buffer and 20 µl was subjected to SDS-PAGE. After running, the gel was dried and exposed to X-ray film.

2.21 Anchorage independent (soft agar) growth assays

Soft agar assays were performed according to the method of Yang *et al.*, (1996). The underlay gel was prepared by mixing 1.7% autoclaved agarose with an equal volume of 2X DMEM and adding 1.25 ml to each 6 cm plate. The overlay gel was prepared with 0.7% autoclaved agarose (cooled to 40°C) mixed with an equal volume of 2X DMEM (containing 10^3 cells per 1.25 ml overlay) supplemented with 10% FBS and 1.25 ml was added to each plate. Colonies containing 50 or more cells were counted after 15 days.

Chapter 3

Results

3.1 Bag-1 interacts with the vitamin D receptor

Previous studies have shown the BAG-1 can interact with a variety of steroid hormone receptors (Zeiner *et al.*, 1995; Froesch *et al.*, 1998; Liu *et al.*, 1998; Kullmann *et al.*, 1998). We therefore decided to determine if BAGp50 could interact with the vitamin D receptor.

3.1.1 Far Western blot

Fig.3.1a shows the result of a far Western blot in which GST and GST-BAGp50 fusion proteins were probed with [^{35}S]-methionine labeled *in vitro* translated VDR (Fig 3.1b). It was observed that the VDR specifically interacted with GST-BAGp50, but not the GST protein.

3.1.2 GST Pull Down Assays

The structure of BAG-1 reveals several domains which have potential to be involved in protein-protein interactions. There are N-terminal and C-terminal α-helical domains, which are commonly the site of protein heterodimerization (Villanueva 1994; Marshall *et al.*, 1993; Oleinikov 1993; Xing *et al.*, 1994). In addition, the centrally located ubiquitin-like domain is a likely candidate for a dimerization domain, since ubiquitin is involved in interactions with many proteins (Ciechanover *et al.*, 1991; Hershko and Ciechanover 1998).

Fig. 3.1a Binding of VDR and BAGp50 using Far western blotting

Far Western blot showing specific binding of the VDR to BAGp50. 10 μg GST (lane 1) or GST-BAG-1 (lane 2) protein was separated on 10% polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was subsequently probed with *in vitro* translated VDR.

Fig. 3.1b *In vitro* translated protein products

5 μl [^{35}S]-methionine labeled *in vitro* translated VDR (lane 1) or hsp70 (lane2) as a positive control, were separated on a 10% polyacrylamide gel.

Fig. 3.1a

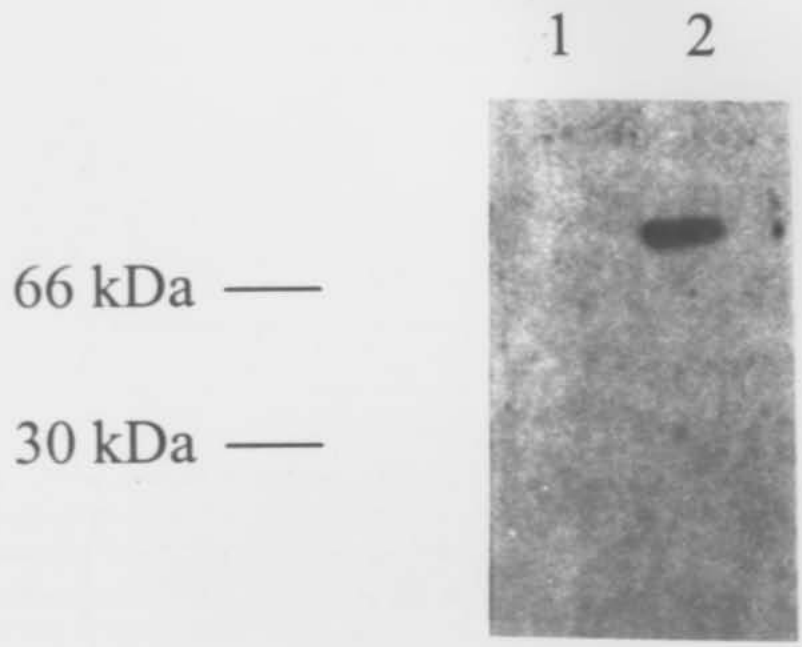


Fig. 3.1b

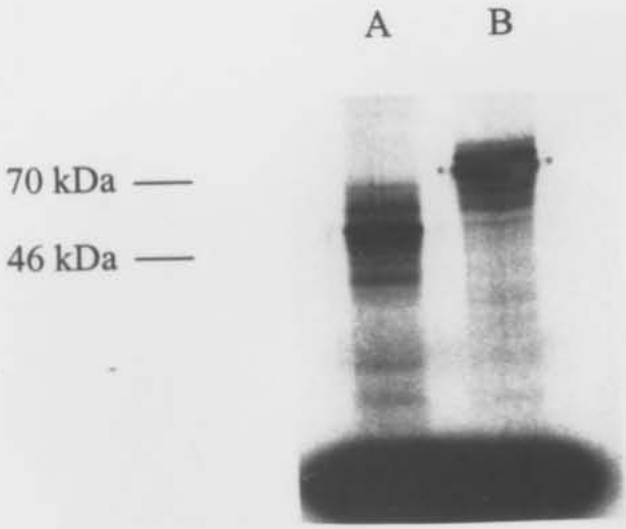
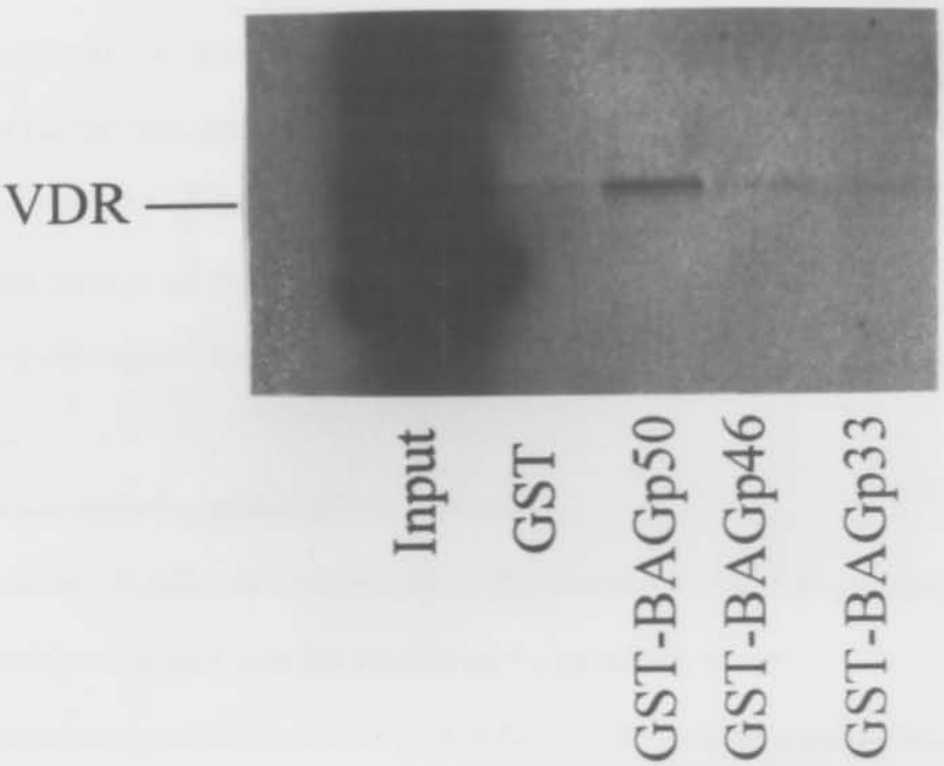


Fig. 3.2 VDR specifically binds the p50 isoform of BAG-1.

The N terminal of BAGp50 is responsible for its interaction with the VDR. Lane 1 contains 5 µl [^{35}S]-methionine labeled *in vitro* translated VDR. 10µg of GST or GST-BAG-1 fusion proteins were immobilized on glutathione-sepharose beads and incubated with 5 µl VDR (lanes 2-5). BAGp50 bound to the VDR (lane 3), but not the shorter p46 (lane 4) lacking 71 amino acids from the N terminus or the p33 isoform (lane 3) lacking 145 N terminal amino acids.


VDR
Input
GST
GST-BAGp50
GST-BAGp46
GST-BAGp33

To analyze the BAG-1 isoform and structural domain necessary for interaction with the vitamin D receptor, full length BAGp50, the p46 and p33 kDa isoforms were expressed in GST-fusion vectors and incubated with *in vitro* translated [^{35}S]-methionine labeled VDR (Fig. 3.1b). It was found that BAGp50 could strongly bind to the VDR, but did not interact with non-specifically [^{35}S] labeled proteins appearing in the input lane (Fig 3.2), but. Neither BAGp46 nor p33 however, could bind the VDR, indicating that the unique N-terminal of the p50 isoform is the site of their interaction. It has been previously shown that BAG-1's binding to the hsp70 is not dependent upon the amino terminus (Takayama *et al.*, 1997), demonstrating that BAG-1's interaction with the VDR is independent of heat shock proteins.

3.2.1 Stable transfection of BAGp50 in U87 cells

To examine the effect of the BAGp50-VDR interaction *in vivo,* U87 glioblastoma cells were stably transfected with the plasmid pcr3.1 containing BAGp50 in the EcoRI cutting site or an empty vector as a control (Fig. 3.3 a,b,c). U87 cells were chosen because they express very low levels of endogenous BAG-1 mRNA or protein (Fig. 3.3 a,b). This result demonstrates that BAG-1 expression, while ubiquitous, can differ greatly in the amount of protein expressed and suggests that it may cell specificity. BAGp50-stably transfected cells will forthwith be described as U87BAG-1 and pcr3.1 stably transfected U87 cells described as U87-3.1.

3.2.2 Morphology of transfected cells

The overexpression of oncogenes in cells can often lead to morphological changes

Fig. 3.3a Northern blot of BAG-1 mRNA in stably transfect U87 cells

20 μg mRNA from wild type U87 (lane 1), U87-3.1 (lane2) or U87BAG-1 (lane3) was separated on a 1% agarose gel and immobilized on a nylon membrane. Membrane was probed with [^{32}P]-labeled BAG-1 cDNA.

Fig. 3.3b Western blot of BAGp50 protein instably transfect U87 cells

10 μg total protein from U87 (lane1) or U87-BAG-1 was separated on 10% polyacrylamide gel and transferred to nitrocellulose membrane. The membranes were probed with 1:100 dilution of the anti-human BAG-1 monoclonal antibody CC9E8 and 1:1500 dilution of the anti-β-actin monoclonal antibody.

