Forschungszentrum Karlsruhe
in der Helmholtz-Gemeinschaft

Wissenschaftliche Berichte
FZKA 6953

Molecular Chaperones and Cochaperones as Regulators of the Transcriptional Activity of the Glucocorticoid Receptor

W. Hong

Institut für Toxikologie und Genetik

Dezember 2003

Forschungszentrum Karlsruhe

in der Helmholtz-Gemeinschaft

Wissenschaftliche Berichte

FZKA 6953

Molecular Chaperones and Cochaperones as Regulators of the Transcriptional Activity of the Glucocorticoid Receptor

Wei Hong

Institut für Toxikologie und Genetik

Von der Fakultät für Chemie und Biowissenschaften

der Universität Karlsruhe (TH) genehmigte Dissertation

Forschungszentrum Karlsruhe GmbH, Karlsruhe

2003

Impressum der Print-Ausgabe:

Als Manuskript gedruckt

Forschungszentrum Karlsruhe GmbH
Postfach 3640, 76021 Karlsruhe

Mitglied der Hermann von Helmholtz-Gemeinschaft
Deutscher Forschungszentren (HGF)

ISSN 0947-8620

Molecular Chaperones and Cochaperones as Regulators of the Transcriptional Activity of the Glucocorticoid Receptor

Zur Erlangung des akademischen Grades eines
Doktors der Naturwissenschaften
an der Fakultät für Chemie und Biowissenschaften
der Universität Karlsruhe (TH)
genehmigte

DISSERTATION

von

Wei Hong

aus Tianjin, China

2003

Dekan: Prof. Dr. M. Metzler

1. **Gutachter:** Prof. Dr. Andrew C. B. Cato
2. **Gutachter:** PD. Dr. Armin Huber

Tag der mündlichen Prüfung: 05.12.2003

Abstract

Glucocorticoids play important roles in anti-inflammation, immnunosuppression and induction of apoptosis. Glucocorticoids function via binding to the glucocorticoid receptor (GR) which is localized in the cytoplasm of glucocorticoid target cells. The action of the GR is modulated by molecular chaperones and cochaperones that interact with the aporeceptor to provide the correct conformation for hormone binding. Recent studies suggest that the molecular chaperones and cochaperones may also act in the nucleus to modulate the transcriptional activity of the GR. Bag-1M, a member of the Bag-1 family of cochaperones, is transported by the GR from the cytoplasm into the nucleus where it inhibits the transcriptional activity by the receptor, but does not affect the action of the structurally related mineralocorticoid receptor. However the mechanism by which it inhibits the action of the receptor is not known.

In this study, the modes of action of Bag-1M in inhibiting GR-mediated transactivation have been analyzed. The hinge region of the GR was found to be essential for Bag-1M-mediated repression of the transactivation by the receptor. Bag-1M binds to lysine 496 and isoleucine 497 in the hinge region of the GR to inhibit DNA binding by the receptor in a process that requires ATP hydrolysis. Using a combination of siRNA and chromatin immunoprecipitation techniques, it has also been demonstrated that inhibition of the DNA binding by the receptor is one of two ways by which Bag-1 negatively regulates GR action. The second mechanism occurs through the recruitment of histone deacetylases 1 and 2 to glucocorticoid regulated genes to repress their action once the GR had bound Bag-1M. This action of Bag-1M is cell type specific, since it was observed in kidney cells but not cervical or hepatoma cells.

The negative regulation of GR activity may therefore be relevant in kidney cells that possess both glucocorticoid and mineralocorticoid action where the action of the glucocorticoid needs to be downregulated for the mineralocorticoid receptor to function.

Molekulare Chaperon und Cochaperon als Regulatoren der Transkriptionellen Aktivität des Glukokorticoid Rezeptors

Zusammenfassung

Glukokortikoide spielen eine wichtige Rolle bei der Hemmung von Entzündungsreaktionen, Immunsuppression und Induktion von Apoptose. Diese Eigenschaften werden durch den Glukokortikoidrezeptor (GR) vermittelt, der im Cytoplasma der Zielzellen lokalisiert ist. Die Wirkung des Glukokortikoidrezeptors kann durch molekulare Chaperone und Cochaperone moduliert werden. Die simple Interaktion dieser Proteine mit dem Aporezeptor gewährleistet die korrekte Konformation des Rezeptorproteins, welche Vorraussetzung für die Liganden-Bindung ist, wobei der Einfluss der Chaperone auf die transkriptionelle Kontrolle durch den GR auf einer mehr aktiven Rolle basiert.

Das Bag-1 Protein, ein Mitglied der Bag-1 Chaperon-Familie, wird mit dem GR aus dem Cytoplasma in den Zellkern transportiert, wo es die transkriptionelle Aktivität von GR-regulierten Genen hemmt. In dieser Arbeit wurde die Wirkungsweise untersucht, die der Hemmung der GR-vermittelten Transaktivierung durch das Bag-1M-Protein zugrunde liegt.

Die hinge Region des Glukokortikoidrezeptors ist für die Bag-1M-vermittelte Repression der GR-abhängigen Transaktivierung essentiell. Das erste Ergebnis dieser Arbeit zeigte, dass Bag-1M mit den Aminosäuren Lysin 496 und Isoleucin 497 in der hinge Region des GR interagiert und dabei die Fähigkeit des Rezeptors an die DNA zu binden in einem ATP-abhängigen Prozess inhibiert. Unter Verwendung von siRNA-Techniken und Chromatin-Immunopräzipitation-Analysen wurde gezeigt, dass Bag-1M GR-regulierte Gene in negativer Weise kontrolliert. Darüber hinaus wurde betätigt, dass die Beeinträchtigung der DNA-Bindung des Rezeptors ein Mechanismus ist, durch den Bag-1M die Wirkung des GR negativ reguliert. Die zweite Entdeckung demonstrierte, dass Bag-1M, im Anschluss an die Bindung des GR Bag-1M, Histon-Deacetylasen zu Glukokortikoid-regulierten Genen rekrutiert, um so deren transkriptionelle Aktivität zu hemmen. Diese Wirkung ist jedoch zelltypspezifisch. Demnach erfolgt die Hemmung der GR-

abhängigen Transaktivierung durch Bag-1M nach zwei unterschiedlichen Mechanismen.

Die biologische Bedeutung dieser Ergebnisse wurde erörtert, wobei postuliert wurde, dass es sich hierbei um ein spezifisches Phänomen kortikosteroid (glukokortikoider und mineralokortikoider) Wirkung handelt. Sowohl der GR als auch der Mineralokortikoidrezeptor (MR), können durch Glukokortkoide aktiviert werden, obwohl sie unterschiedliche Funktionen besitzen. In Geweben, die beide Rezeptorproteine aufweisen, ist ein Weg zwischen den Rezeptortypen zu unterscheiden, die Aktivität des eine, jedoch nicht des anderen Rezeptors zu herabzusetzen. Dies scheint die Aufgabe des Bag-1M Proteins zu sein, da es ausschließlich die Wirkung des GR und nicht die des MR hemmt.

TABLE OF CONTENTS

1. Introduction

1.1. Glucocorticoid and Glucocorticoid Receptor

Glucocorticoids are steroid hormones that are involved in diverse physiological processes in vertebrates. Produced and secreted by the adrenal cortex, levels of circulating glucocorticoids are regulated by the adrenocorticotrophic hormone (ACTH) largely under the control of the hypothalamic-pituitary-adrenal axis (HPA). Glucocorticoids are named for their role in glucose homeostasis, they are also important throughout physiology, with regulatory roles in development, metabolism, proliferation, anti-inflammation and programmed cell death. In addition to these physiological roles, glucocorticoids have been clinically used as therapeutic agents since it was initially used in the treatment of rheumatoid arthritis (Hench et al, 1949). The pharmacological benefits of glucocorticoids are primarily anti-inflammatory and immunosuppressive, for instance, in the treatment of rheumatoid arthritis (Hench, 1949), asthma (Walsh and Grant, 1966) and allergy (Sarkany et al., 1965). Glucocorticoids are also therapeutically used in leukemias, lymphomas and cancers due to their critical role in the induction of apoptosis (Robertson et al, 1978; Dutta et al, 1989). The physiological response and sensitivity to glucocorticoids varies among species, individuals, tissues, cell types and even during the cell cycle (Hsu and DeFranco 1995; Lim-Tio SS et al., 1997). Long period of high doses of glucocorticoid administration exerts a wide variety of side effects such as osteoporosis, aseptic osteonecrosis, diabetes, psychotic disterbances and atrophy of the skin (Capewell et al, 1990; Abma et al, 2002; Dendukuri et al, 2002; Ismail et al, 2002; McLaughlin et al, 2002). Additionally, pathological conditions can result in glucocorticoid resistance (Kino and Chrousos, 2001; Hillmann et al., 2000; Bronnegard et al., 1996; Bamberger et al., 1996; Werner and Bronnegard, 1996).

Glucocorticoids reach their target cells through the blood. They enter into the cells by free diffusion due to their lipophilic character. Within the cells,

glucocorticoids function by binding to glucocorticoid receptor (GR) which is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors. In the absence of hormone, GR localizes in the cytoplasm in an inactive form. Upon binding hormone, GR translocates into the nucleus and binds the promoter of glucocorticoid responsive gene to mediate the transcription of the gene.

Several discrete domains of the GR have been identified including the N-terminal transactivation function 1 (AF1) domain, the DNA-binding domain (DBD), the hinge region and the ligand-binding domain (LBD) (Wrange and Gustafsson, 1978; Carlstedt-Duke et al., 1982,) (Fig 1.1). The N-terminal AF1 domain is the most divergent region and functions as a hormone-independent transactivation domain. The DBD, the most conserved region of GR, comprises two zinc fingers responsible for recognizing specific glucocorticoid response element (GRE) and high affinity of DNA binding (Weinberger et al., 1985). The DBD is also involved in the dimerization of the receptor. The hinge region, named for its localization between the DBD and the LBD, functions in modulation of DNA-binding activity and harbors nuclear localization signals. The carboxy-terminal LBD domain is a multifunctional domain that, in addition to the binding of ligand, mediates homodimerization, interaction with heat shock proteins, nuclear localization and ligand dependent transactivation.

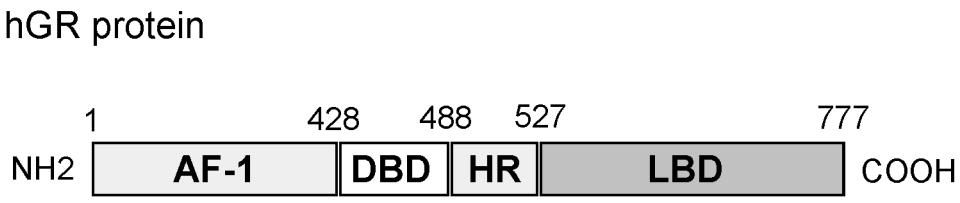


Fig 1.1 Domain structure of the human glucocorticoid receptor. Schematic diagram of the hGR represents the N-terminal AF-1 domain which functions as a hormone independent transactivation domain, the DNA binding domain (DBD), the hinge region and the ligand binding domain harboring hormone dependent transactivation function.

1.2. Regulation of gene expression by glucocorticoid

1.2.1 Positive regulation of gene expression by glucocorticoid

The GR in its unliganded state is located primarily in the cytoplasm associated with heat shock proteins (Joab et al, 1984; Catelli et al, 1985; Denis et al, 1988), and molecular cochaperones, such as p23 and Hop (Hsp70 and Hsp90 organizing protein) (Beato and Sanchez-Pacheco, 1996). After binding to its ligand, the GR undergoes conformational change, dissociates from the heat shock proteins, homodimerizes, and translocates into the nucleus where it binds to glucocorticoid response element (GRE) in the promoter region of target genes. GRE is an inverted repeat (corresponding to the consensus sequence AGAACAnnnTGTTCT, n is any nucleotide) of a hexameric nucleotide sequence, spaced by three base pairs (Scheidereit et al, 1983).

Once localized to the promoter, the receptor mediates transcriptional activation of the target gene by interaction with general transcription factor and by recruitment of transcriptional coactivators. Coactivators are molecules that form either a functional or physical bridge between the receptor and components of the basal transcription machinery that make up the constitutive core of the RNA polymerase II complex. Studies showed that coactivators including SRC-1 (steroid receptor coactivator 1) (Kurihara et al, 2000; Kucera et al, 2002), p300 (Kino et at, 1999) and GRIP-1 (glucocorticoid receptor interacting protein 1) (Eggert et al, 1995) stimulate GR-mediated transcription. The structure of coactivators and their manner of transcriptional activation have been studied (Horwitz et al., 1996; Darimont et al., 1998; McKenna et al., 1999; Freedman, 1999; Glass and Rosenfeld, 2000; Robyr et al., 2000). SRC-1 and GRIP-1 belong to a p160 family of coactivators because of their molecular mass of 160 KDa. Both of these coactivators interact with nuclear receptors in a highly ligand dependent manner (Cavailles et al, 1994; Halachmi et al, 1994; Kurokawa et al, 1995). SRC-1 and GRIP-1 possess a nuclear receptor interacting domain in their central region. This domain contains three highly conserved LXXLL motifs, where L represents leucine and X is any amino acid, and are necessary and sufficient to mediate

association of coactivators to ligand-bound receptors (LeDouarin et al, 1995; Ding et al, 1998; Heery et al, 1997; Torchia et al, 1997; Voegel et al., 1998). Analysis of the LXXLL motifs has revealed that they form amphipathic □-hilices with the leucines forming a hydrophobic surface on one face of the helix (Nolte et al., 1998; Shiau et al., 1998). The structure of these motifs is necessary for the interaction with helix 12 in the LBD of the receptors, which is required for ligand-dependent interaction with coactivators, and also forms an amphipathic □-helix. It is conceivable that these motifs have evolved to provide a critical mode of assembling the ligand-dependent nuclear receptor-coactivator complexes.