Fig. 3.3a

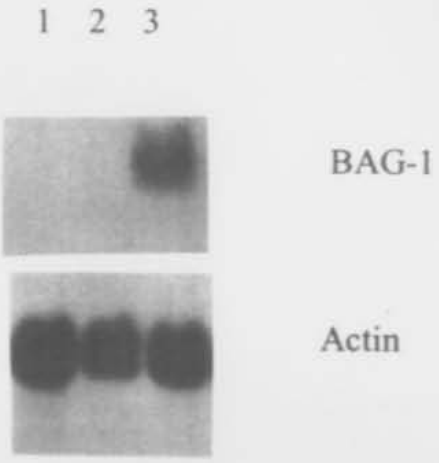


Fig. 3.3b

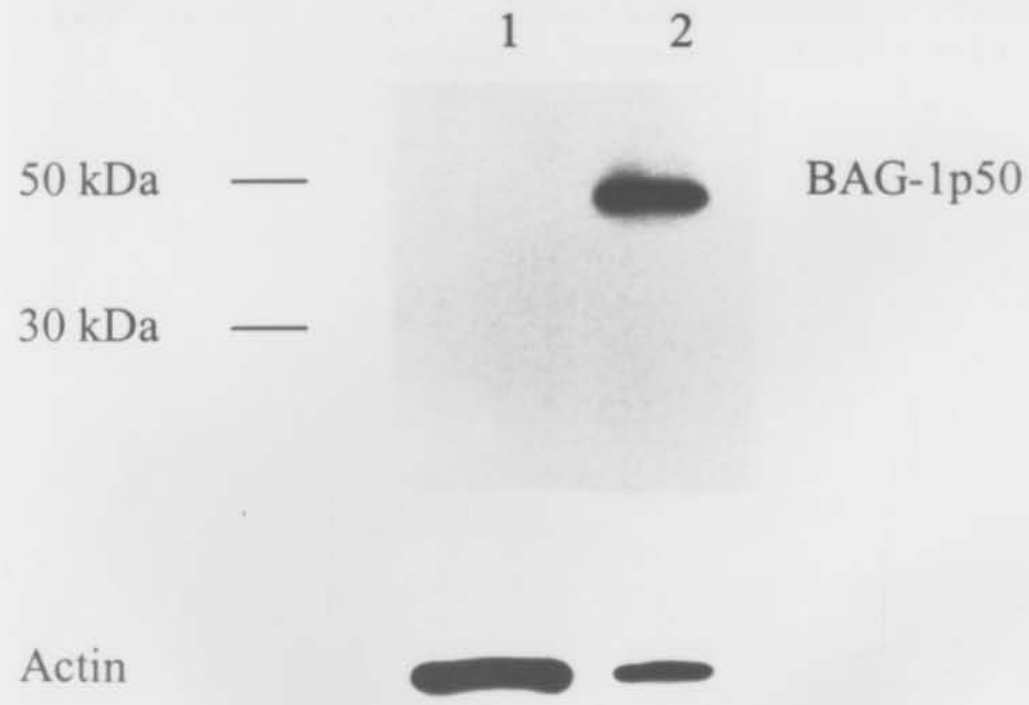


Fig. 3.3c PCR product from U87 cells stably transfected with the empty vector pcr3.1

PCR3.1 specific primers were used to amplify 50 ng of pcr3.1 plasmid DNA (lane 3) or 50 ng total DNA from U87 wildtype (lane2) or pcr3.1 stably transfected U87 cells (lane 4). PCR products were separated on a 2% agarose gel along with a 100 bp DNA ladder (lane 1). Only lanes 3 and 4 show the 180 bp amplification product.

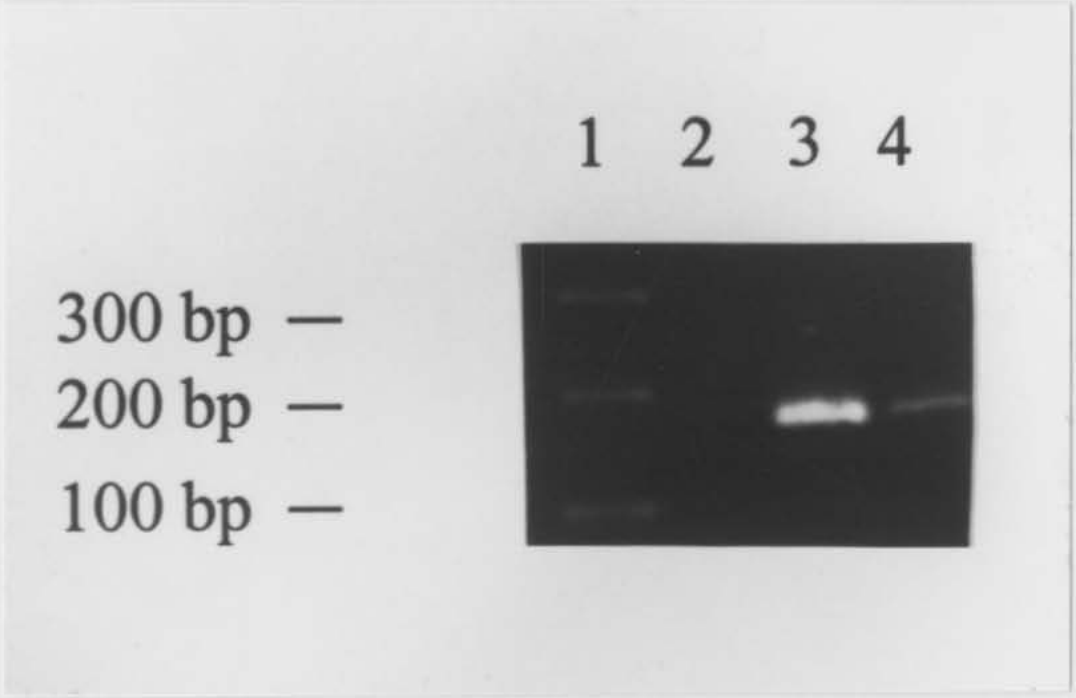
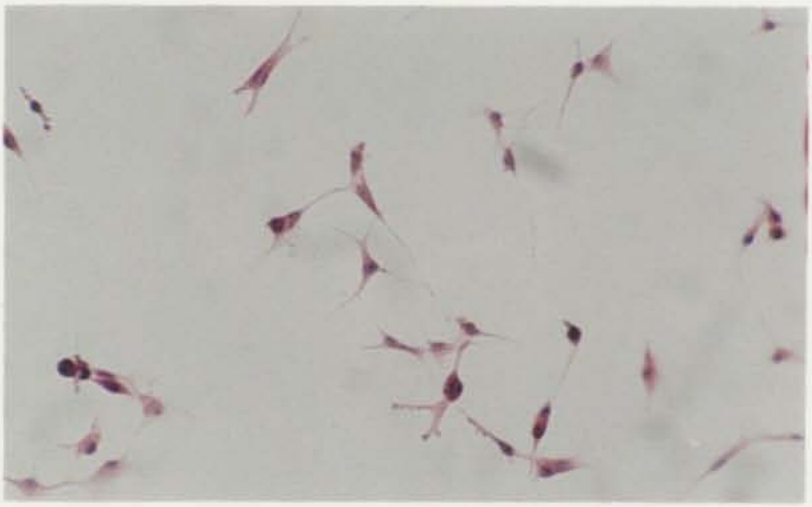

1 2 3 4
300 bp —
200 bp —
100 bp —

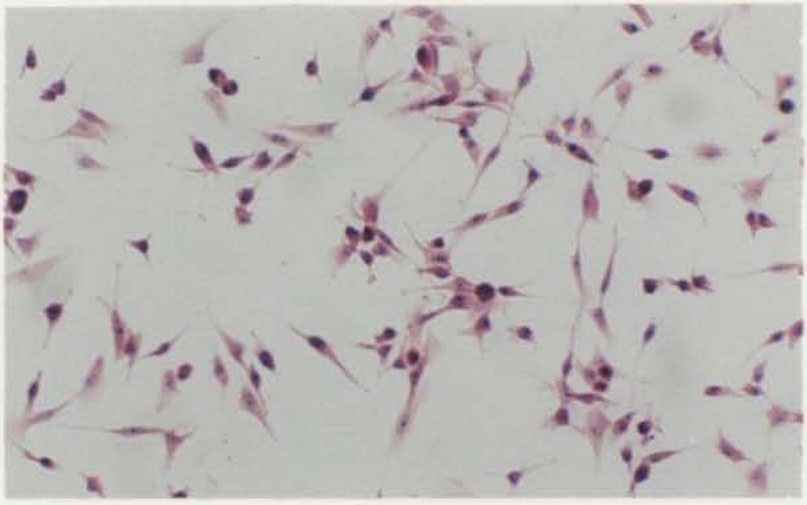
Fig. 3.4 Morphology of U87 wildtype, U87-3.1 and U87BAG-1 cells

Cells were grown on 6-well slides, stained with Eosin and Haematoxylin and photographed at 100X magnification. U87BAG-1 cells are approximately 3X larger than U87-3.1 cells. In addition, U87BAG-1 cells have a broader, more flattened morphology when not in contact with other cells.

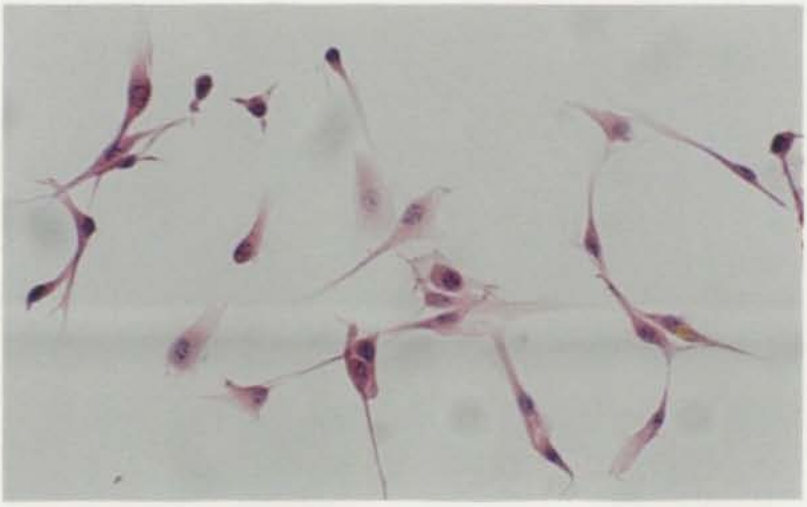
U87



U87-3.1



U87BAG-1



(Kato and Maeda 1997; Schuebel *et al.*, 1998; Sugiyama *et al.*, 1998). Because BAG-1 is thought to be a potential oncogene, the morphology of the stably transfected BAG-1 cells was studied using Eosin and Haematoxylin stained cells. Clearly, there are dramatic changes in the BAGp50 overexpressing cells (Fig. 3.4). Many of the U87BAG-1 cells are three times larger than those transfected with an empty vector (U87-3.1). In addition, before the U87BAG-1 cells come in contact with other cells, many cells lose their angular profile and become more rounded and flattened. The cells generally return to a more normal morphology once cell-cell contact occurs. The cytoplasmic to nuclear ratio appears to have remained the same. These cells are similar to a type of brain tumor referred to as giant glioblastoma cells (McKeever 1998; Katoh *et al.*, 1995).

3.3 EMSAs

The diverse cellular effects of the vitamin D hormone are carried out through its activation of the VDR. Once activated by 1,25-vitamin D3 (the metabolically active form of vitamin D), the receptor can bind to its DNA response element as either a homodimer or heterodimer with various other hormone receptors including the RAR (Nishikawa *et al.*, 1994b) or RXR (Liu *et al.*, 1996a). The VDR can then help regulate transcription of the gene in which its response element is located. It should also be noted that the VDR has the ability to bind to its response element as a homodimer in the absence of 1,25-vitamin D3 (Cheskis and Freedman 1994). To determine if the binding of BAG-1 to the VDR had any affect on the ability of the VDR to bind to its response element, whole cell extracts were collected from U87BAG-1 and U87-3.1 cells and EMSAs were performed using an oligonucleotide

Fig. 3.5a EMSA for consensus and mutant VDRE

BAGp50 interferes with binding of the VDR to consensus VDRE. EMSA reactions performed with a consensus DR-3 type VDRE. Lane 1 was loaded with free probe. Lanes 2,3 and 5 contain 15μg U87-3.1 whole cell extract. Lane 3 also had 300X excess cold oligo. The reaction in lane 5 was performed with a mutated VDRE oligo. Lane 4 contained 15 μg U87BAG-1 whole cell extract. The top arrow points toward the VDR/RXR complex and the bottom arrow indicates the position of unbound probe.

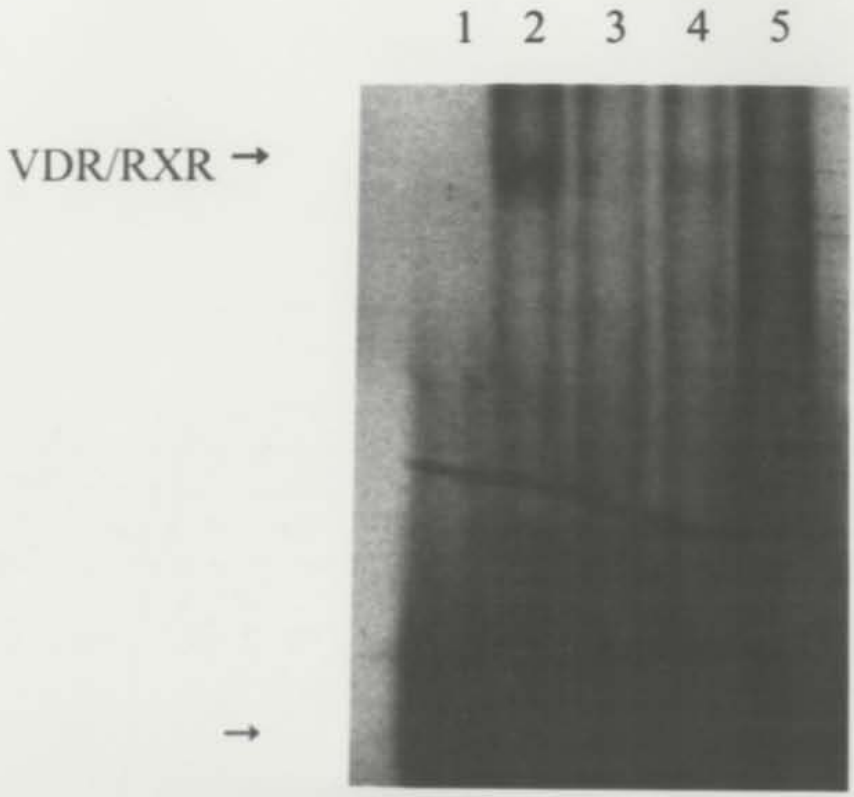
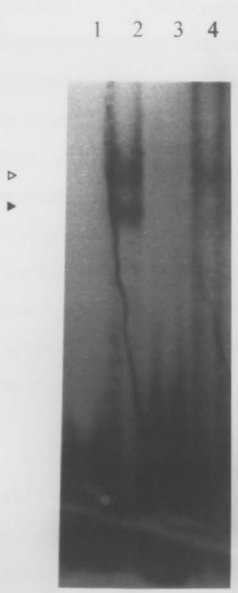

1 2 3 4 5
VDR/RXR →
→

Fig. 3.5b EMSA for non-consensus VDRE from p21^{waf1} promoter region

EMSA reactions performed using a VDRE containing oligo from the p21^{waf1} promoter. Lane 1 was loaded with free probe. Lane 2 and 3 contain 10 μg U87-3.1 whole cell extract. Lane 3 also had 300X excess cold oligo. Lane 4 contained 10 μg U87BAGp50 whole cell extract. The open triangle points toward a putative VDR/RXR complex and the closed triangle a VDR homodimer.


1 2 3 4

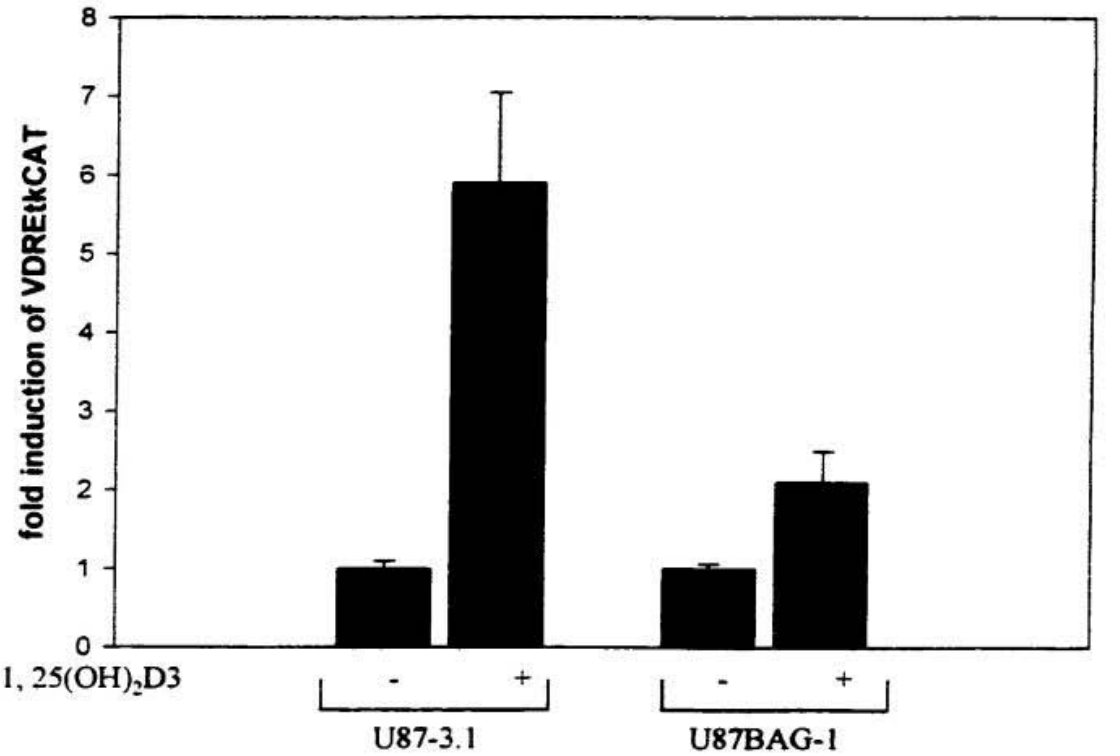
containing a consensus VDRE. The complex bound to this consensus VDRE was likely a VDR/RXR heterodimer (Fig. 3.5a lane 2), which was previously shown to bind a consensus VDRE (Nishikawa *et al.*, 1994a; Jin *et al.*, 1996). EMSAs performed with extracts from U87BAG-1 however, showed a greatly reduced amount of VDR/RXR complex binding to the VDRE (lane 4). To determine if BAG-1 could also inhibit the VDR from binding to DNA as homodimer, a VDRE from the $p21^{waf1}$ promoter was employed. It was previously demonstrated that the VDR could bind this VDRE as a homo or heterodimer (Liu *et al.*, 1996a). It was found that two complexes could bind this response element (Fig. 3.5b), corresponding to the complexes previously described (Liu *et al.*, 1996). Specific competition for the oligo was shown by adding 300X excess unlabeled oligonucleotide (lane 3). Extracts from U87BAG-1 however, showed significantly less VDR DNA binding as either a homodimer or heterodimer (Fig. 3.5b, lane 4). This suggests that BAGp50 can bind to the VDR in cell extract and that this binding abrogates the ability of the VDR to bind to its response element.

3.4 CAT assays in U87-3.1 and U87BAG-1 cells

The previous experiments suggest that BAGp50 is capable of down-regulating 1,25-vitamin D3-mediated transcription by blocking the VDR from binding to its response elements. To investigate this hypothesis, U87-3.1 and U87BAG-1 cells were transfected with the CAT reporter plasmid VDREtkCAT, containing multiple copies of the VDRE belonging to the osteocalcin gene. Twenty four hours after transfection, 10^{-7} M 1,25-vitamin D3 was added to the cells. CAT activity normalized with the luciferase reporter plasmid PGL3 or by

Fig. 3.6 Comparison of vitamin D3-induced CAT activation in U87-3.1 and U87BAG-1 cells.