SRC-1 and GRIP-1 have been reported to harbor intrinsic histone acetyltransferase (HAT) activity and modulate chromatin (Spencer et al, 1997; Chen et al, 1997). They can also associate with other HAT-containing proteins such as CBP/p300 (Kamei et al, 1996; Yao et al, 1996).

An important coactivator that bears no sequence homology to p160 family members is CREB-binding protein (CBP). A number of studies have implicated CBP as a coactivator for a wide variety of transcription factors including GR. Apart from CBP, p300 represents another cointergrator molecule that bears a high degree of sequence and functional homology to CBP. Like p160 family members, both CBP and p300 possess HAT activity (Bannister and Kouzarides, 1996; Ogryzko et al., 1996), which likely contributes to its ability to stimulate receptor-mediated transcription.

These coactivators interact with the GR in a ligand-depended manner, individually or together, and thereby activate GR-mediated transcription, at least in part, through histone acetyltransferase-mediated nucleosome remodeling (Ogryzko et al., 1996). This in turn allows receptor/coactivator–mediated recruitment of the basal transcriptional machinery into a functional preinitiation complex.

In addition to binding to the unique GRE of glucocorticoid responsive genes and stimulating the transcription of these genes, GR can bind as well to DNA elements that consist of a non-overlapping GRE and a binding site for a different transcriptional factor (composite GREs) and enhances the transcription of the gene modulated by the different transcription factor. For

example, in the proliferin gene promoter a GRE and a binding site for the transcription factor complex AP-1 form such a composite GRE. On this element, the GR can enhance AP-1-induced transactivation depending on the cell type and the composition of the AP-1 dimer (Diamond et al, 1990; Pearce et al, 1993; Harrison et al, 1995).

1.2.2 Negative regulation of gene expression by glucocorticoid

Besides transactivating gene transcription, GR is also able to regulate gene expression negatively, which is termed transrepression. One way of the GR to inhibit gene expression is to bind to a DNA sequence called negative GRE (nGRE) in the promoter of specific genes. (Drouin et al, 1993; Saatcioglu et al, 1994; Lefstin and Yamamoto 1998). nGREs have been described for few genes. Well characterized nGREs include the binding elements in the promoters of the pro-opiomelanocortin (POMC) (Drouin et al., 1993), osteocalcin (Morrison et al., 1993), and bovine prolactin genes (Sakai et al., 1988; Subramaniam et al., 1997). A comparison of GR response elements showed that GREs and nGREs have different sequences (Beato et al., 1989). Although the sequence of the nGREs are too heterogeneous to determine a consensus sequence, binding of GR presumably replaces or prevents binding of a transcription factor that would induce transcription (Drouin et al, 1989; Nakai et al, 1991). Thus,it has been suggested that not only ligands but also the different response elements can act as an allosteric effector for the nuclear receptor (Lefstin and Yamamoto, 1998).

Studies of the anti-inflammation role of the GR have demonstrated that in addition to the proper function as a DNA-binding transcription factor, the GR has been found to modulate other transcription factors, essentially without the need for DNA binding. This was named “cross-talk”. GR modulates negatively, without DNA contact, the activities of AP-1 (Jonat et al., 1990; Schüle et al., 1990; Yang-Yen et al., 1990). The GR probably inhibits AP-1 by direct protein-protein interaction and this interaction appears to be mutual, and is not mediated by competition for coactivators CBP (De Bosscher et al,

2001). In addition, activated GR is able to repress the activity of the transcription factor NF-kB (Caldenhoven et al, 1995; Ray and Prefontaine, 1994; Scheinman et al, 1995; McKay and Cidlowski, 1998). The GR inhibits the activity of the p56/RelA subunit, with which it can physically interact. The interaction of the GR with AP-1 and NF-kB is particularly interesting as these transcription factors control the expression of most proinflammatory cytokines and chemokines genes. The negative regulation of the action of AP-1 and NF-kB has therefore turned out to be a paradigm for the anti-inflammatory action of glucocorticoids (Beato et al., 1995; Cato and Wade, 1996; Barnes, 1998; Göttlicher et al., 1998).

1.3 Modulation of the action of GR by molecular chaperones and cochaperones

1.3.1 Chaperones and cochaperones in the maturation of GR

Glucocorticoid receptor exists in the absence of hormone in target cells in an inactive form in the cytoplasm. Maintenance of the inactive state is brought about by a large group of nuclear receptor associated proteins: the chaperones and cochaperones. Chaperones are a group of highly conserved proteins that bind to and orchestrate the folding of other proteins, in association with cochaperones that dictate the affinity of the chaperone for its substrate (Hartl 1996; Morimoto 1998; Luders 1998). The chaperones have ATP dependent protein folding activity the cochaperones have only supporting or regulating functions. The most important chaperones for nuclear receptors are the heat shock proteins Hsp70 and Hsp90. A typical mature aporeceptor complex capable of hormone binding contains a Hsp90 dimer, a Hsp70 monomer, a p23 monomer, Hop (Hsp70 and Hsp90 organizing protein) and Hip (Hsp70 interacting protein) (Buchner 1999; Pratt & Toft 1997). Upon hormone binding the receptor is thought to dissociate from the multiprotein chaperone complex and is translocated into the nucleus where it binds specific DNA sequences. Chaperones and cochaperones are therefore presumed to exert an exclusive cytoplasmic function in the maintenance of

the receptor in an inactive state. Recent findings, however, deviate from this notion and demonstrate that some molecular chaperones are recruited to the nucleus or exist in nuclear isoforms and may have a nuclear function downstream of hormone binding. For instance, Hsp70 and Hsp90 have been found colocalized or complexed with nuclear receptors in the nucleus (Landel et al, 1994; DeFranco et al, 1998; Meng et al, 1996). Members of a family of cochaerones termed Bag-1 possess the ability of regulating nuclear receptor functions downstream of hormone binding in the nucleus (Schneikert et al, 1999). These proteins additionally inhibit apoptosis induced by a variety of reagents including glucocorticoids. Thus they may serve as molecular links between steroid hormone action and apoptosis.

1.3.2 Bag-1 proteins

Bag-1 was first identified as a bcl-2 –binding protein from the mouse embryo cDNA library in a phage expression vector screen with recombinant human Bcl-2 protein (Takayama et al, 1985). Its interaction with bcl-2 gave it the name Bag-1 (Bcl-2associated athanogene-1). A human protein of 46 KDa with high homology to Bag-1 was identified in an interaction screening of a liver cDNA expression library with the liganded GR (Zeiner & Gehring 1995). A slightly smaller human cDNA encoding a protein homologous to Bag-1 was identified from a human fetal brain cDNA library (Takayama et al, 1996). Sequence analysis revealed an open reading frame and a predicted protein of 274 amino acids (34 KDa). Since the 5´ untranslated region of this mRNA contained no stop codon in any of the 3 reading frames, it has concluded that larger isoforms of this protein exist. This has since been confirmed by other investigators (Packham et al, 1997; Yang et al, 1998; Takayama et al, 1998). In human, the Bag-1 gene encodes four isoforms of the Bag-1 proteins, all of which are expressed through alternative translation initiation sites form the same mRNA. The largest isoform (Bag-1L) starts at a noncanonical CUG codon followed by inframe downstream AUG start sites giving rise to Bag-1M and Bag-1S(p33). However, p29, the smallest isoform is thought to be a post-

translational product because there is no corresponding AUG found within the open reading frame (Doong et al, 2002).

Various domains have been identified within Bag-1 proteins (Fig 1.2). Bag-1L harbors a unique nuclear localization signal (NLS) that results in a nuclear localization of this protein. All Bag-1 isoforms contain a carboxy-terminal " BAG domain " which plays a key role in mediating many Bag-1 functions. The BAG domain consists of two anti-parallel alpha-helices that mediate interaction with Hsp70/Hsc70 (Sondermann et al, 2001; Briknarova et al, 2001) and regulates the chaperone function of Hsp70 (Hohfeld 1998). Bag-1 also interacts with the serine/threonine kinase Raf-1, which is normally activated by Ras to stimulate the mitogen-activated protein kinase (MAP) signaling pathway. The interaction of Bag-1 and Raf is Ras independent (Song et al, 2001). Thus, Bag-1 overexpression provides a potential mechanism by which tumors lacking oncogenic Ras mutations might activate MAP kinase cascade mediated proliferation and survival. Bag-1 contains a ubiquitin-like domain (UBL) with which it interacts with the E3 ligase CHIP (Carboxyl terminus of Hsc70 interacting protein) and Siah-1 and stimulates CHIP-mediated ubiquitination of substrates (Demand et al, 2001).

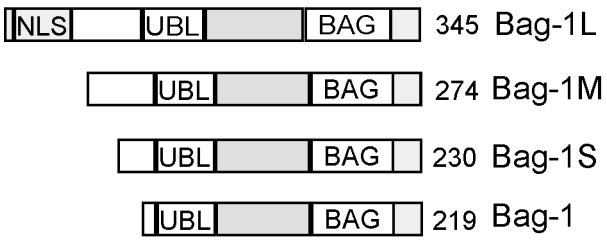


Fig 1.2 Human Bag-1 family proteins. Schematic diagram of Bag-1 proteins represents the conserved carboxy-terminal BAG domain, the ubiquitin like domain and a nuclear localization domain in Bag-1L.

Bag-1 proteins have been found expressed in various tissues (Takayama et al, 1998). Overexpression of Bag-1 was found to suppress activation of apoptosis induced in different cell types (Hohfeld, 1998; Takayama and Reed, 2001) and to enhance cell migration (Naishiro et al, 1999). Suppression of apoptosis may be responsible for the ability of Bag-1 to promote metastatic spread (Takaoka et al, 1997; Yawata et al, 1998).

1.3.3 Bag-1 proteins regulate the nuclear receptors

The other function of Bag-1 of likely importance for cancer is regulation of nuclear receptors. Bag-1 isoforms have different effects on the action of steroid receptors. Bag-1 downregulates the activity of the vitamin D3 receptor (VDR) (Witcher et al, 2001) and represses the activity of the retinoic receptor heterodimer (RAR/RXR) but not the homodimers (RXR/RXR) (Liu et al, 1998). Regulation of nuclear receptors is frequently specific for the Bag-1L and Bag-1M proteins. The androgen receptor (AR) is important in prostate cancer and both the Bag-1L and Bag-1M increase sensitivity of AR expressing cells to androgen and decrease sensitivity to anti-androgen clinically used in the treatment of prostate cancer (Froesch et al, 1998). Bag-1M downregulates transactivation function of the GR (Schneikert et al, 1999; Kullmann et al, 1998) and the progesterone receptor (PR) (Schneikert and Cato, unpublished observations) but not the mineralocorticoid receptor (MR) (Schneikert et al, 1999). It has been shown that Bag-1M could be transported from cytoplasm into the nucleus by the liganded GR and this process needs the involvement of Hsp70. Bag-1M utilizes the Bag domain to bind the ATPase domain of Hsp70. The hinge region of the GR was also found to be essential for the binding of Bag-1M to GR (Schneikert et al, 1999; Kullmann et al, 1998). Thus, the effect of Bag-1 on glucocorticoid receptor require the BAG domain and is likely to involve chaperone molecules which are known to be important for nuclear receptor function. The effects on nuclear receptor may be partly through Bag-1 triggered conformational changes mediated by the heat shock proteins which may be essential for altering hormone binding capacity. However, this is not the only case for nuclear receptors, as for the example

the Hsp70 binding region within the C-terminus of Bag-1 is not required for binding to the RAR (Cato and Mink, 2001). Biochemical and mutagenic studies therefore suggest that there may not be a single mechanism to account for Bag-1-mediated regulation of nuclear receptor activity (Cato and Mink, 2001).

Aim

The aim of this study was based on the previous discovery of the laboratory, i.e. Bag-M downregulates the transactivation activity of glucocorticoid. Glucocorticoid, which functions via binding to the glucocorticoid receptor localized in the cytoplasm of the target cells, is involved in a variety of physiological regulations and is frequently prescribed in the clinical treatment of pathological disorders, for example, cancers. Bag-1M was found overexpressed in neoplastic cells and in turn inhibits the transactivation function by the GR. Bag-1M, however, has no effect on the closely related MR although both receptors can be functionally activated by glucocorticoid. Thus, investigation of the mechanism by which Bag-1M inhibits the transactivation activity by the GR may elucidate one of the reasons of glucocorticoid resistance and may feature prominently in the control of GR action in the mineralocorticoid target tissues.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals were of the highest quality, and were purchased from:

Acetic Acid	Merck, Darmstadt
Agarose	Peqlab, Erlangen
Ammonium persulfate	Sigma, Deisenhofen
Ampicillin	Roche Diagnostics, Mannheim
Bacterial Petri dishes	Greiner Labortechnik, Nürtingen
Bacto-Agar	Roth, Karlsruhe
Bacto-Yeast Extract	Roth, Karlsruhe
Bacto-Peptone	Roth, Karlsruhe
2- Mercaptoethanol	Roth, Karlsruhe
Bromophenol blue	Roth, Karlsruhe
Chloroform	Merck, Darmstadt
Coomassie Brilliant Blue R250	Serva, Heidelberg
Dexamathasone	Schering AG, Berlin
2-Deoxyglucose	Sigma, Deisenhofen
Dithiothreitol	Sigma, Taufkirchen
Dulbecco´s modified Eagle´s medium	Gibco BRL Life Trch., Eggenstein
DMSO (Dimethyl sulfoxide)	Fluka, Neu-Ulm
Easy Pure DANN-Elution Kit	Biozym GmbH, Hess. Oldendorf
ECL™ Western blotting detection reagents	Amersham Pharmacia Biotech, Freiburg
EDTA	Merck, Darmstadt
Ethanol	Roth, Karlsruhe
Ethidium bromide	Sigma, Taufkirchen
Fetal bovine serum (New Sealand)	Gibco BRL Life Tech., Eggenstein

Filter paper 3MM	Bender & Hobein, Karlsruhe
Fish sperm-DNA	Sigma, Taufkirchen
Formaldehyde	Merck, Darmstadt
FuGene6 transfection kit	Roche Diagnostics, Mannheim
Glycerol	Merck, Darmstadt
D-Glucose	Roche Diagnostics, Mannheim
HCl	Merck, Darmstadt
Luciferase-Luciferin	Sigma, Taufkirchen
Methanol	Roth, Karlsruhe
Mount Medium	Shandon, Pittsburg
Nonidet P-40	Boehringer, Mannheim
Passive lysis Buffer (5x)	Promega
PMSF	Sigma, Taufkirchen
2-Propanol (Isopropanol)	Merck, Darmstadt
Qiagen DNA purification Kit	Qiagen GmbH, Hilden
QIAquick PCR purification Kit	Qiagen GmbH, Hilden
Rotiphorese Gel30: Acrylamide/bis-acrylamide (30% / 0.8%)	Roth, Karlsruhe
Sodium chloride	Roth, Karlsruhe
SDS (Sodiumdodecylsulfate)	Roth, Karlsruhe
Sodium hydroxide	Roth, Karlsruhe
Skimmed milk powder	Saliter, Obergünzburg
TEMED	Bio Rad Laboratories GmbH, München
Tris	Roth, Karlsruhe
Tris Hydrochloride	Roth, Karlsruhe
Triton-X100	BioRad, Müchen
Tween-20	Roth, Karlsruhe

All other chemicals were, unless otherwise stated, purchased from Carl Roth GmbH+Co (Karlsruhe, Germany), Merck AG (Darmstadt, Germany), Sigma Aldrich (Taufkirchen, Germany), Roche Diagnostics (Munich, Germany). All radiochemicals were supplied by Amersham Pharmacia Biotech (Freiburg, Germany). All antibiotics were obtained from Roche Diagnostics (Munich, Germany).

2.1.2 Oligonucleotides

2.1.2.1 DNA oligonucleotides

All DNA oligonucleotides were, unless otherwise stated, synthesized by MWG biotech GmbH (Ebersberg, Germany) with HPLC-purified grade.
Oligonucleotides for the GR mutations:
5'-GCT CGA AAA ACA AAG AAA CTA GGA AAA GGA ATT CAG CAG GCC AC-3' (K496L and I497G for)
5'-GTG GCC TGC TGA ATT CCT TTT CCT AGT TTC TTT GTT TTT CGA GC-3' (K496L and I497G rev)
5'-GGA AAG CAG CAG GCC GAG ACA GGA GTC TCA CAA G-3' (I500K and T504E for)
5'-CTT GTG AGA CTC CTG TCT CGG CCT GCT GCT TTC C-3' (I500K and T504E rev)
Oligonucleotides used for chromatin immunoprecipitation:
5´-CCG GTT ACT GTG ATG CTG CA-3´ (hMTIIa for)
5´-GCG GGA GGA CAC AGT GTA CC-3´ (hMTIIa rev)
5´-GGC CTA TTT CCC ATG ATT CC-3´ (snRNA for)
5´-ATT TGC GTG TCA TCC TTG C-3´ (snRNA rev)
5´-CAT AAA TAA CAG GAA GCC CAA GGT T-3´ (TAT for)
5´- ACC CAG AAA CCG ACA GGC GAC T-3´ (TAT rev)
5´-TCT GAG CCT GTT GTT GGC CCA-3´ (5'-flanking for)
5´-TGT TCT AGC AGG ATT TGT CTG TG-3´ (5'-flanking rev)
Oligonecleotides used for Real-time PCR:
5´-TCG GAT ACG TCA TCA GCA CC-3´ (hMTIIa for)

5´-TCC CTC CTG TCC TGT ACT CGA-3´ (hMTIIa rev)
5´-TCA CCC ACA CTG TGC CCA T-3´ (human β-actin for)
5´-CTC TTG CTC GAA GTC CAG GG-3 (human β-actin rev).
5´- CCA ATG TTC CCA TCC TGT CC-3´ (TAT for)
5´-GGG TTC GCT GAA GGA TGC T-3´ (TAT rev).
5´-GCC ATC CAG GCT GTG TTG T-3 (Rat β-actin for)
5´-GGT GAA GCT GTA GCC ACG CT-3 (Rat β-actin rev)
Oligonucleotides used for RT-PCR:
5´-AAG AAG AAA ACC TGG CGC CGC TCG A-3 (Bag-1 for)
5´- TTC ACC CTG GGT CGC CTC TTC ACT-3 (Bag-1 rev).

2.1.2.2 RNA oligonucleotides

RNA oligonucleotide were synthesized by Dhamacon Reserch, Inc (Lafayette, CA) The specificity of siRNA was proven by BLAST-search (NCBI-program).
Bag-1 siRNA, 5'-AAG CAC GAC CUU CAU GUU ACC-3'

2.1.3 Plasmids and Constructs

Constructs with point mutatioins resulting in a double exchange of amino acids within the hinge region of the GR were obtained with the PCR-based QuickChange site-directed Mutagenesis Kit from Stratagene. Oligonucleotides used to obtain a lysine-leucine exchange at position 496 together with an isoleucine –glycine exchange at position 497 of the GR and to obtain exchanges from isoleucine to lysine at position 500 and threonine to glutamic acid at position 504 of the GR are described in section 2.1.2.1. The mutagenesis was performed according to the manufacture´s instructions (Stratagene, TX). Mutations were confirmed by nucleic acid sequencing.
pGL3MMTV encodes the firefly luciferase gene under the control of the mouse mammary tumour virus (MMTV) long terminal repeat cloned as a BamHI/BglII fragment from the plasmid pHCwtCAT (Kaspar et al., 1993).
pTOPO TA plasmids were generated in the pCR2.1 vector from the TOPO TA cloning kit (Invitrogen, Munich, Germany). A PCR product encompassing part

of the coding region of the gene of interest was generated using the appropriate forward and reverse oligonucleotides and cloned in the pCR2.1 TA vector following the manufacturer's instructions. The identity of the inserts was ascertained by sequencing. pRenillaluc was obtained from Promega (Mannheim, Germany).

PSG5Bag-1M has been described by Schneikert et al. (1999)

PGex4T, pGex-2T-Bag-1M, pGex-2T-Bag-1MΔC47, pGex-2T-Bag-1MΔC47ΔN71, pGex-2T-Bag-1MΔN131, pGex-2T-Bag-1MΔN71 were described and constructed by Schneikert et al. (2000).

pRshGR and pBK7GR have previously been reported by Schneikert et al (1999).

pBATGRI500K/T504E and pBATGRK496L/I497G were constructed by inserting a KpnI/DraI fragments of the pSG5GRI500K/T504E and pSG5K496L/I497G into the KpnI/SmaI site of pBAT vectors.

The hMTIIa reporter plasmid H1S CAT was generously provided by M. Karin.

The pCIneoHDAC-1 and pCMVHDAC-2 were obtained from W.M. Young and T. Heintzel respectively.

2.1.4 Enzymes

All restriction enzymes and other enzymes were purchased from Invitro GmbH (Karlsruhe, Germany), Promega (Mannheim, Germany) or Roche (Mannheim, Germany).

2.1.5 Antibodies

2.1.5.1 Primary antibodies

The following antibodies were used for immunoblotting: GR antibody (PA1-512, Affinity Bioreagents INC, CA), monoclonal GR antibody (NCL-GCR, Novocastra Lab Ltd, UK), Bag-1 antibody (C-16, Santa Cruz, CA), Actin antibody (I-19, Santa Cruz) and Hsp-70 antibody (K-20, Santa Cruz). For ChIP assay, GR (P-20), Bag-1 (FL-274), Hsp70 (W-27), Hsp90 (H-114), NcoR (H-

303), SMRT (C-19), HDAC1 (H-51), HDAC2 (C-19), CBP (A-22), SRC1 (M-341) antibodies and rabbit IgG, goat IgG and mouse IgG (all from Santa Cruz) were used.

2.1.5.2 Secondary antibodies

All the HRP conjugated goat anti mouse IgG, goat anti rabbit IgG, rabbit anti goat Ig G were purchased from DAKO, Glostrup, Denmark.

2.1.6 Cell lines

All culture media and other reagents for cell culture were, unless otherwise indicated, purchased from Invitrogen GmbH (Karlsruhe, Germany). All media were supplemented with 100U/ml penicillin and 100 mg/ml streptomycin. Trypsin was purchased from Difco Laboratories (Detroit, USA) and was diluted to 0.25% in 15 mM sodium citrate, 134 mM potassium chloride prior to use.
COS-7 siman kidney cells belongs to the cell stock of Prof. Cato´s lab.
Human HeLa cells belongs to the cell stock of Prof. Cato´s lab.
Rat FTO cells belongs to the cell stock of Prof. Cato´s lab.

2.1.7 Bacteria

E.coli DH5□: supE44□lacU169(□80lacZ□M15)hsdR17recA1 endA1 gyrA96 thi-1 relA1, belonged to the cell bank of ITG.

TOP10F´: F^-mcrA □(mrr-hsdRMS-mcrBC) □lacX74(□80lacZ□M15) recA1 endA1 deoR araD139 □(ara-leu)7697 galU galK rpsL (Str^R) nupG, were part of the TOPO TA cloning kit from Invitrogen (Groningen, The Netherlands).

E. coli Xl-1 blue: F^-Tn10proA+b+$lacI^q$, d(lacZ)M15, recA1, endA1, gyrA96, (Nal^r), thi, hsdR17 (r_K^-,m_K^+), supE44, relA1, lac, belonged to the cell bank of ITG.

E. coli BL21 (DE3) pLysS: F^-; ompT; $hsdS_B$; (r_B^-,m_B^-); dcm; gal; (DE3); pLysS (Cm^r), bacterial strain for expresion of the GST-fusion proteins, belonged to the cell bank of ITG.

2.1.8 Other materials

Glutathione Sepharose 4B beads and protein A agarose beads were bought from Amershem Pharmacia, Uppsala, Sweden.

2.2 Methods

The majority of protocols and recipes for commonly used buffers in this work were taken from Molecular Cloning (Maniatis et al.,1989) and Current Protocols in Molecular Biology (Ausubel et al., 1989) unless otherwise stated.

2.2.1 Small scale plasmid preparation from bacteria (Modified Qiagen MaxiPrep kit)

Single bacterial colony was picked, inoculated in 3 ml of LB medium (10g Tryptone, 5g Yeast extract and 10g Nacl for Ll) containing the appropriate antibiotics and grown for 6h at 37°C. 1.5 ml of the culture was transferred to a new vial and the bacteria were pelleted by centrifugation at 13,000 rpm for 30 sec and resuspend in 150 □l of P1 buffer (10mM EDTA, 50mM Tris-HCl , pH 8 and 0.4 mg/ml Rnase A). The bacteria were then lysed by addition of 300□l of P2 buffer (200mM NaOH and 1% SDS), and mixed with 225□l of P3 buffer subsequently. The lysate was kept on ice for 15 min and was then centrifuged at 13,000 rpm for 10 min. The supernatant containing the plasmid DNA was transferred and precipitated with 2.5 volumes of ethanol. The plasmid DNA was collected by centrifugation at 13,000 rpm for 10 min and was subsequently washed with 70% ethanol. The air-dried pellet was finally resuspended in 50□l TE buffer.

2.2.2 Large scale plasmid preparation

A 300ml overnight culture of bacteria transformed with a plasmid was used for the Maxi-preparation using the Qiagen Plasmid Maxi Kit following the manufacture's instructions.

2.2.3 Restrication endonuclease digestion of DNA

1-2 units of restriction enzyme were used for every μg plasmid DNA. The reactions were carried out according to the instructions of the manufactures. The quality of the digest was determined by the gel electrophoresis.