Vitamin D3-mediated transactivation is down-regulated by BAGp50. 2 μg of the plasmid VDREtkCAT was transfected into U87-3.1 and U87BAG-1 cells in 6 well plates. Total DNA was normalized to 5μg. Vitamin D3 was seen to activate the VDRE containing CAT construct VDREtkCAT 5.9 fold in U873.1 cells stably transfected with empty vector. In U87BAG-1 cells that overexpress the p50 isoform of BAG-1 however, this activation was reduced to 2 fold.


fold induction of VDREtkCAT
8
7
6
5
4
3
2
1
0
1, 25(OH)2D3
-
+
-
+
U87-3.1
U87BAG-1

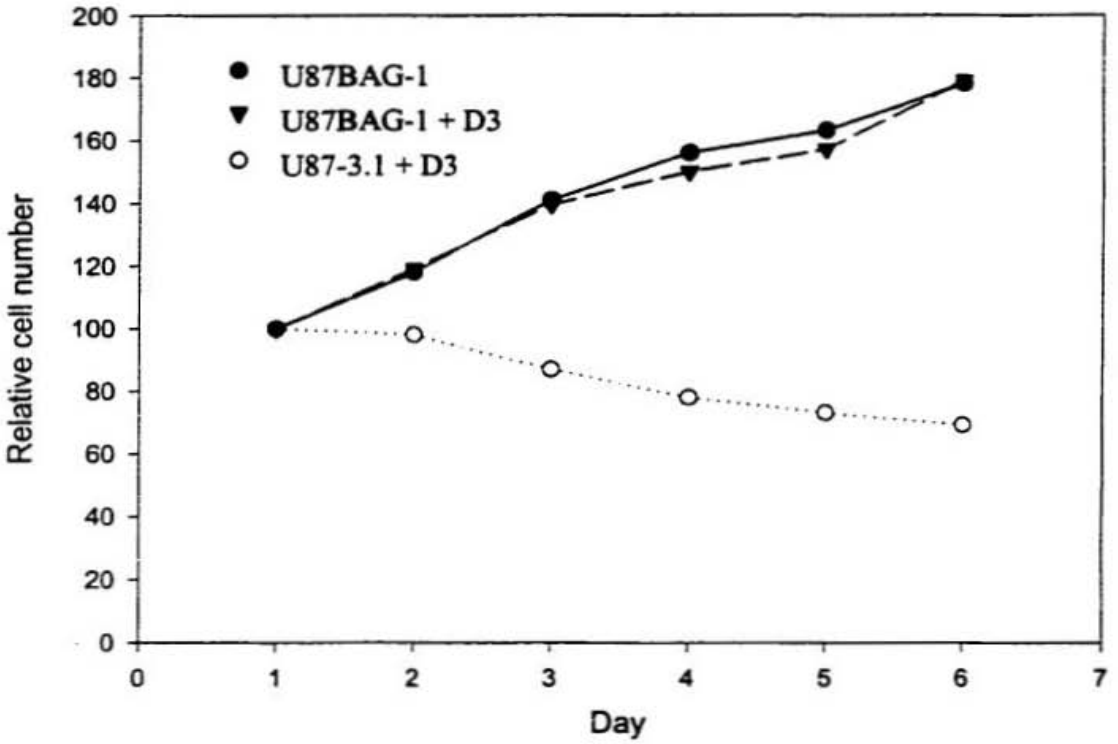
equalizing total amount of protein used in each CAT assay (Kullman *et al.*, 1998). In the presence of hormone, CAT activity was increased over 5 fold in U87-3.1 cells (Fig.3.6). In cells containing BAG-1 however, activation by 1,25-vitamin D3 was strongly repressed and only an approximate 2-fold induction was observed. This suggests that BAGp50 is a negative regulator of VDR-mediated transcription *in vivo*. These experiments were also repeated in C33A-3.1 and C33A-BAG-1 cervical carcinoma cells (these cells were provided byYang *et al.*, unpublished data), but 1,25-vitamin D3 had no effect on CAT activity in this system. These results point towards BAGp50 being a novel repressor of VDR activity.

3.5 Proliferation Assays

1,25-vitamin D3 has been reported to have anti-proliferative effects on numerous cell lines, including MCF-7 breast cancer cells (Simpson *et al.*, 1987), lymphocytes (Rigby *et al.*, 1985) and others. We next decided to investigate if vitamin D had any affect on the growth of U87 cells, and if so, whether the overexpression of BAGp50 could inhibit this effect. 10^{-7} M 1,25-vitamin D3 was added to U87-3.1 and U87BAG-1 cells plated at a density of 10^4 cells per well in 6 well plates. 1,25-vitamin D3 was found to inhibit the growth of U87-3.1 cells by over 30% over a six day period (Fig. 3.7). Compared to U87-3.1 cells, U87BAG-1 cells grew at a much faster rate. The untreated BAG-1 cells grew 79% faster than their U87-3.1 counterparts after a six day period. Interestingly, 1,25-vitamin D3 had no effect on the growth of U87BAG-1 cells over a 6 day period, demonstrating that BAGp50 rendered these cells resistant to the growth inhibitory actions of 1,25-vitamin D3. This also lends supporting evidence that BAG-1 and the VDR can bind *in vivo* and that this binding abrogates the DNA

Fig. 3.7 Growth assays of U87-3.1 and U87BAG-1 in presence and absence of vitamin D3.

BAGp50 increases proliferation of U87 cells and induces resistance to vitamin D3-mediated proliferation inhibition. Growth of U87-3.1 cells under normal conditions is taken to be 100%. Vitamin D3 is shown to reduce the proliferation of U87-3.1 cells (O). Overexpression of BAGp50 in U87 cells is seen to enhance the rate of proliferation (●) and confers vitamin D3 resistance upon the cells (▼).


U87BAG-1
U87BAG-1 + D3
U87-3.1 + D3
Relative cell number
Day
0
20
40
60
80
100
120
140
160
180
200
0
1
2
3
4
5
6
7

binding potential of VDR.

3.6 Western blotting of the VDR

Studies have shown that cellular VDR levels increase in response to 1,25-vitamin D3 and that this increase is necessary to observe the anti-proliferative effects of 1,25-vitamin D3 (Wiese *et al.*, 1992; Santiso-Mere *et al.*, 1993; Solvsten *et al.*, 1997). Protein levels are thought to be increased through both an increase in transcription (Taoka *et al.*, 1993 and Cornet *et al.*, 1998) and enhanced protein stabilization (Wiese *et al.*, 1992). VDR levels were assayed in the BAG-1 negative, U87 glioblastoma and C33A cervical cell lines after the addition of 1,25-vitamin D3 for 6 and 24 hour periods. A monoclonal VDR antibody (Affinity Bioreagents) at a 1:1000 dilution was used to detect VDR protein levels. The level of VDR was seen to rise in both cell lines after the addition of 1,25-vitamin D3 (Fig. 3.8a). The faint upper band seen in U87 cells after the addition of 1,25-vitamin D3 is probably due to VDR phosphorylation, as previously described (Iwata *et al.*, 1996; Jurutka *et al.*, 1993).

As mentioned above, increased VDR levels are thought to be necessary for the cellular effects of 1,25-vitamin D3 to occur. Therefore, it was believed that if VDR levels were unable to rise in U87 cells in response to 1,25-vitamin D3 stimulation, that the resulting decrease in proliferation may not occur. Therefore, we hypothesized that a possible mechanism of action by BAG-1 to inhibit the cellular actions of 1,25-vitamin D3 could involve decreasing VDR levels via binding and inhibiting the normal function of the VDR. To test this hypothesis, we again utilized Western blotting to detect VDR protein levels in

Fig. 3.8a Western blot of VDR in U87 and C33A cells.

10 μg of protein from C33A and U87 cells was resolved on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. Blot was probed with 1:1000 dilution of VDR monoclonal antibody. Lane 1 contains wild type protein. Lanes 2 and three contains protein from wild type cells after the addition of 10^{-7} M vitamin D3 for 6 and 24 hours respectively. The same blot was stripped and subsequently reprobed with 1:1500 X dilution of β-Actin.

Fig. 3.8b Western blot of VDR from U87-3.1 and U87BAG-1 cells.

10 μg protein from U87-3.1 (lanes 1 and 2) or U87BAG-1 (lanes 3 and 4) was resolved on 10% polyacrylamide gel and transferred to nitrocellulose membrane. Membranes were probed with 1:1000 X dilution of VDR monoclonal antibody. Lanes 2 and 4 contain protein from cells grown in the presence of 10^{-7}M vitamin D3 for 24 hours.

Fig. 3.8a

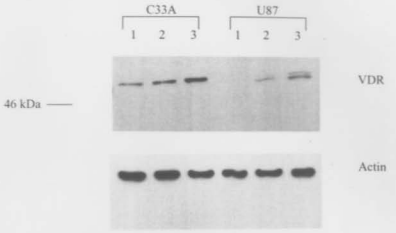


Fig. 3.8b

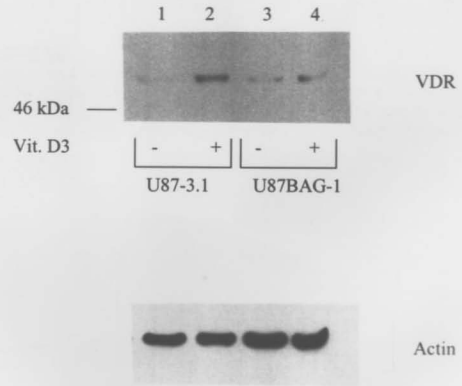


Fig. 3.9 Growth of U87-3.1 and U87BAG-1 in soft agar

1 x 10^3 U87-3.1 or U87BAG-1 cells were seeded in soft agar and grown for 15 days. U87BAG-1 cells formed 84% ±13% more colonies. The colonies formed from U87BAG-1 cells were also significantly larger in diameter. Photographs were taken at a magnification of 25X.

U87-3.1

U87BAG-1

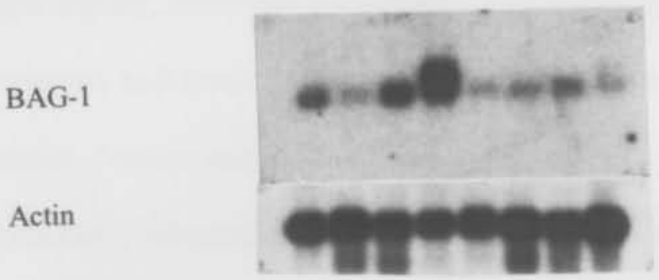
U87-3.1 and U87BAG-1 cells after the addition of 1,25-vitamin D3. It was found that the presence of BAG-1 significantly reduced the increase of VDR in response to ligand stimulation (Fig. 3.8b).