2.2.4 Size separation of nucleic acid by agarose gel eletrophoresis

Given amounts of agarose were dissolved in TAE buffer (0.04M Tris pH 7.2, 0.02M Sodium acetate, 1mM EDTA). Ethidium bromide was added at a concentration of 0.3 μg/ml. The gel was set in horizontal electrophoresis chambers and was run at 35-45 mA (50-100V) at RT for the required time. Samples were loaded onto the gel in loading buffer (0.5M EDTA, 50% glycerol, 0.01 g bromophenol blue). DNA was visualised by transillumination with 320 nm UV light and photographs were taken by using an Eagle Eye (Stratagene) photocamera system.

2.2.5 Isolation and purification of DNA from agarose gel

To isolate an appropriate DNA fragment from an agarose gel, Easy Pure DNA Purification Kit (Biozym Diagnostik GmbH, Oldendorf, Germany) was used as recommended by the manufacturer. Briefly, the DNA band of choice was cut out from the gel under long wave UV light with the aid of a scalpel. The gel piece containing DNA was melted at 55 °C in the salt buffer and the DNA –binding resin was added .After two subsequent washing steps with the

washing buffer, the resin with bound DNA was air-fried and the DNA eluted by addition of bi-distilled water. The presence of the DNA fragment in solution was confirmed by agarose gel electrophoresis.

2.2.6 DNA ligation

All the DNA ligation reactions were performed in a total volume of 20 µl and incubated overnight at 15 °C, with the use of the ligase and buffers supplied by the manufactures.

2.2.7 Sub-cloning

The cloned DNA fragment was released from the vector by using the appropriate restriction enzymes, resolved on an agarose gel by electrophoresis, isolated from the gel and purified with the Easy Pure Kit (Biozyme, Oldendorf, Germany), and subsequently cloned into the new vector by the compatible sites or blunt ends ligation.

2.2.8 Phenol/Chloroform extraction of nucleic acids

To remove unwanted protein contaminants from nucleic acids solutions an equal volume of Tris-buffered phenol and chloroform at 1:1 (v/v) was added and the mixture was vortexed. The two phases were separated by centrifugation at 1200 rpm for 10 min. The upper aqueous nucleic acid-containing phase was transferred to a fresh reaction tube and subjected to a further round of extraction with phenol/chloroform.

2.2.9 Precipitation of nucleic acids

In order to recover nucleic acids from solution, the salt concentration was brought to 200mM with 3 M sodium acetate pH 5.0 and 2 volumes of cold ethanol was added. After 2 h to overnight incubation at –20 °C or 30 min at

–80 °C , the precipitate was centrifuged at 12000 rpm for 15 min. The pellet was washed with 70% ethanol, centrifuged for another 3 min to remove the salt and was then dried. DNA was resuspended in either water or TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

2.2.10 Determination of nucleic acid concentration

The concentration of nucleic acids was determined by measuring their optical density (OD) at 260 and 280 nm, respectively. An OD_{260}=1 is equivalent to 50 μg/ml double stranded DNA or 40 μg/ml RNA or 20 μg/ml single-stranded oligonucleotide. Pure DNA or RNA in aqueous solution should have an OD_{260}/OD_{280} ratio of 1.6-1.8 and 1.8-2.0, respectively.

2.2.11 Preparation of chemically competent bacteria (E.coli)

A single bacterial colony was inoculated in 5ml LB medium and incubated overnight under appropriate shaking at 37 °C . 4ml was then transferred to 400ml fresh LB medium and allowed to grow to reach an OD_{600} of 0.4. The bacteria were kept on ice for 10 min and then centrifuged at 3500 rpm for 10 min at 4 °C. The bacteria were resuspended in 20 ml of ice-cooled 0.1 M $CaCl_2$ and kept on ice for 10 min. After centrifugation, the pellet was resuspended in a 20 ml ice-cold $CaCl_{2m}$. This process has to be repeated for one more time. Finally, the pellet was resuspended in 2 ml ice-cooled $CaCl_2$ with 10% glycerol. The bacteria were dispensed in 200μl aliquots after 5 min extra incubation on ice and frozen at -80°c.

2.2.12 Transformation of bacteria (E.coli)

2.2.12.1 Chemical transformation

0.05-1μg of plasmid DNA or 5μl of ligation mixture was applied to 100μl ice-thawed chemically competent bacteria. The bacterial mixture was left on ice

for 30 min before heat-shock at 42 °C for 1min. After heat-shock, the bactria were then put back on ice for an extra 2 min. The transformed bacteria were mixed with 450µl SOC medium (2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5Mm $MgCl_2$, 10mM $MgSO_2$ and 20mM glucose) and incubated with shaking for 30 min at 37 °C. Finally the bacteria were spread on LB agar plate containing appropriate antibiotics and further incubated for 16-20 hours at 37 °C.

2.2.12.2 Transformation by electroporation

Up to 1µg plasmid DNA or appropriate amount of 70% Ethanol washed NaAC (1:10 to the volume of ligation mixture) precipitated ligation was added to 40µl competent DH5α. The bacteria were transferred into electroporation cuvettes and were subjected to electroporation at 1.8 KV. 1 ml SOC was added to the cuvette after electroporation and the mixture was transfered into a fresh vial and incubated for 30 min at 37 °C. The bacteria were then plated on LB agar plate supplied with appropriate antibiotics and incubated for 16-20 h at 37 °C.

2.2.13 Polymerase chain reaction (PCR)

All PCR reactions were carried out in a total volume of 50 µl in the presence of 0.25mM dNTPs, 1 pmol of primers, 1 U of Taq polymerase, 1× supplier's buffer and 2mM MgCl2. The reactions were performed in a PCR thermocycler (Perkin Elmer, Norwalk, USA), using the following cycing parameters: 94 °C, 1 min, 1 cycle; 94 °C, 30 sec, 55 °C, 45 sec, and 72 °C, 45 sec for 27-30 cycles. Analysis of the PCR products were performed by a gel electorphoresis

2.2.14 Cloning into pCR2.1-TOPO TA vector

The plasmid vector (pCR2.1) is supplied linearised with single 3'-thymidine (T) overhangs for TA cloning and with a covalently bound topoisomerase I. Because Taq polymerase has a non-template dependent terminal transferase

activity, it adds single deoxyadenodine (A) to the 3' end of PCR products. This allows PCR inserts to efficiently ligate with the vector's overhanging 3'-deoxythymidine residues. The reactions for TA cloning were performed according to the TOPO (TA) cloning Kit (Invitrogen, Groningen, The Netherlands) instructions.

2.2.15 Manual (radioactive) DNA sequencing method

The Thermo Sequenase Radiolabelled Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech, Braunschweig, Germany) was used for the sequencing of DNA templates. The label is incorporated into the DNA reaction products by the use of four (^{33}P)-ddNTP terminators. By use of an engineered Thermo Sequenase DNA polymerase, efficient incorporation in cycling sequencing protocols is allowed. Sequencing of desired templates was carried out according to the manufacturer's guidelines. Approximately 1 µg of plasmid DNA was taken together with 3 pmol of an appropriate sequencing primer with 8 U of the Thermo Sequenase polymerase in a total volume of 20 µl. From this mixture 4.5 µl was aliquoted into each 'termination' PCR vial ('G', 'A', 'T', 'C') containing a mixture of 2 µl dGTP and 0.5 µl (^{33}P)-ddNTP and cycled in a PCR machine using the following parameters: 95°C, 30 s, 55°C, 30 s and 72°C, 1 min for a total of 40 cycles. The reactions were stopped by the addition of stop solution (provided). After a denaturation step (i.e. incubation at 70°C for 5 min), 3 µl samples were loaded in each lane and resolved over a 6% polyacrylamide 6 M urea/TBE (90 mM Tris-base, 90 mM boric acid, 2.5 mM EDTA, pH 8.3) gel. Once the run had reached the desired length, the gel was removed, dried on Whatmann 3MM paper at 80°C for 2 hours on a vacuum gel dryer before autoradiography. Films were developed after 18-36 hours exposure.

2.2.16 Radioactive labeling of DNA probe for EMSA

To label the DNA probe, 8□l of [^{32}P] □ATP was applied to a reaction mixture containing 1.5□l 10□PNK buffer, 1.5□l of T_4 PNK (30 U), 2□l of each of the

single stranded GRE oligonucleotides (10pmol/µl). The mixture was gently mixed and incubated at 37°C for 1 hour and then was denatured at 95°C for 15 min. The vial should be cooled down gradually in a beaker filled with hot water. The labeled DNA was separated by running a 15% non-denaturing acrylamide gel in 1×TBE (89mM Tris base, 89mM boric acid, 2mM EDTA pH 8.0) and then the gel was dried. A MP film (Amersham Pharmacia, Buckinghamshire, UK) was exposed to identify the position of the labeled DNA before it was cut out of the gel and resolved in water.

2.2.17 Determination of protein concentration

Protein concentration was determined by the Lowry method. 100 volumes of Lowry I reagent (2% Na_2CO_3 in 0.1 N NaOH) were added to 1 volume of Lowry II reagent (2% Na-K-tartrate) and to 1 volume of Lowry III reagent (1% Cu sulfate) immediately before use and strictly in the order indicated. To 495µl of this mixture, called Lowry IV reagent, 5µl of the protein solution to be measured were added, vortexed and let 10 min at RT in the dark. 0.1 ml of Lowry V (50% Folin solution) reagent were then added. The mixture was immediately vortexed and let at RT for 30 min in the dark. Finally, it was vortexed and 300 µl were transferred to a 96-well plate. Absorbance at 600 nm was measured. To establish the exact amount of protein present in the probe a standard curve was generated by measuring the absorbance of known amounts of BSA (typically from 0 to 50 µg).

2.2.18 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were electrophoretically separated on the basis of size using the method of Laemmli (1970). The resolving gels containing between 6-15% acrylamide depending on the experiment and 5% stacking gel were cast according to Sambrook et al. (1989). Samples were run into the stacking gel at 80-100 V and then run at 150-200 V in the separating gel with a Mini-gel system (Hoefer, San Francisco, USA).

2.2.19 Coomassie staining

SDS-PAGE gels were immerged with Coomassie staining solution (0.25% Coomassie brilliant blue R-250, 50% methanol and 10% acetic acid) for several hour at RT with shaking. To destain, the gels were incubated firstly in a solution containing 50% methanol, 10% acetic acid for 1 hour and then in 10% methanol, 10% acetic acid for several hours and several changes of the destaining solution until the signal was clear enough for observation.

2.2.20 Western blot

Proteins in SDS-PAGE gels were transferred onto methanol pre-soaked Immobilon-P membranes (Millipore, Bedford, USA) in a transfer chamber (Bio-Rad, Hercules,USA) containing blotting buffer (20mM Tris, 192 mM glycine and 10% v/v methanol) at 30 V overnight (4°c). A Semi-dry blotter (H.Hörzel, Wörth/Hörlkofen, Germany) was used for blotting using the same transfer buffer at a current of 1mA/cm^2 for 1 hour. After the transfer was completed, membranes were incubated in TBST buffer (10 mM Tris-HCl pH7.6, 100 mM NaCl, 0.05% Tween 20) supplemented with 5% non-fat milk powder at RT for 1-2 hours to reduce unspecific binding. Primary antibodies were diluted in the blocking TBST buffer at a concentration recommended by the supplier (generally 1:500-1:1000). Then the membranes were incubated in the primary antibody-containing buffer for a further 1-2 hours at RT or 12 hours at 4°C. Thereafter the membranes were washed three times with TBST buffer for 5 min each. An appropriate secondary HRP-conjugated antibody was added and incubated for additional 1 hour at RT. The membranes were further washed three times with TBST buffer. Detection of specific protein signals was achieved by enhanced chemiluminescence using ECL Western blotting detection reagents and ECL Hyperfilm (Amersham Pharmacia, Buckinghamshire, UK) following the manufacturer's instructions. The Aida 2.11 program was used for quantification of the density of the signals.

2.2.21 Stripping Western blot membrane

To utilize more than a single use of the Western blot membranes, they were stripped by the following method. They were incubated in a stripping solution (62.5 mM Tris-HCl pH 6.8, 2% SDS and 100mM 2-mercatoethanol) at 50°C for 40 min with shaking. The membranes were then washed twice with TBST for 5 min each time, blocked with 5% milk buffer as usual and used for Western blot probing again.

2.2.22 Cell culture

Cells were maintained at 37°C in an incubator (Forma Scientific, labortect GmbH, Göttingen, Germany) with 5% CO_2 and 95% humidity. All cells were grown in petri dishes, plates or flasks (Greier, Frickenhausen, Germany) depending on the application. In general, the cells were allowed to grow until a confluence of 80-90% had been reached, whereupon the cells were subsequently split by trypsinisation and re-seeded at a lower density. Trypsin treatment of cells was performed by removal of the culture medium from the cells, followed by one wash with PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO4, 1.5 mM KH_2PO4). After removal of PBS, 0.25% trypsin was added to the cells and the cells were incubated at 37°C until they become detached. Fresh medium was then directly added and the cells re-plated at the desired density in new petri dishes.