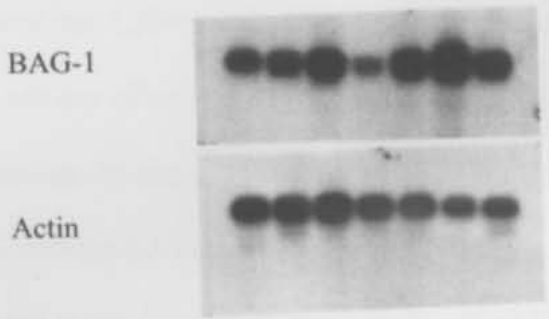
3.7 Soft Agar Assays

It has previously been reported that proliferation-promoting factors such as the epidermal growth factor receptor and interleukin 6 can enhance the colony formation of transformed cells in soft agar (Okamoto *et al.*, 1998).To further investigate the pro-proliferative ability of BAG-1, we subjected U87-3.1 and U87Bag-1 cells to soft agar assays. Increased rates of cellular proliferation and tumorigenicity are known to be related (Chen *et al.*, 1998; Popik and Inglot 1987). Therefore, an increase in soft agar colony formation by BAG-1 overexpressing cells would lend support to its oncogenicty, as well as confirming its pro-proliferative capability. In addition, we investigated whether the addition of 1,25-vitamin D3 to the cells in soft agar could inhibit their clonal growth, since it has been reported to do so in other cell lines (Yoneda *et al.*, 1984; Dokoh *et al.*, 1984, Haussler *et al.*, 1986). U87Bag-1 cells formed 84% ±13% more colonies than U87-3.1cells, of 50 cells or more (fig. 3.9), after a period of 15 days. Interestingly, the colonies formed by BAG-1 overexpressing cells were significantly larger than their U87-3.1 counterparts (Fig. 3.9). 1,25-vitamin D3 had no effect on the growth of the cells. These results demonstrate again that BAGp50 can increase the rate of cell proliferation and may confer an increased tumorigenic potential upon cells.

Fig. 3.10 Expression of BAG-1 mRNA in normal tissue

Nylon membranes containing mRNA from a variety of normal tissue were probed with BAG-1 cDNA. Membrane was subsequently stripped and reprobed with β-Actin cDNA.


PBL
Colon
Small intestine
Ovary
Testis
Prostate
Thymus
Spleen
BAG-1
Actin


Stomach
Thyroid
Spinal Cord
Lymph Node
Trachea
Adrenal Gland
Bone Marrow
BAG-1
Actin

3.8 Expression of BAG-1 in normal tissue

The classical 1,25-vitamin D3 target tissues are the bone, skin, kidney and intestine. The VDR has also recently been found at highly variable levels in the prostate (Kiviniva *et al.*, 1998). To determine if BAG-1 expression corresponded with VDR expression, multiple tissue Northern blots (Clontech) were probed with BAG-1 cDNA (Fig 3.10). BAG-1 mRNA expression showed tissue tropism, but no obvious pattern of expression could be discerned. BAG-1 was found at relatively high levels in bone marrow and the prostate but was virtually absent in the small intestine. Previous studies have found BAG-1 at high levels in the kidney but very low levels in the skin (Takayama *et al.*, 1998). Our results indicate that high level of BAG-1 correlates with high VDR levels only in bone marrow tissue.

3.9 Effect of BAGp50 on 1,25-vitamin D3-mediated induction of $p21^{waf1}$

1,25-vitamin D3 is known to be an inducer of $p21^{waf1}$ transcription and this is thought to be one of the mechanisms through which 1,25-vitamin D3 can inhibit proliferation (Liu *et al.*, 1996). Our results showed that 1,25-vitamin D3 can inhibit U87 cell growth, so we decided to test if the hormone had any effect on $p21^{waf1}$ transcription. This was achieved by transfecting cells with luciferase reporter plasmids containing the $p21^{waf1}$ promoter/enhancer. Transfections were normalized with the CAT reporter plasmid PSV2CAT or by normalizing the total amount of protein used in each luciferase assay as described by Kullman *et al.*, (1998). We found that 1,25-vitamin D3 had no effect on the $p21^{waf1}$ promoter in U87 cells, but could induce $p21^{waf1}$ transcription in C33A cells (Fig. 3.11). We next investigated if BAGp50 could inhibit this induction. To accomplish this, C33A cells stably trasfected with

Fig. 3.11 Effect of BAG-1 on vitamin D3-induced upregulation of $p21^{waf1}$ transcription.

1 μg of the plasmid p21-4 containing the $p21^{waf1}$ promoter in a luciferase vector was transfected into C33A cells stably transfected with empty vector (C33A-3.1) or BAGp50 (C33A-BAG-1). Transfections were normalized to total of 5 μg plasmid DNA. 10^{-7} M vitamin D3 was added to cells 24 hours post transfection where applicable.

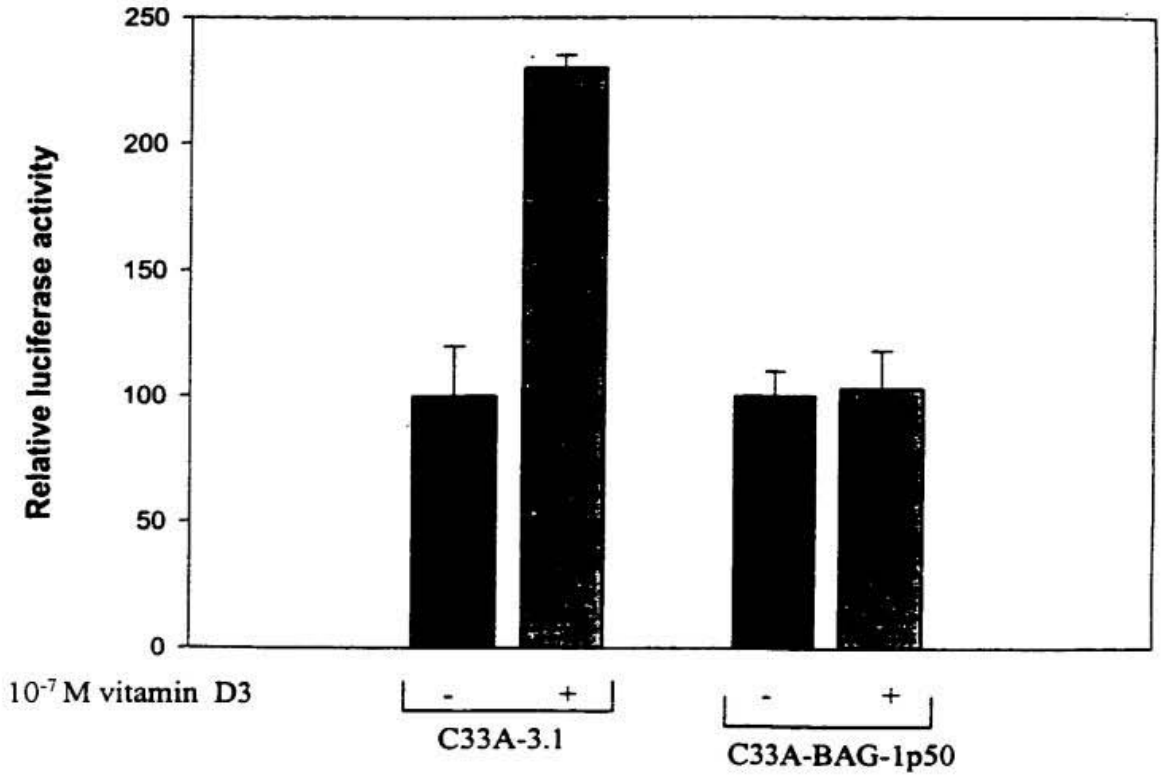
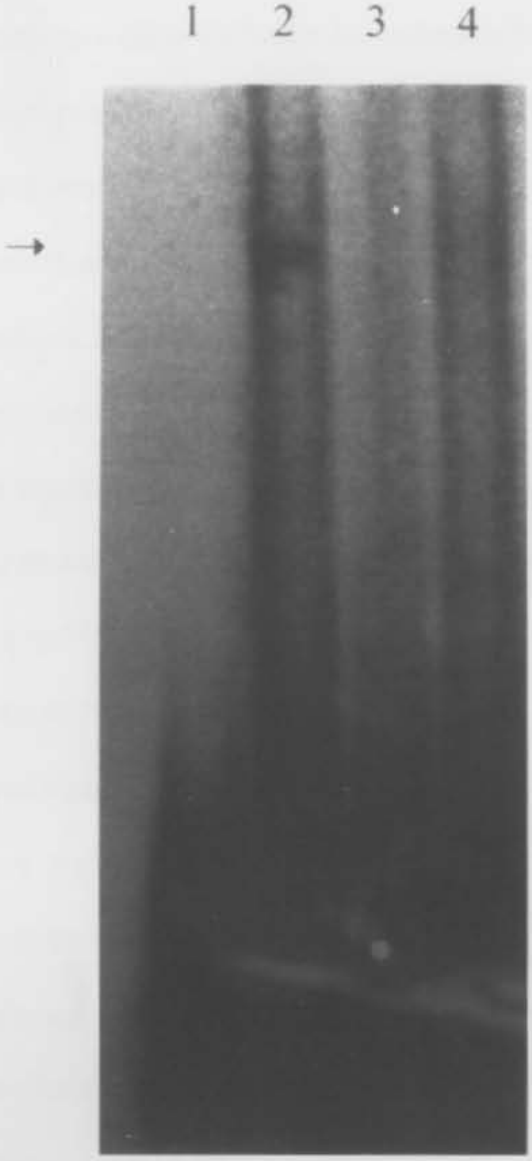

250
200
150
100
50
0
Relative luciferase activity
10-7 M vitamin D3
-
+
-
+
C33A-3.1
C33A-BAG-1p50

Fig. 3.12 EMSA for p21^{waf1} VDRE with extract from C33A-3.1 and C33A-BAG-1 cells.

10 μg extract from C33A-3.1 (lanes 2 and 3) and C33A-BAG-1 (lane 4) was incubated with 50,000 cpm radiolabeled p21^{waf1} VDRE. Lane 1 contains free probe and lane 3 contains 300X excess cold oligo. Arrow indicates bound VDR complex.


1
2
3
4

BAGp50 were utilized. We found that BAGp50 could inhibit the transcriptional increase of $p21^{waf1}$ promoter by 1,25-vitamin D3 (Fig.3.11).

Next we investigated whether BAGp50 could inhibit the binding of the VDR to the $p21^{waf1}$ VDRE in C33A cells as it did in U87 cells. Interestingly, it was found that only one complex from C33A extract bound to this VDRE (Fig.3.12). The presence of BAGp50 inhibited its binding. Further experiments showed that no binding to the consensus VDRE could be observed (data not shown). The complex bound to the $p21^{waf1}$ VDRE in this cell line is likely a VDR homodimer and not a VDR/RXR heterodimer, since the VDR/RXR heterodimer is necessary to activate the osteocalcin VDRE found in the VDREtkCAT vector (Sone *et al.*, 1991). The fact that 1,25-vitamin D3 has no effect on this CAT construct in these cells (as mentioned above) and that no binding to the consensus VDRE is observed in C33A cells, suggests that the VDR/RXR heterodimer does not form in C33A cells and that the complex bound to the $p21^{waf1}$ promoter is a VDR homodimer. This also proposes that $p21^{waf}$ transcription can be regulated by a VDR homodimer in this cell line.

It has been shown that glucocorticoids can down-regulate cellular BAG-1 levels (Clevenger *et al.*, 1997). In turn, BAG-1 can inhibit the cellular actions of glucocorticoids. (Kullman *et al.*, 1998). Thus, it appears that BAG-1 and glucocorticoids act in opposition to one another in a negative feedback loop. To investigate whether BAG-1 and 1,25-vitamin D3 can work in this fashion, the BAG-1 promoter/enhancer contained in the luciferase vector PGL2 (Yang *et al.*, 1999b) was utilized. This construct was transfected into C33A and U87 cells with and without the addition of 1,25-vitamin D3. It was found that 1,25-vitamin D3 had no effect on the level of BAG-1 transcription in these cell lines (data not shown). This

assay also demonstrates that 1,25-vitamin D3 is not a nonspecific simulator of transcription.

3.10 Effect of BAGp46 on the $p21^{waf1}$ promoter

To investigate whether BAGp46 could inhibit the effects of 1,25-vitamin D3, we cotransfected the $p21^{waf1}$ promoter region with this isoform of BAG-1 and added 1,25-vitamin D3. Interestingly, we found that BAGp46 could decrease $p21^{waf1}$ transcription in C33A cells by 75% (Fig. 3.13). When 10^{-7} 1,25-vitamin D3 was added there was an increase in $p21^{waf1}$ promoter activity, which was repressed by the presence of BAGp46. However, it is also possible that the addition of 1,25-vitamin D3 abrogated the transcriptional repression of $p21^{waf1}$ by BAGp46 or that the two effects were additive.

Next, we decided to determine if the BAGp46 could repress $p21^{waf1}$ transcription in another cell line. It was found that BAGp46 could also inhibit $p21^{waf1}$ transcription in U87 cells (Fig. 3.14). Transcriptional repression in these cells was slightly weaker than in C33A cells.

Fig. 3.13 Negative regulation of $p21^{waf1}$ transcription in C33A cells.

5 μg of the plasmid p21-luc containing the $p21^{waf1}$ promoter in a luciferase reporter vector was transfected into C33A cells grown in 5cm plates with varying amounts of BAG-1p46. Total plasmid DNA was normalized to 15 μg. 10^{-7} M vitamin D3 was added 24 hours post transfection where applicable.

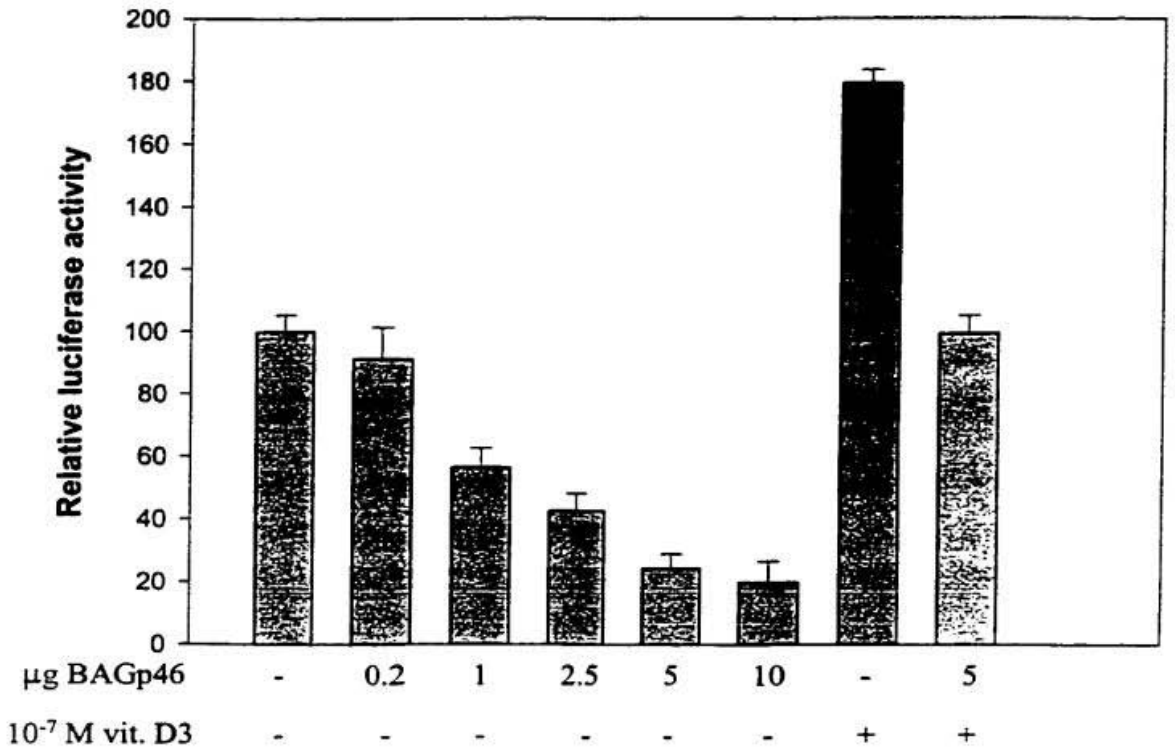
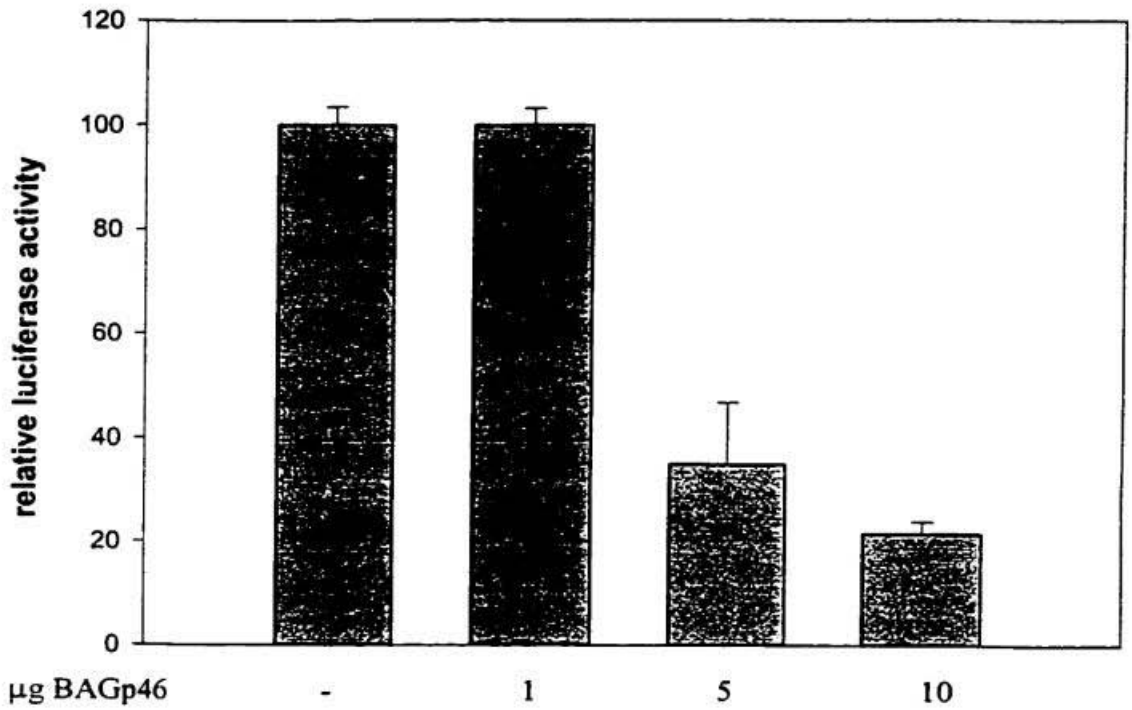

Relative luciferase activity
200
180
160
140
120
100
80
60
40
20
0
µg BAGp46 - 0.2 1 2.5 5 10 - 5
10-7 M vit. D3 - - - - - - + +

Fig. 3.14 Negative regulation of $p21^{waf1}$ transcription in U87 cells.

5 μg of the plasmid p21-luc containing the $p21^{waf1}$ promoter in a luciferase reporter vector was transfected into cells grown in 5 cm plates with varying amounts of BAGp46. Total plasmid DNA was normalized to 15μg.


120
100
80
60
40
20
0
relative luciferase activity
µg BAGp46
-
1
5
10

CHAPTER 4

DISCUSSION

4.1 BAG-1 interacts with the VDR

Human BAG-1 was originally isolated due to its ability to bind the GR (Zeiner and Gehring 1995). Since that time, it has been found to bind, and also modulate the function of the GR (Kullman *et al.*, 1998), AR (Froesch *et al.*, 1998) and RAR (Takayama *et al.*, 1998). In this study, it was found that BAGp50 could bind the VDR.

Each of the previous reports described a different isoform of BAG-1 binding to a steroid hormone receptor. BAGp46 was found to bind the GR and BAGp33 was found to bind the RAR. It has not been investigated whether other isoforms can bind these receptors. The AR was found to bind to BAGp50 but not to shorter BAG-1 isoforms.

Here, Far Western blotting (Fig. 3.1a) and GST-fusion protein pull-down assays (Fig. 3.2) were employed to demonstrate the VDR-binding potential of BAGp50. As with the AR, the VDR bound specifically to the N-terminus of BAG-1 as was determined in GST-fusion protein assays. The C-terminal of BAG-1 is responsible for binding to hsp70 (Takayama et et, 1997). Therefore, it appears that BAG-1 binds to the VDR, RAR and AR independently of hsp70. This is important, because Zeiner *et al.*, (1997) found that BAG-1 could interact with a variety of proteins indirectly via hsp70. The N-terminus of BAGp50 has no readily apparent secondary structures that may be involved in protein-protein interactions. Site-directed mutagenesis of BAG-1's N-terminus would be useful for determining which amino acids are necessary for hormone receptor binding.

Far Western and GST-fusion protein binding show that the VDR does not need to bind 1,25-vitamin D3 to bind BAG-1 *in vitro*. It has been found that several VDR coactivators, such as steroid receptor coactivator 1, interact with VDR in a 1,25-vitamin D3-dependent manner (Gill *et al.*, 1998). Further work using the yeast two hybrid binding system and immunoprecipitation is necessary to determine if BAGp50 can bind the VDR independently of 1,25-vitamin D3 *in vivo*.