2.2.23 Long term storage of cells (freezing and thawing of cells)

To prepare cells for long term storage, logarithmically growing cells were trypsinised and collected by centrifugation at 800-1500 rpm. The medium was removed and the cells re-suspended in culture medium with 50-90% FCS and 10% DMSO (Fluka Chemie AG. Switzerland) and placed in 1 ml aliquots in

cryovials. After incubation on ice for 1 hour, the cells were placed at -80°C for 16-24 h before finally being stored in liquid nitrogen. To re-propagate cells, the vials were removed from the liquid nitrogen and placed at 37°C until the cells had thawed. The cells were then removed and mixed with 10 ml fresh medium; cells were then collected by centrifugation and seeded on Petri dishes in fresh medium.

2.2.24 Transient transfection of mammalian cells

Cells to be transfected were split one day before transfection to allow them to reach a logarithmic phase. Fugene 6 (Roche, Mannheim, Germany) was used for transfection of plasmid DNA, according to the instruction of the manufacturer.

2.2.25 Whole cell extract for EMSA

Cells were harvested in Ca/Mg free PBS by scraping with a rubber policeman and then centrifuged at 12,000 rpm for 5 min. The lysis buffer (10mM Na_2HPO4/NaH_2PO4 pH 7.4, 1mM EDTA, 400mM KCl, 15% glycerol) was added to the pellet and the vial was put into liquid nitrogen to be frozen and thawed for 5 times. The lysate was spined down at 12,000 rpm for 15 min before ready to be used or frozen at -80°c.

2.2.26 Whole cell extract for Western blot

Cells were lysed directly in the SDS-PAGE sample buffer (120 mM Tris-HCl, pH 6.8, 4% SDS, 100mM 2-mercaptoethanol, 20% v/v glycerol and 0.01% bromophenol blue). The samples were boiled for 5 min before loading on a SDS-PAGE gel.

2.2.27 Preparation of GST (Glutathione-S-Transferase) and GST fusion proteins (large scale)

Inoculate 20 ml 2□LB or 2□YT (16g tryptone, 10g yeast extract, 5g NaCl pH 7.0) media with the bacteria containing GST or GST fusion constructs and incubate them at 37°C under shaking until the bacteria had reached the logarithmic phase. 1:50 dilution of these bacteria by fresh media was performed and further incubation should be carried out until the OD_{600}=0.5. The bacteria were induced with IPTG (0.5mM) for 16 hours and then centrifuged at 5000 rpm for 5 min. The pellet was resuspended with 10 ml (for 200 ml culture medium) Ca/Mg free PBS containing 2mM EDTA and 200mM isopropanol. Lysozyme was applied to the mixture and the bacteria were kept on ice for 10 min. Appropriate sonication was performed by using the Branson Sonifier (Heinemann, Germany) and the solution was left on ice for 10 min before centrifugation at 12,000 rpm for 10 min, 4°C. The supernatant was transfered to a fresh tube containing 800 ml 50% slurry of Sepharose 4B beads (Amershem Pharmacia, Uppsala, Sweden) the tube was allowed to rotate for 1 hour at 4°C. The supernatant was discarded and the beads were washed three times with PBS for 3 times. Proteins bound to the beads were eluted with 1 ml elution buffer (10 mM glutathione, 50 mM Tris-HCl) and then pooled in one vial. The eluted proteins can be used immediately or frozen at -80°C.

2.2.28 Cleavage of the protein fused to GST

In the GST-pull down experiment, purified Bag-1M is needed and, therefore, the GST tag should be removed from the GST-fusion protein containing a Thrombin recognition site. 10ml of Thrombin solution (10 units) was added (Amershem Pharmacia, Uppsala, Sweden) per mg fusion protein, mixed gently and incubated at RT for 2-16 hours depending on the cleavage efficiency monitored in between. Once digestion is complete, GST can be removed by extensive dialysis (e.g. 2000 volumes) against 1□ PBS followed by purification on Glutathione Sepharose 4B. The purified protein of interest will be found in the flow-through fraction.

2.2.29 Preparation of radioactively labeled in vitro translated GR and GR mutants

GR expression plasmid DNA containing a T7 promoter or GR mutants expression plasmids containing T3 promoters were used for in vitro transcription/translation with the TNT Quick Coupled Transcription/Translation System or TNT Coupled Reticulocyte Lysate System (Promega, Madison, WI, USA) to generate [^{35}S]-metnionine- labeled GR or GR mutants according to the manufacturer's instruction manual.

2.2.30 ATP depletion of COS-7 cells

Before harvesting the transfected COS-7 cells, they were washed with PBS. 10 mM 2-Deoxyglucose in PBS was supplied to the cells and the cells were incubated for 180 min at 37°C. The cells were then washed twice with PBS and harvested in 1× lysis buffer (Passive Lysis Buffer, Promega, Mannheim, Germany) and were boiled for 45 sec at 95°C to get the ATP released from the cells. The debris of the cells was spun down at 12,000 rpm for 5 min and the supernatant was transferred to a fresh vial and kept on ice for use. The ATP depletion efficiency was detected by measuring the ATP-dependent luciferase activity using the luciferase-luciferin substrate (Sigma, Louis, MO, USA) diluted in sterile water according to the manufacturer's instructions. The method used is identical to that of the firefly luciferase activity mentioned previously.

2.2.31 Isolation of total RNA from cultured cells

Cultured cells were grown to sub-confluency. After removing the medium, cells were lysed by addition of 1 ml (per 3,5 cm dish) of the TRIFAST Reagent

(Peqlab, Erlangen, Germany). The cell lysate was homogenised by passing it several times through the pipette tip. Upon completion of homogenisation the lysates were incubated for 5-10 min at room temperature in order to allow dissociation of nucleoproteins. This was followed by addition of chloroform (0.2 ml per 1 ml of TRIFAST reagent used) and vigorous shaking by hand. The samples were then allowed to stand at room temperature for another 5-10 min and centrifuged at 12000 rpm for 15 min at RT. Centrifugation resulted in separation into two phases. The top aqueous RNA-containing layer was carefully decanted and transferred to a fresh tube. In order to precipitate the RNA, an equal volume of isopropanol was added, followed by centrifugation for 15 min at 12000 rpm at 4°C. The RNA pellet was washed twice in 70% ethanol before being air-dried. Finally, the RNA was re-suspended in bi-distilled water at a final concentration of 1 mg/ml and stored at -80°C.

2.2.32 Separation of RNA by agarose gel electrophoresis

To prepare the gel, 1 g of Agarose (Peqlab, Erlangen, Germany) was re-suspended in 100 ml of MOPS buffer (0.02 M 4-morpholinepropanesulfonic acid, 5 mM Na-acetate, 1 mM Na_2EDTA, pH 7.0) The buffer was made Rnase-free by autoclaving. Just before pouring the gel the agarose/MOPS mixture was melted in a microwave oven. After brief cooling, 3 ml of 37% formaldehyde were added. Gels were electrophoresed in a RNAse-free horizontal chamber at 90 V for 2.5 h in 1× MOPS buffer. Prior to loading RNA samples were denatured for 15 minutes at 55°C in formamide loading buffer (1× MOPS, 22.6% formaldehyde, 64.5% formamide, 3 µg/ml ethidium bromide, 0.01 g bromophenol-blue) and loaded onto the gel. The integrity of the mRNA was visualised by illumination with UV light and photographs were taken using an Eagle Eye (Stratagene) photocamera system.

2.3. Analytical Methods

2.3.1 Measurement of firefly luciferase activity

Cells were growing for 20-24 hours after transfection and were treated with hormone for an additional 16 hours to allow the expression of the transfected reporter gene. Typically, they were grown in 6-well plates, Before harvest, the cells were washed twice with Ca/Mg free PBS. The cells were then harvested in 350ml 1□lysis buffer (passive lysis buffer, Promega, Mannheim, Germany). The cells were kept on ice for 10 min and collected by a rubber policeman and finally transferred to a fresh vial. The vial was vortexed and centrifuged at 12,000 for 3 min and the supernatant was transferred to another vial. Typically, 100ml of cell lysate was applied to a reading tube supplemented with 300ml of assay buffer consisting of 1 mM DTT, 2 mM ATP in Glycylglycine buffer (25 mM glycylglycine,15 mM $MgSO_4$ and 4 mM EGTA)) and 100ml of luciferin assay solution (1 mM luciferin,; stock solution: 0.28 mg/ml) were then added. Luciferase activity was measured by using of a Luminometer (Berthold, Wildbad, Germany).

2.3.2 Measurement of Renilla luciferase activity

100ml of cell lysate obtained as described in the former paragraph were mixed with 400ml of coelenterasin buffer (0.1 M Kpi-buffer (0.2 M KH_2PO_4 and 0.2 M K_2PO_4 pH 7.6), 0.5 M NaCl, 1 mM EDTA, pH 7.6) together with substrate solution (25 nM final concentration coelenterazine, Byosinth AG, Gstaad, Switzerland). Luminescence was measured by using a luminometer.

2.3.3 GST-pull down assay

For 1 reaction, 80□l of 50% slurry of Sepharose A beads were washed with 10-fold volumes of Ca/Mg free PBS for 3 times and then were diluted to 300ml with PBS. 20mg of GST or GST fusion proteins were applied to the

beads and rotated for 1 hour at 4°c. The beads were washed with PBS for 3 times before blocking with 5% milk in PBS for half an hour at 4°c. Equilibration of the beads with LBST buffer (20 mM Hepes-KOH pH 7.9, 100 mM NaCl, 2.5 mM MgCl2, 0.1 mM EDTA, 0.05% NP40, 1.5% Triton X-100) containing 1 mM DTT and 1 mM PMSF was then carried out. The addition of 20ml radioactively labeled in vitro translated protein to the beads was performed and rotated for 1 hour at 4°C. Thereafter, the beads were intensively washed with LBST buffer for 3 times and supplied with 25ml of LBST buffer. The beads were then supplied with SDS-PAGE sample buffer and heated at 95°C for 3 min before loading onto the SDS-PAGE gel. After electrophoresis, the proteins in the gel were fixed with 10% acetic acid and 40% methanol for 30 min and washed with water and further treated with 1 M sodium salicylate to increase the signal. The gel was dried and an ECL film was subjected to exposure.

2.3.4 Electrophoresis mobility shift assay (EMSA, Band Shift)

This assay was used to detect the binding of glucocorticiod receptor to sequence-specific DNA probe (GRE). 4ml of whole cell extract (WCE) containing the GR was applied to a reaction mixture containing 10ml of 2□ reaction buffer (20 mM Hepes pH 7.9, 2 mM EDTA, 2 mM DTT, 5% Ficoll 100), 0.5ml of polydIdC (Roche, Mannheim, Germany), or the other components depending on the requirements of the experiments. The maximum volume of the reaction mixture should not exceed 19ml. The mixture was kept on ice for 10 min before the application of 1ml of the radioactively labeled DNA probe and further incubation at RT for 15 min. The mixture was then loaded on a 15% non-denaturing acrylamide gel (acrylamide 15%, TBE 0.5□, APS 0.1%, TEMED 0.1%) and subjected to SDS-PAGE. A MP film was exposed by the dried gel to detect the signals of interest.

2.3.5 Chromatin Immunoprecipitation (CHIP)

Eight to ninety percent confluent cells at the appropriate time after transfection, they were treated with hormone (10^{-7} M) for different times

depending on the experiments. The cells were then washed with PBS (-Ca/-Mg) and X linking buffer (100 mM NaCl, 50 mM HEPES pH 7.9, 0.5 mM EGTA, 1 mM EDTA) respectively before crosslinked with X linking buffer supplemented with 1% Formaldehyde for 15 min at RT. Crosslinking was stopped by withdraw of the crosslinking solution and addition of 0.125 M glycine in X linking buffer with appropriate shaking.

The cells were washed twice with PBS and detached from the plate by trypsinisation . After harvesting and washing of the cells with PBS, they were lysed by 1 ml lysis buffer (50 mM Hepes-KOH, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 10% glycerol, 0.5% NP40, 0.25 Triton-X.100, 1 mM PMSF). The supernatant was discarded after centrifugation at 2,500 rpm for 10 min at 4°C and the pellet was washed with sashing buffer (10 mM Tris pH 8.0, 1 mM EDTA, 0.5 mM NaCl, 1mM PMSF). The cells were resuspended in RIPA buffer (10 mM Tris-HCl, 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl,1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, 1 mM PMSF) and subjected to sonication (5-7 times, 20 times of each in pulse mode) by using the Branson sonifrer.

An aliquot of the solution was taken out to check the length of the DNA fragment by agarose gel after decrosslinking at 95°C for 20 min. The average size should be smaller than 1 kb. The rest of the liquid was centrifuged at 13,000 rpm for 10 min in an outswing rotor. The supernatant was transferred to a new vial and precleared with 50 ml 50% Sepharose A slurry (Amershem Pharmacia, Uppsala, Sweden) for 1 hour at 4°C. Centrifugation was performed at 3000 rpm for 3 min before the supernatant was aliquoted depending on the requirement of the experiment. 2mg of each of the antibodies was applied to every aliquot and the aliquots were incubated overnight at 4°C. 50 ml 50% Sepharose A beads slurry was then added to the aliquot and further incubated for at least 3 hours at 4°C. The beads were span down and underwent washing procedures as follow: RIPA twice; RIPA containing 100mg/ml salmon sperm DNA 5 min with rotation; RIPA supplemented with both 100mg/ml salmon sperm DNA and 500 mM NaCl ; RIPA finally. After washing, 150ml Elution buffer (50 mM Tris pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.5% SDS) was applied to the beads and the DNA-

protein complexes were eluted from the beads for 15 min at 37°c. For digestion, 100 mg/ml Proteinase K was added to the elute and it was incubated at 55°c for several hours before it was digested at 65°c overnight.