BAG-1 contains two potential amphipathic α-helical domains. These motifs have been shown to be important for binding of other proteins to the TFIIB and TFIID components of the basal transcriptional machinery (Latchman 1990). In addition, the steroid receptor coactivator GRIP1 has been shown to interact with receptors through an amphipathic α-helical domain (Darimont *et al.*, 1998). This suggests that BAG-1 may form a novel link between nuclear receptors and the basal transcription machinery. It would be interesting for future studies to explore the potential relationship of BAG-1 with TFIIB and TFIID. A N-terminus α-helical domain is found in both the p50 and p46 isoforms of BAG-1. This motif was shown not to be necessary for *in vitro* binding to the VDR, but may be important for proper binding or joining accessory proteins to the VDR complex *in vivo*. This is possible, since the binding of nuclear hormone receptors to DNA and subsequent transcriptional regulation involves a number of coactivators, corepressors and other proteins involved in chromatin restructuring (Horwitz *et al.*, 1996; Haussler *et al.*, 1997). To identify any VDR accessary proteins or other new proteins that interact with the N-terminus of BAGp50, the yeast two-hybrid screening system could be utilized using a truncated BAG-1 containing only the N-terminus as bait protein fused to the Gal4 DNA binding domain.

4.2 Stable transfections of U87 glioblastoma cells

To further study the interaction of BAG-1 and the VDR, stable transfections of cells with the BAGp50 protein (Fig. 3.3b and Fig. 3.4) were be made. Cells lacking BAG-1 were considered most suitable for constructing stable transfectants, because there would be no background effects of endogenous BAG-1. In addition, it would be clear in subsequent studies with these cells, that any cellular or molecular changes would be due solely to the presence of high levels of the BAGp50, but not other isoforms. U87 cells were considered to be ideal because, of the two available BAG-1 negative cell lines (U87 and C33A), U87 was the only one which responded to 1,25-vitamin D3 with a reduced rate of proliferation. Past studies also have shown different glioblastoma cell lines to be sensitive to 1,25-vitamin D3 (Margrassi *et al.*, 1995).

The most obvious change in U87BAG-1 cells was their altered morphology (Fig. 3.4). Many of the cells were larger, rounder and flatter than their U87-3.1 counterparts. This is similar to the morphological changes that were reported when primary cells were immortalized by oncogenic factors such as human papillomavirus type 16 (Tsutsumi *et al.*, 1992) and the K-ras protein (Sugiyama *et al.*, 1998). It is not yet understood what intracelluar changes occur that lead cells to have this altered morphology. A change in structural protein expression seems possible. Undoubtedly however, there must be numerous changes in gene expression to account for these features. Differential display would be a useful technique to help reveal genes having altered expression in U87BAG-1 cells. The fact that BAG-1 transfected cells exhibit a morphology similar to other cells overexpressing oncogenic factors also implies that BAG-1 itself may be a proto-oncogene.

Interestingly, a rare type of giant cell glioblastoma exists. The cells from these tumors are enormous, measuring up to 400 μm in diameter (Katoh *et al.*, 1995). Giant cell glioblastoma cells are often mulitinucleated but are not formed from cell fusion (McKeever 1998). There have been few studies performed on these rare tumors. One study found that 12 of 16 tumors had mutated p53 proteins (Peraud *et al.*, 1997) and others have found high immunohistochemical staining of proliferation markers such as proliferating cell nuclear antigen (McKeever 1998; Katoh *et al.*, 1995). Mutated p53 alone however couldn't explain the strange phenotype exhibited by these cells. It would be worthwhile to examine these tumors for BAG-1 expression, since they show a morphology similar to BAG-1 overexpressing glioblastomas.

In addition to the morphological changes, BAGp50 was shown to increase the basal proliferative rate of these cells (Fig. 3.7). BAG-1 overexpression was previously shown to have some correlation with survival and proliferation in white blood cells, but BAG-1 overexpression alone did not directly increase the rate of proliferation of these cells (Clevenger *et al.*,1997). BAG-1 was demonstrated to make an interleukin-3-dependent cell line proliferate independently of interleukin-3. BAG-1 protein levels were also shown to be upregulated by interleukin-2 (Adachi *et al.*, 1996) and Interferon-γ. Both interleukin-2 and interferon-γ have been shown to increase cell proliferation (Mehrotra *et al.*, 1995 and Widschwendter *et al.*, 1995) in T lymphocytes. Hence, BAG-1 may be involved in the induction of proliferation by these, and maybe other, cytokines.

The involvement of BAG-1 in cell growth was further supported by the fact that BAG-1 transfected cells could form more colonies in soft agar than non-transfected cells.

A potential future study would involve stably transfect non-tumorigenic baby rat kidney cells with the various BAG-1 isoforms and investigate whether BAG-1 could confer immortilization and the ability to grow in soft agar or form tumors in nude mice upon these cells. Since BAG-1 can inhibit apoptosis in several cell lines, it is reasonable that U87BAG-1 too, could be resistant to apoptosis. If U87BAG-1 cells do have heightened protection from apoptosis, it would be interesting to study what agents could overcome this resistance. These may include apoptosis-inducing drugs such as cisplatin, etoposide, etc.

Proliferation is a complex biological process and involves a large number of interrelated factors, such as cell stimulation by hormones and cytokines and their related signal transduction pathways. Also, cell cycle-related proteins, such as cyclins and their inhibitors, play an essential role in proliferation. Taking the number of factors involved in proliferation into account, it is difficult to speculate on the exact role that BAG-1 is playing in proliferation. However, regulation of proliferation through modulation of hormone receptor function may certainly be one mode of action.

4.3 BAGp50 can inhibit the binding of DNA by the VDR

Transcriptional transactivation by steroid receptors is influenced by accessory factors through several mechanisms. These accessory factors can form a direct link between the receptor and the basal transcriptional machinery, remodel chromatin structure, or interfere with the ability of the receptor to bind DNA. After observing the interaction between BAG50 and the VDR, the next question that needed to be addressed was whether the binding of the VDR by BAGp50 had an effect on the ability of the VDR to bind its DNA responsive

element.

It was found that the VDR contained in cell extracts from U87-3.1 could bind to a consensus VDRE and a VDRE from the $p21^{waf1}$ promoter with high affinity (Fig. 3.5a and b). The presence of BAGp50 in the U87BAG-1 cell extracts greatly diminished the binding of the VDR to VDRE containing oligonucleotides. BAGp50 could apparently inhibit the binding of VDR as either a homodimer or heterodimer. BAGp50 may abrogate DNA binding by the VDR via several mechanisms. BAG-1 may bind to the DNA binding domain of the VDR, thus blocking the functionality of this domain. The binding of BAG-1 to the VDR could change the conformation of the VDR which could result in an inability to bind DNA or form dimers. Finally, BAGp50 may simply sequester the receptor to some region in the nucleus. Further work could explore the exact mechanism of this inhibition.

4.4 Inhibition of 1,25-vitamin D3-mediated transactivation by BAG-1

A corepressor is a protein that can repress the activity of a nuclear receptor through one of several mechanisms. These mechanisms include: 1) binding of the corepressor to a response element that overlaps the hormone response element, thus competing out the hormone receptor 2) the repressor competes with the receptor for binding to the basal transcriptional machinery thereby interferes with the receptor activity 3) The repressor binds the receptor and inhibits its ability to bind DNA. There have been several corepressors discovered that antagonize steroid hormone receptors (Horwitz *et al.*, 1996). Up to the present, the calcium binding protein calreticulin is the only known corepressor of the VDR that binds the VDR and blocks its DNA binding ability (Wheeler *et al.*, 1995).

Based on the aforementioned results, we decided to investigate whether BAGp50 had any corepressor-like influence on 1,25-vitamin D3-mediated transcription *in vivo*. A CAT reporter plasmid containing multiple VDREs in tandem, transiently transfected into U87-3.1 cells was stimulated over 5-fold by the addition of 10^{-7} M 1,25-vitamin D3. However, the same construct transfected into U87BAG-1 cells showed less than a 2-fold induction when stimulated by the same hormone (Fig. 3.6). This suggests that BAGp50 can regulate 1,25-vitamin D3-mediated transactivation *in vivo*. In addition, it also suggests that the interaction between BAGp50 and the VDR can also occur *in vivo*.

Similarly, it was found that BAGp50 could block 1,25-vitamin D3-induced transactivation of the $p21^{waf1}$ promoter in C33A cells (Fig. 3.11) demonstrating that inhibition of the VDR by BAGp50 is not promoter or cell specific. Transcription of $p21^{waf1}$ could not be stimulated by 1,25-vitamin D3 in U87 cells suggesting that other transcriptional factors were blocking, or necessary for, 1,25-vitamin D3-mediated action on this promoter and these factors were not present in C33A cells. This result also suggests that 1,25-vitamin D3 may utilize a $p21^{waf1}$ independent pathway in U87 cells to inhibit proliferation or alternatively, may regulate $p21^{waf1}$ expression on a post-transcriptional level as previously reported to occur in a prostate cancer cell line (Zhuang and Burnstein, 1998) . Of course, the actual transcription of genes from chromosomal DNA is very different than that from plasmid DNA in that chromatin structure and DNA methylation play an important role.

In agreement with the binding and transactivation studies were results showing that BAGp50 could confer resistance to 1,25-vitamin D3-mediated reduction of proliferation (Fig. 3.7). This also proposes that BAG-1 can interact with, and regulate the function of the

VDR *in vivo*. Further work is needed to show that the BAGp50-VDR interaction has significance in other cell lines. The fact that BAGp50 is primarily localized in the nucleus indicates that it may function as a regulator of steroid receptor function under normal physiological circumstances. The overexpression of BAG-1 may play a role in the resistance of cells to new 1,25-vitamin D3 analog-based therapies that are being developed.

It seems possible that the physiological role of BAG-1 is to regulate the function of steroid hormone receptors. BAGp50 was previously reported to bind, and enhance AR-mediated proliferation. BAGp46 and p33 were reported to bind and inhibit the functions of the GR and RAR respectively. Under normal circumstances, BAG-1 could be regulated at the level of transcription or translation to help regulate the cellular response to hormones. For example, if a cell is at a stage in which it needs to resist the actions of 1,25-vitamin D3, the cellular levels of BAGp50 could be upregulated. Conversely, if a cell needs to enhance the effect of 1,25-vitamin D3, such as increasing calcium absorption in the intestine or slowing the rate of cell growth, BAG-1 could be down-regulated. Recently, it was discovered that BAG-1 protein levels could be lowered by the addition of glucocorticoids to a lymphocyte cell line (Clevenger *et al.*, 1997). Considering the inhibitory effects of BAG-1 on GR-mediated transcription (Kullman *et al.*, 1998), this suggests that BAG-1 may be involved in a negative feedback loop within the glucocorticoid pathway. Similarly, it has been found that the promoter region of BAG-1 contains a consensus VDRE (Yang *et al.*, 1999b). It is unknown at this time what, if any, role this response element plays in the regulation of the BAG-1 protein. Further work might find that BAG-1, and the VDR, are involved in a negative feedback loop. If this is true, and BAG-1 is overexpressed as the result of some

cellular disruption, it could have a snowball effect in increasing its own protein levels. By repressing the action of its own inhibitors (steroid hormone receptors), BAG-1 would be actively increasing its own transcription. This may be a factor in BAG-1's potential role as an oncogene. In addition, BAG-1 was found to be localized in the nucleus and cytoplasm at different ratios at different times in the same cell line (Takayama *et al.*, 1998). This may potentially be due to the action of hormones. Future work could study the effect of hormones on BAG-1 expression in various types of cells.

As previously discussed, BAG-1 has been found at higher levels in breast tumors than the surrounding wild type tissue (Zapata *et al.*, 1998). Steroid hormone receptor status is often used as a prognostic marker for breast cancer (Ravaioli *et al.*, 1998). If BAG-1 were up-regulated in cells, this could lead to malfunction of hormone receptors and a loss of proliferation control. This could explain why the status of hormone receptors in breast carcinomas is not always a reliable predictor of of tumor status (Ravaioli *et al.*, 1998; Allred *et al.*, 1998). Even if hormone receptors are present, their function may be altered by increased BAG-1 levels. Conversely, in the case of the AR, increased BAG-1 levels may increase the receptor activity, resulting in resistance to androgen antagonists that are used for prostate cancer treatment. Further work is necessary to study the correlation of BAG-1 levels with hormone/hormone antagonist resistance.

4.5 BAGp50 can lower VDR protein levels

One possible explanation for the inhibition of 1,25-vitamin D3 action by BAGp50 is the fact that increasing BAG-1 levels results in the inability of 1,25-vitamin D3 to up-

regulate its own receptor (Fig. 3.8b). As previously mentioned, 1,25-vitamin D3 has been reported to increase its receptor's level by increasing VDR stabilization and through heightened transcription (Wiese 1992 ; Taoka 1993). Some studies suggest that the binding of 1,25-vitamin D3 to its receptor induces a conformational change, resulting in increased stabilization of the protein (van den Bemd *et al.*, 1996). It is also possible that the VDR upregulates the transcription of proteins that activate VDR transcription or increase VDR stabilization. However, the precise mechanism of vitamin D-mediated up-regulation of its own receptor is unknown at this time since the VDR promoter/enhancer does not contain a VDRE (Jehan and DeLuca 1997; Crofts *et al.*, 1998). This study suggests that BAGp50 could block 1,25-vitamin D3-mediated up-regulation of its own receptor through abrogation of VDR DNA binding ability. But the exact mechanism of this inhibition is unknown. Future work could examine VDR RNA levels in response to 1,25-vitamin D3 in U87-3.1 cells. If unchanged, the increase in VDR would be due to increased protein stabilization. If this were the case, the binding of BAG-1 to the VDR may result in an inability of the VDR to bind its ligand, thereby negating it's stabilizing effect. Future work could determine the domain of the VDR involved in BAG-1 binding. If BAG-1 bound to the DNA or ligand binding domain of the VDR, it would help explain how it blocks VDR function.

4.6 BAGp46 can repress transcription of $p21^{waf1}$

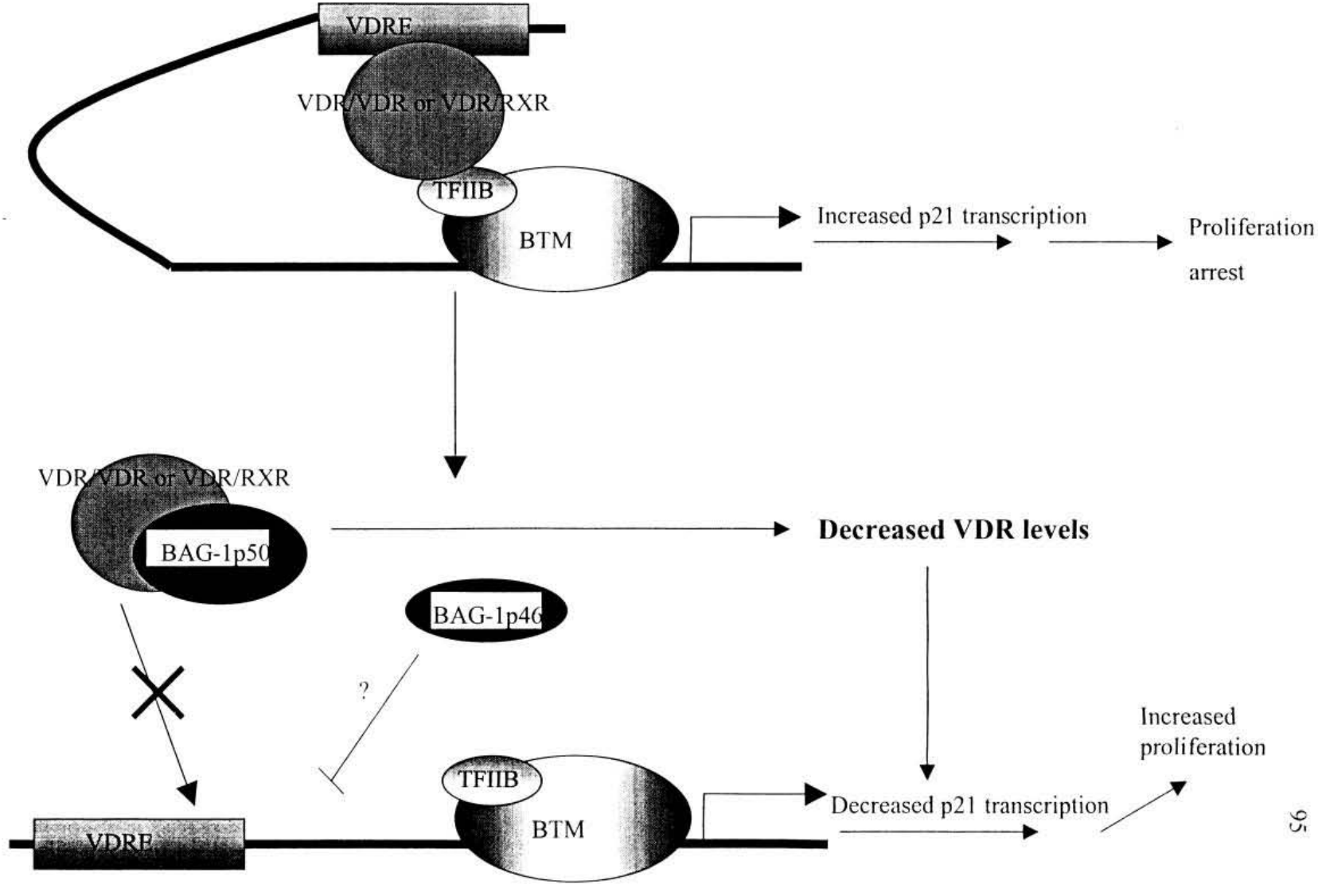
The experiments presented in this thesis, show that BAGp46 can transrepress the cell cycle inhibitor $p21^{waf1}$ (Fig. 3.13 and 3.14). The exact mechanism of this repression remains unknown. BAGp46 has previously been reported to inhibit RAR transactivation (Liu *et al.*,

Fig 4.1 Possible mechainism of BAG-1 action

Upper figure demonstrates transcriptional activation by the VDR. The VDR is thought to bend the DNA of the promoter containing a VDRE enabling it to bind the TFIIB protein of the basal transcriptional machinery. This results an increase in the rate of transcription for such genes as $p21^{waf1}$.

The lower figure displays a proposed mechanism by which BAGp50 may inhibit vitamin D action. BAGp50 disables the ability of VDR to bind DNA and interact with TFIIB, thus preventing an increase in the rate of transcription. BAGp46 may complement the action of BAGp50 by down-regulating $p21^{waf1}$ transcription through an unknown mechanism.

Possible mechanism of BAG-1 action



1998). In addition, the $p21^{waf1}$ promoter region is known to contain an RARE and is transactivated through this motif by the RAR (Liu *et al.*, 1996). Therefore, it is possible that BAGp46 may inhibit the transactivation of $p21^{waf1}$ indirectly via inhibition of the RAR. Further experiments using various deletions of the $p21^{waf1}$ promoter region cotransfected with BAGp46 could confirm this theory. Also, further work should be performed using Northern blots (RNA from BAGp46 transfected cells probed for endogenous $p21^{waf1}$ expression) to further demonstrate that this transcription repression may have physiological relevance. Overall, these results also suggests that different isoforms of BAG-1 may work collaboratively to achieve a common goal (Fig. 4.1). Different isoforms of BAG-1 may be able to promote proliferation through regulation of the various steroid hormone receptors. BAG-1 may have evolved to make multiple isoforms so that it could regulate multiple hormone receptors simultaneously. It would be interesting to know if BAG-1 transcription is regulated by hormone receptors, since this would create a mechanism of receptor auto-regulation.

4.7 BAG-1 expression

BAG-1 was found to be expressed in a wide variety of tissue types (Fig. 3.10). It is difficult to draw conclusions as to the physiological function of BAG-1 from this expression pattern. BAG-1 was found at high levels in several hormone responsive tissues, such as the prostate, testis, thyroid and adrenal gland indicating that BAG-1 may play a role in hormone signal transduction in these tissues. A good model for this would be in glands such as the prostate, in which cell growth can be inhibited by 1,25-vitamin D3 (Zhuang and Burnstein

1998) and enhanced by androgens (Schuurmans *et al.*, 1991). It is possible that BAGp50 may simultaneously enhance AR function (Froesch *et al.*, 1997) and suppress VDR function. In this manner, cells could regulate hormone pathways, at least in part, by controlling BAG-1 levels.

Of the four immune system-related components (peripheral blood leukocytes, thymus, lymph nodes and spleen) studied, BAG-1 was observed at very low levels in peripheral blood leukocytes and the thymus, with lymph node tissue being the only one with significant amounts of BAG-1 mRNA present. All of these tissues are known to be targets of glucocorticoid action with an end result of apoptosis in the white blood cells involved (Cohen 1992, Horigome *et al.*, 1997 and Wang *et al.*, 1999). The thymus has recently been found to express the GR at a level four times higher than splenic immune tissue. BAG-1 protein levels have been reported to be controlled by glucocorticoids (Clevenger *et al.*, 1997). Our results and the results of past studies indicate the pertinence of this regulation occurring *in vivo*.

CHAPTER 5

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