2.3.5.3 DNA purification and PCR

After digestion, the DNA was purified by using the QIAquick PCR Purification Kit (QIAGEN, GmbH, Hilden, Germany) according to the manufacture's protocol. One half of the purified DNA was used for PCR and the PCR reaction was run for 27 cycles. The amplified DNA fragments were checked with the use of 2% agarose gel.

2.3.6 RNAi

Bag-1 siRNA, located 460 nucleotides downstream from the initiation codon (CTG) of Bag-1L and the luciferase GL3 siRNA used as control, were purchased from Dharmacon, Lafayette, CO. Transfection of HeLa cells with the siRNA was performed using an Oligofectamine reagent (Invitrogen, Karlsruhe, Germany) at a final concentration of 150 nM. The culture medium was changed and the cells were re-transfected every 24 h and allowed to grow for different periods of time. The cells were finally collected for RT-PCR and immnunoblotting. For the RT-PCR, cDNA, was synthesised with oligo-dT and amplified for 28 cycles with primers of Bag-1M.

2.3.7 Real-time PCR

1-2 μl Dnase I (Roche) was added to 20 μg of total RNA (final volume to 100 μl) for digestion of DNA, the reaction mixture was incubated at 25°C for 15-20 min . After digestion, the temperature was increased to 75°C for 5 min to inactivate the Dnase. The volume was amplified to 200 μl and then remove the proteins by phenol/chloroform/isoamyl ethanol (identical volume) extraction. Recovery of RNA was done by using 1/10 vol of sodium acetate, 1 μl of carrier and ethanol. Finally, dissolve RNA in 12.5 μl H_2O.

1 μl of OligodT or 1 μl primer pd(N)6 (random primer) was applied to 11 μl of RNA and incubated at 70 °C for 10 min in a thermocycler. During this period, prepare a reaction mixture containing 4 μl of 5× first strand buffer, 2 μl of DTT and 1 μl of 10mM dNTPs mix. The denatured RNA was moved on ice immediately after incubation and added with the prepared reaction mixture and 1 μl of reverse transcriptase. Thereafter, the mixture was moved to a thermocycler and incubated at 42°C for 90 min. The reaction was then carried out at 70 °C for 15 min and amplified to 100 μl.

3 μl of synthesized template was mixed with 10 μl of SYBR-Green 2× mix, 4 μl of water and 2 μl of primers as described previously. The ABI PRISM 7000 sequence detection system and the SYBR Green PCR Master Mix (Applied Biosystem, Warrington, UK) and 40 cycles were used for the amplification.

3. Results

3.1 Bag-1M functions via distinct amino acid sequences in the GR

Bag-1 family members have different effects on the action of steroid hormone receptors. Bag-1M negatively regulates transactivation activity of both the GR (Schneikert et al, 1999; Kullmann et al, 1998) and the progesterone receptor (PR) (Schneikert & Cato, unpublished observation) but not the mineralocorticoid receptor (MR) (Kullmann et al, 1998) and the androgen receptor (AR) (Kullmann et al, 1998; Froesch et al, 1998). Among all the functional domains of the GR, the hinge region (amino acids 490-515) was found the site of interaction with Bag-1M and to contribute to Bag-1M mediated negative regulation of glucocorticoid action (Kullmann et al, 1998). This fact suggested that the hinge region sequence of these receptors might be important for Bag-1M-mediated effect on these receptors. Thus, the hinge region sequence of these receptors were compared and demonstrated that apart from a nuclear localization sequence that is conserved in all these receptors there are some other amino acids that are conserved only in the MR and AR but not in the GR and PR. It therefore assumed that the difference in response of the four receptors to Bag-1M occurs from the difference in the primary amino acid sequence of their hinge regions. Six amino acids within the GR hinge region were selected as possible targets of action of Bag-1M on the transactivation by this receptor and were replaced with the amino acids that are conserved at the equivalent positions in the MR and AR. Thus, four GR hinge region mutants, K496L/I497G, G499L/I500K, T504E and E513P were constructed by the PCR-based QuickChange site-directed mutagenesis method. However, among these mutants only K496L/I497G could not exert Bag-1M-mediated negative effect. Another double changed mutant, I500K/T504E, confirmed that its transactivation could be repressed by Bag-1M. Hence, the K496L/I497G (GRmt2) and I500K/T504E (GR mt1) GR

mutants were chosen and analyzed for possible mechanism of action of Bag1M.

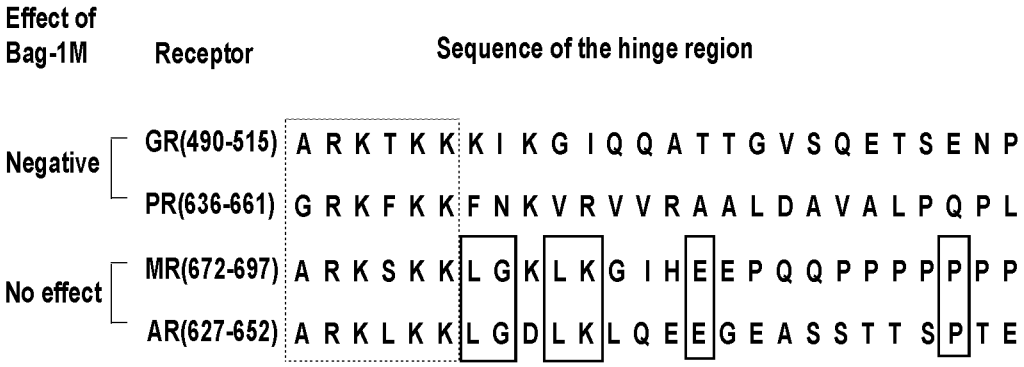


Figure 3.1 Alignment of the sequence of the hinge region of the selected steroid receptors. The dotted area is the conserved nuclear localization sequence (NLS) of these receptors. The squared areas are the conserved amino acids within the hinge region of the MR and AR. The GR hinge region mutants are: K496L/I497G (GRmt2) and I500K/T504E (GR mt1).

3.1.1 Lysine 496 and isoleucine 497 in the hinge region of the GR are essential for the negative regulation of Bag-1M on the transactivation by the GR

To investigate whether Bag-1M affects the activities of the K496L/I497G and I500K/T504E GR mutants, the effect of Bag-1M on the transactivation of the mutant GRs was examined using a luciferase reporter gene assay. The luciferase reporter assay is a sensitive method for determining the level of luciferase expression in cells transfected with firefly luciferase reporter gene driven by a promoter specific for transcription factor of interest. Meanwhile, a renilla luciferase reporter gene driven by a promoter that the transcription factor can not bind should also be transfected into the cells as an internal control. Thus, the activity of the transcription factor through its specific promoter on the reporter gene can be evaluated by the relative level of firefly

luciferase over renilla luciferase. COS-7 cells that are devoid of both the GR and Bag-1M were cotransfected with expression vectors for either the wild type GR or the mutant GRs, expression vector for Bag-1M and an indicator construct containing a luciferase gene driven by the mouse mammary tumor virus (MMTV) promoter. The transactivation functions of these two mutant receptors in transfected COS-7 cells were not much different from that of the wild-type GR following treatment with the synthetic glucocorticoid dexamethasone (Fig 3.2). However overexpression of Bag-1M had different effects on the activities of the wild-type and mutant receptors. While Bag-1M repressesd the action of the wild-type GR as previously described (Kullmann et al, 1998; Schneikert et al, 1999) as well as the activity of the I500K/T504E (GRmt1) receptor mutant, transactivation by the K496L/I497G (GRmt2) was not downregulated (Fig. 3.2). This result showed that the lysine 496 and isoleucine 497 in the hinge region of the GR are essential for Bag-1M mediated downregulation of the transactivation by the GR.

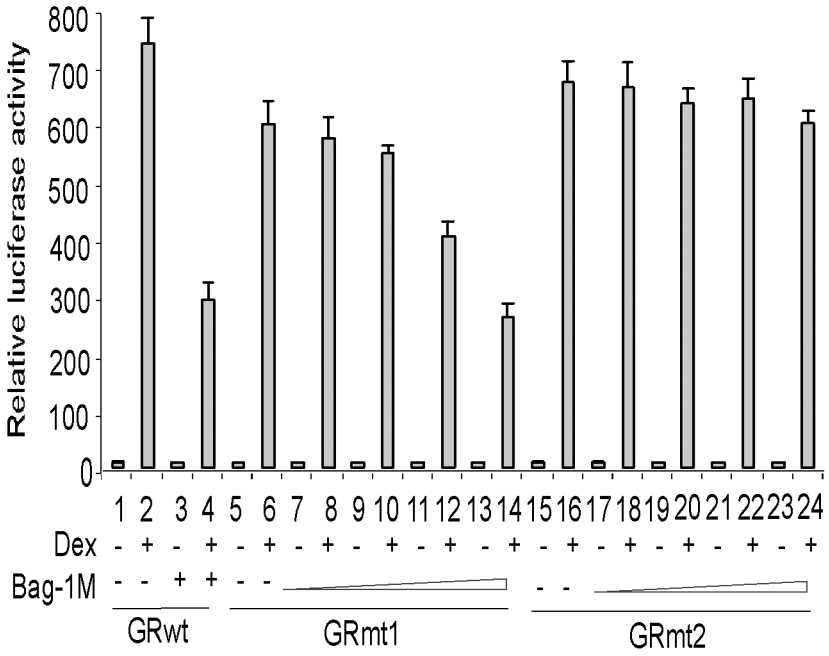


Figure 3.2. Transactivation by the I500K/T504Emutant GR but not the K496L/I497G is inhibited by Bag-1M. $1.5x10^5$ COS-7 cells were transiently cotransfected with 0.15□g of GR-responsive MMTV-luciferase (pGL3MMTV) indicator gene and 50ng of an internal control plasmid encoding the Renilla luciferase under control of the ubiqutin promoter. In addition ,0.2□g of the expression vector encoding either the wild type GR, or the GR hinge region mutants, GRI500K/T504E or GRK496L/I497G were cotransfected with either an empty vector (Bag-1M-) or different amount of expression vectors encoding BAG-1M ranging from 10 ng to 0.2 □g (Bag-1M+). Twenty-four hours after transfection, the cells were treated with vehicle (ethanol) alone (Dex-) or with vehicle containing 0.1□M dexamethasone (Dex+) for 16h, Thereafter the cells were lysed for mesurement of luciferase activity. The bar chart represents the mean value of the relative firefly luciferase activity over renilla luciferase activity of three independent experiments.

3.1.2 Bag-1M does not inhibit the DNA binding by the K496L/I497G (GRmt2)

It is known that Bag-1M represses both the transactivation function and the DNA binding ability by the GR (Kullmann et al, 1998). As transactivation is dependent on the ability of the GR to bind DNA, the effect of Bag-1M interferes on DNA binding ability by the GR mutants was investigated. COS-7 cells were cotransfected with either the wild-type or the mutant GRs expression vectors in the presence or absence of everexpession for Bag-1M. Whole cells extracts from transfected cells were prepared for electric mobility shift assay (EMSA) where the extracts are allowed to interact with a radioactively labeled GRE oligonucleotide and the interaction of a protein and the oligonucleotide can be detected after electrophoresis because the mobility of the oligonucleotide is retarded by the bound protein. The result showed that the ineffectiveness of Bag-1M to suppress transactivation by the K496L/I497G (GRmt2) mutant GR was also reflected by its inability of this protein to downregulate DNA binding by the mutant GR in the EMSA. (Fig. 3.3 lanes 7 and 8). In contrast, DNA binding by the wild-type GR and the GRmt1 was repressed by overexpression of Bag-1M (Fig 3.3 lanes 3-6). Extracts from cells transfected with the empty vector or with Bag-1M alone did not bind DNA (Fig. 3.3 lanes 1 and 2). Control immunoblots showed that the levels of the GR and Hsp70 in the extracts used for the EMSA were not altered following overexpression of Bag-1M in the transfected cells. Thus the 496 lysine and 497 isoleucine in the hinge region of the GR contribute to Bag-1M-mediated repression on the DNA binding by the receptor and the negative regulation of Bag-1M on DNA binding by the GR is most likely one of the means used by this protein to inhibit transactivation by the GR.

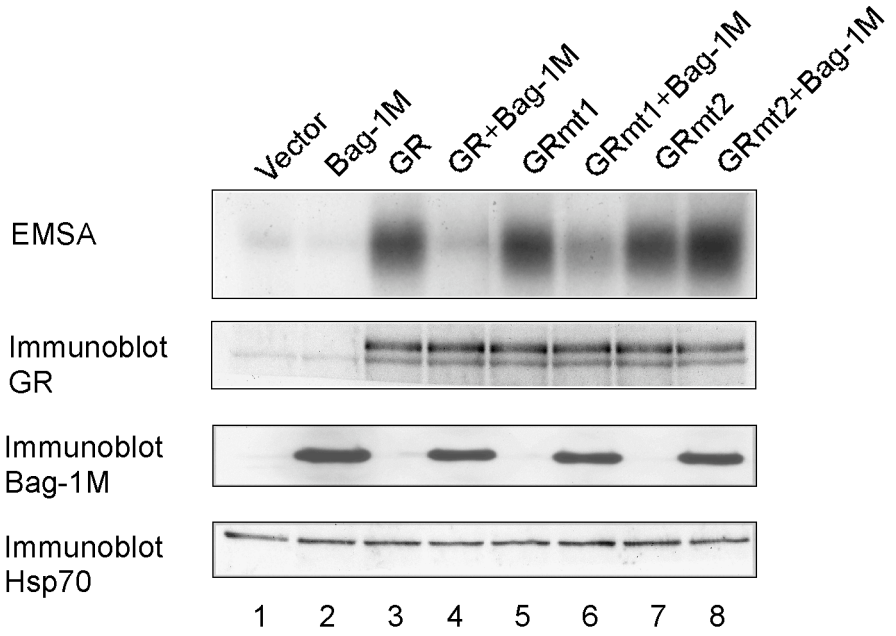


Fig.3.3 DNA binding by the K496L/I497G mutant GR is not suppressed by Bag-1M. Cos-7 cells were transiently cotransfected with either control plasmids (lanes 1 and 2) or plasmids encoding either the wild type GR or the mutant GRs. Twenty-four hours after transfection the cells were treated with 0.1□M dexamethasone for 16h. Whole cell extracts were prepared for EMSA with radioactively labeled glucocorticoid response element (GRE) probe. Aliquots of the reaction mixtures were applied for immunoblotting with anti-GR, anti-Bag-1 and anti-Hsp70 antibodies.

3.1.3. Lysine 496 and isoleucine 497 are sites for the interaction of GR with Bag-1M

The negative effect of Bag-1M on transactivation and the DNA binding activity of the GR occurs through an interaction of this protein with the GR and it is also known that a GR mutant lacking the hinge region abrogates interaction of GR with Bag-1M (Kullmann et al, 1998). Thus the hinge region is required for the physical contact of the GR with Bag-1M. To further determine whether the different effects of Bag-1M on the GR mutants arose from differences in the binding of Bag-1M to the receptors, GST pull-down experiments were carried out. In this experiment bacterially expressed and immobilized Bag-1M was allowed to interact with [^{35}S]-methanine labelled wild type GR and GR mutants. After interaction the nonspecifically bound protein was washed off and the specifically bound protein was eluted and detected by autoradiography. The wild-type GR and the GRI500K/T504E (GRmt1) mutant, preferentially interact with GST-Bag-1M as opposed to GST, but not the K496L/I497G (GRmt2) mutant. (Fig. 3.5 compare lanes 8 and 10 with 6). It was therefore concluded that the 496 lysine and 497 isoleucine are the sites of interaction with Bag-1M. This GST-pull down result was consistent with those of luciferase assay and EMSA. Thus, these results together identify amino acids at position 496/497 as important residues for interaction of Bag-1M with the GR and for the downregulation of the activity of the receptor. However, it remains to be determined which region of Bag-1M are necessary for interaction with the GR.

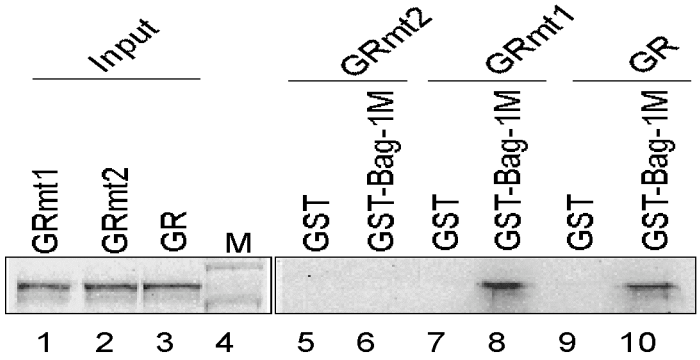


Fig 3.5. The 496 lysine and 497 isolucine residuces within the hinge region of the GR are important for interaction with BAG-1M in vitro. Twenty □L radioactively labeled rabbit reticulocyte lysate-translated wild type GR, $GR_{K496L/I497G}$ and $GR_{I500K/T504E}$ were incubated respectively with 20□g bacterially expressed GST and GST-BAG-1M bound to glutathione sepharose 4B beads. After extensive washing, the immobilized protein were eluted and analysed by SDS-polyacrylamide gel electrophoresis. Lanes 1-3 show one-tenth of the radioactively labeled proteins as input. Lane 4 indicates the radioactively labeled protein markers.

3.2 Domains of Bag-1M involved in the physical contact with GR

The N- and C- termini of Bag-1M are essential for its inhibition of the DNA binding ability and transactivation activity by the GR (Schneikert et al, 1999; Schneikert et al, 2000). To investigate whether both termini of this molecule are involved in the physical interaction with the GR, GST-pull down experiments were performed using in vitro translated radioactively labeled GR and bacterially expressed GST and GST fused to Bag-1M or to Bag-1M mutants. At first, experiments were performed with Bag-1M and two mutants Bag-1M lacking either the first 71 N-terminal amino acids (Bag-1M□N71) or the last 47 C-terminal amino acids (Bag-1M□C47). The interaction of Bag-1M□C47 and GR was drastically decreased compared with that of the wild type Bag-1M and GR(Fig. 3.6 compare lanes 5 and 7 with 9 and 11), indicating that the C-terminus of Bag-1M is essential for binding the GR. The

N-terminus of Bag-1M also showed an affinity for the GR as shown by the reduction of the interaction of the N-terminal deletion mutant of Bag-1M with the GR (Fig. 3.6 compare lanes 13 and 15 with 9 and 11). Taken together, it is suggested that both the amino and the carboxyl termini of Bag-1M are involved in the interaction with the GR.

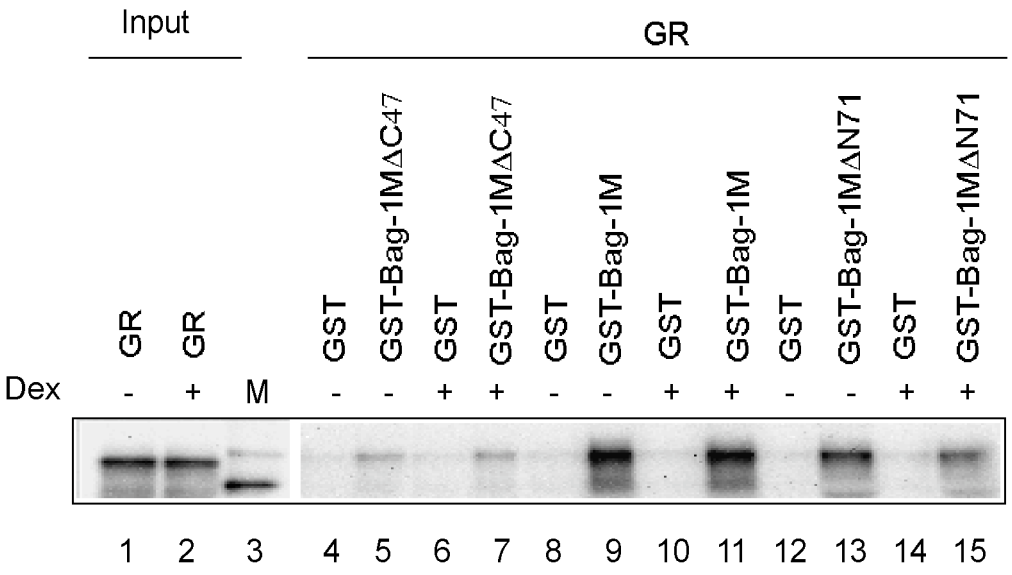


Fig 3.6 Both the N- and C- termini of Bag-1M are involved in the interaction with GR. Twenty □L radioactively labeled rabbit reticulocyte lysate-translated GR was treated with or withnot dexamathasone (10^{-7}M) for 10 min before incubating with 20□g bacterially expressed GST, GST fused Bag-1M and mutants Bag-1M bound to glutathione sepharose 4B beads. After extensive washing, the immobilized proteins were eluted and analysed by SDS-polyacrylamide gel electrophoresis. Lanes 1-2 show one-tenth of the radioactively labeled proteins as input. Lane 4 indicates radioactively labeled proteins used as a marker.

3.3 ATP hydrolysis is required for the regulation of GR activity by Bag-1M

3.3.1 ATP hydrolysis promotes Bag-1M-mediated repression of DNA binding by the GR

Previous studies from this laboratory on modulation of glucocorticoid action by Bag-1M showed that Bag-1M only binds the GR after prior interaction of the receptor with the molecular chaperone Hsp70/Hsc70 (Schneikert et al, 2000) and it is known that Bag-1M uses its carboxyl-terminus to bind the second lobe in the ATPase domain of Hsp70 (Briknarova et al, 2001). As Bag-1 binding promotes dissociation of the GR from the Hsc 70/GR complex and this dissociation requires the presence of ATP (Sondermann et al, 2001), the effect of this nucleotide on Bag-1M-mediated downregulation of DNA binding by the GR was examined. EMSA was therefore carried out with extracts of COS-7 cells transfected with the GR and bacterially purified Bag-1M in the presence or absence of ATP and ADP.

DNA binding by the GR was repressed by the addition of Bag-1M (Fig. 3.7 lanes 2 and 3). Addition of ATP and an ATP degenerating agent (glycerol kinase) responsible for the hydrolysis of ATP to ADP, showed a much stronger inhibition of DNA binding by the GR in the presence of Bag-1M (Fig. 3.7 compare lanes 8, 9 to 2, 3). Addition of the ATP degenerating agent or ATP on their own did not have a drastic effect on the downregulation of GR binding to DNA by Bag-1M (Fig. 3.7 lanes 4-7) neither was the effect of Bag-1M on DNA binding by the GR significantly affected by ADP (Fig. 3.7 lanes 10 and 11). Immunoblotting demonstrated that addition of Bag-1M and the chemical reagents did not alter the receptor and Hsp70 levels. Therefore, Bag-1M mediated inhibition of DNA binding by GR is significantly influenced by ATP hydrolysis.

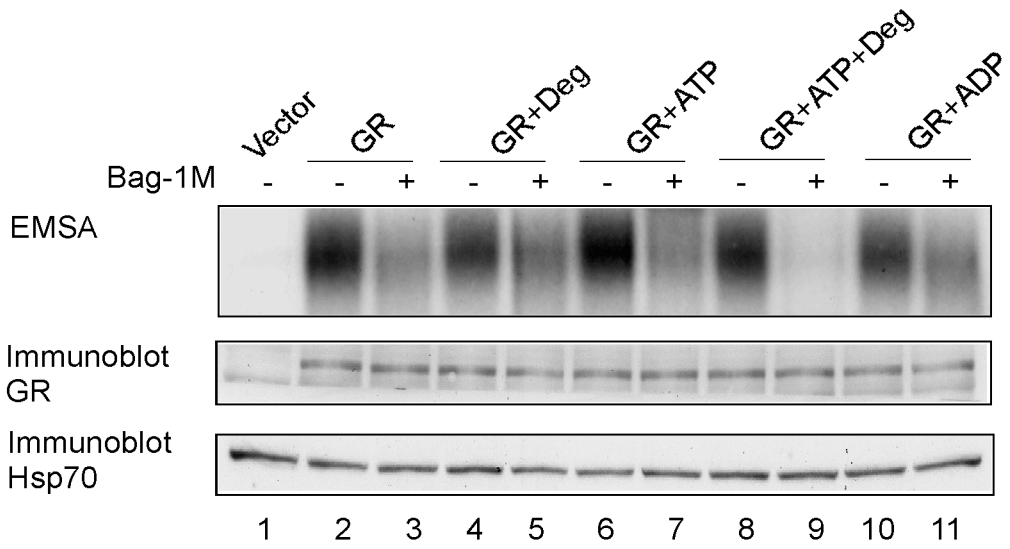


Fig 3.7 Bag-1M inhibits DNA binding by the GR under conditions of ATP hydrolysis. COS-7 cells were transiently transfected with empty expression vector or expression vector for the GR. Twenty-four hours after transfection, the cells were treated with 10^{-7}M dexamethasone for 16 h. Whole cell extracts were incubated with bacterially purified Bag-1M (500 ng), in the absence or presence of ATP (1mM), ADP (1mM), or ATP together with glycerol kinase (3□10^{-2} units/□l). The reaction mixture was incubated on ice for 10 min and then was incubated with radioactively labelled GRE and the bound fragments were separated by gel eletrophoresis. Aliquots of the reaction mixture were used for immunoblotting with anti-GR and anti-Hsp-70 antibodies.

3.3. 2 ATP hydrolysis is necessary for Bag-1M to inhibit DNA binding by the GR

To confirm the role of ATP hydrolysis in the action of Bag-1M, the effect of this protein on GR binding to DNA was determined under conditions of diminished cellular ATP levels. Cells were depleted of ATP and asked whether DNA binding activity of the GR in extracts of such cells could be inhibited by Bag-1M. For the depletion of ATP, the transfected COS-7 cells were treated with 2-deoxyglucose, a non-hydrolyzable analogue of glucose used extensively to produce rapid and severe ATP depletion (Bacallao et al, 1994). Intracellular ATP concentration measured by a modified luciferase assay showed a 70% reduction of the ATP concentration in the COS-7 cells in 4 h following the addition of 2-deoxyglucose.

Ligand-bound GR from the ATP depleted cells interacted with DNA but the addition of Bag-1M had virtually no effect on the DNA binding by the GR (Fig. 3.8 lanes 2-7). Addition of glycerol kinase alone, ATP or ADP also had no significant effect on the ability of Bag-1 to downregulate DNA binding by the GR (Fig. 3.8 lanes 4-7 and lanes 10-11). However addition of ATP and glycerol kinase together showed a strong inhibition of binding of the receptor to DNA in the presence of Bag-1M (Fig. 3.8 lanes 8-9). When the same experiment was performed with the K496L/I497G mutant GR (GRmt2) that does not bind Bag-1M, no effect of Bag-1M was observed on DNA binding by the mutant GR even in the presence of ATP and glycerol kinase (Fig. 3.9 lanes 10-11). Glycerol kinase alone, ATP or ADP did not affect Bag-1M to inhibit the DNA binding by the GRmt2 (Fig. 3.9 lanes 6-9 and lanes 12-13). Thus Bag-1M needs interaction with the GR and ATP hydrolysis to exert its negative regulatory action on DNA binding by the GR. It is most likely that ATP hydrolysis and binding of Bag-1M to the GR produces an altered Bag-1M-GR conformation that is less efficient in DNA binding.

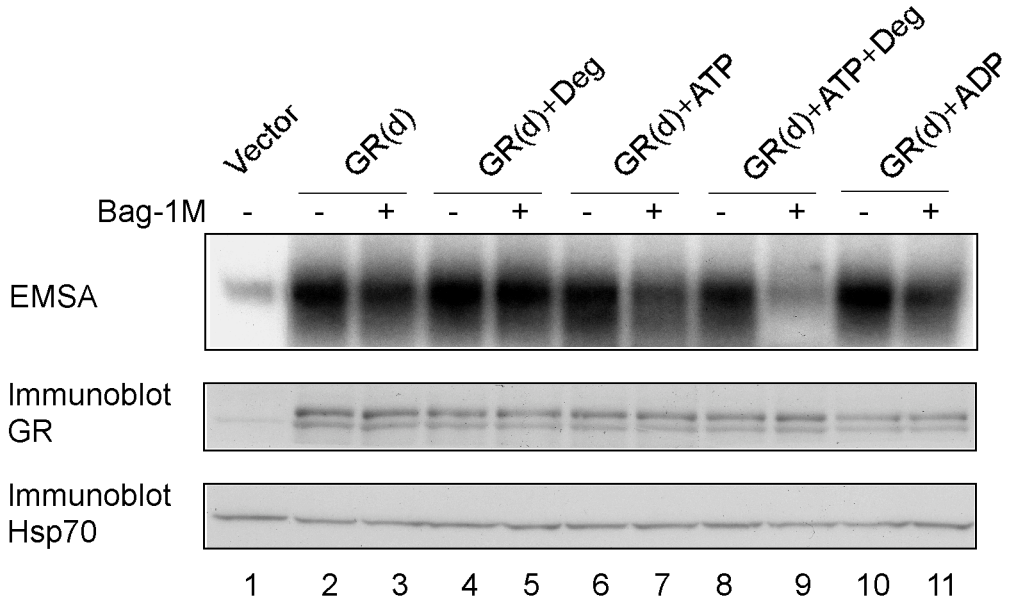


Fig 3.8 ATP depletion prevents BAG-1M from downregulating the DNA binding activity of the GR. COS-7 cells were transiently transfected with empty vector or expression vector for the GR. Twenty-four hours after transfection, the cells were treated with 10^{-7}M dexamethasone for 16h then the cells were incubated with 2-deoxyglucose (10 mM) for 4h. Whole cell extract was prepared for EMSA and immunoblotting was performed with anti-GR and anti-Hsp70 antibodies.

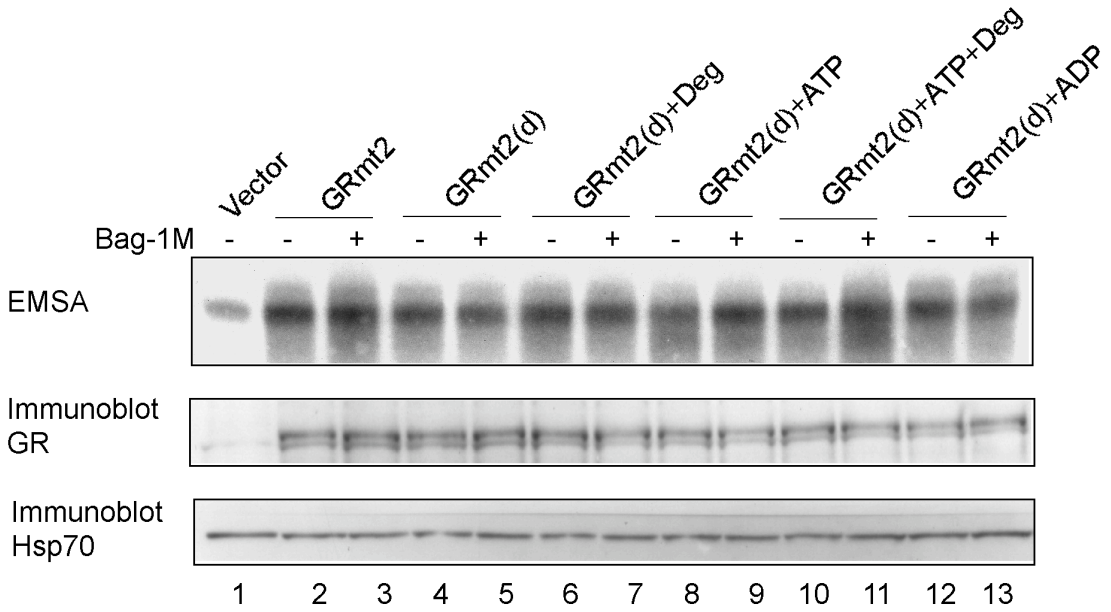


Fig 3.9 Lysine 496 and isoleucine 497 are required for the downregulation of the DNA binding by the GR. COS-7 cells were transfected with an expression vector encoding the mutated GR (GRmt2) and cellular extracts were incubated with bacterially purified Bag-1M and used for EMSA in the presence of ATP, ADP and ATP plus glycerol kinase as described previously. Aliquots of the extracts were also used for immunoblot assay with anti-GR and Hsp70 antibody.

3.4 GR, Bag-1, molecular chaperones are present on glucocorticoid response element

To determine whether Bag-1mediated alteration of the DNA binding properties of the GR occurs prior to the interaction of the receptor with DNA or on DNA, chromatin immunoprecipitation assay (ChIP) was performed to find out whether the GR and Bag-1 are both present on the hormone response element (HRE) of a glucocorticoid regulated gene. ChIP is a sensitive assay to examine the binding of proteins of interest to a fragment of DNA, normally the promoter of a gene. This assay combines several steps — first, in vivo formaldehyde cross-linking of whole cells that freezes protein-protein and protein-DNA interactions, followed by immunoprecipitation of protein-DNA complexes with specific antibodies from sonicated extracts. The immunoprecipitated cross-linked complexes will then be reversed and the DNA can be monitored after amplification by PCR. Thus it confirms that the DNA fragment is bound by a protein of interest. The glucocorticoid regulated human metallothionine IIa gene (Karin et al, 1984) in HeLa cells and the rat tyrosine amino transferase gene in FTO hepatoma cells (Rigaud et al, 1991) were analysed. Following the use of different antibodies to immunoprecipitate non-histone chromatin proteins from these cells, it was shown in a PCR reaction that distinct proteins are bound to the HREs of these genes. In the absence of hormone none of the antibodies used immunoprecipitated the HRE fragment indicating that none of the factors examined bound to the HRE (Fig. 3.10). Thirty minutes after treatment with the glucocorticoid dexamethasone, the GR, Bag-1, Hsp70 and Hsp90 were shown to be present at the promoter (Figs. 3.10 a and b, lanes 3-6). The proteins present at the promoter were identical for the two different genes in the different cell lines analysed. In addition, it could be shown that the coactivators CBP/p300 and SRC-1 were also present at the two HREs (Figs 3.10 A and B lanes 11-12). However corepressors or histone deacetylases 1 and 2 that are known to associate with the promoter of repressed genes were not detected (Collingwood et al, 1999). Thus Bag-1 and the GR as well as the molecular

chaperones Hsp70 and Hsp90 all seem to be present at the HRE of the glucocorticoid regulated genes.

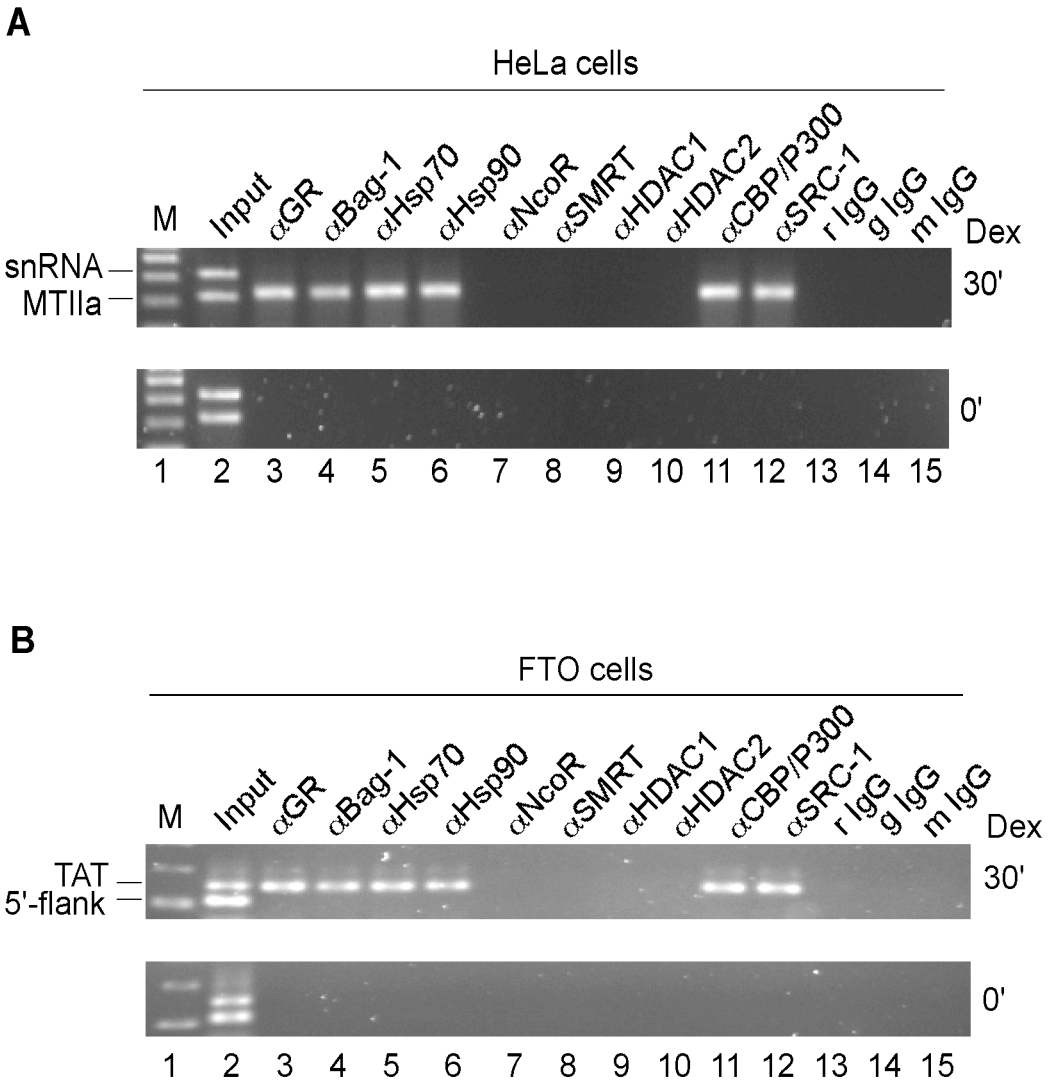
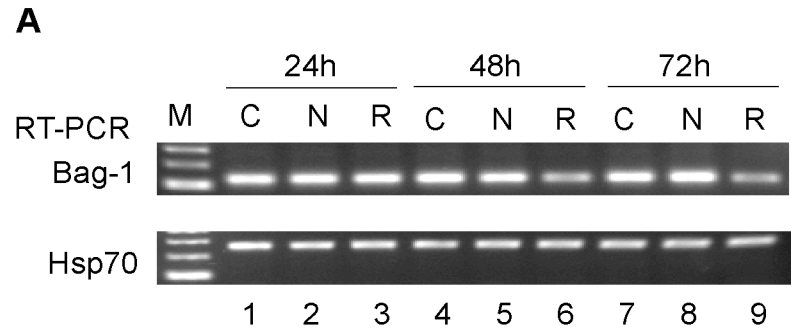


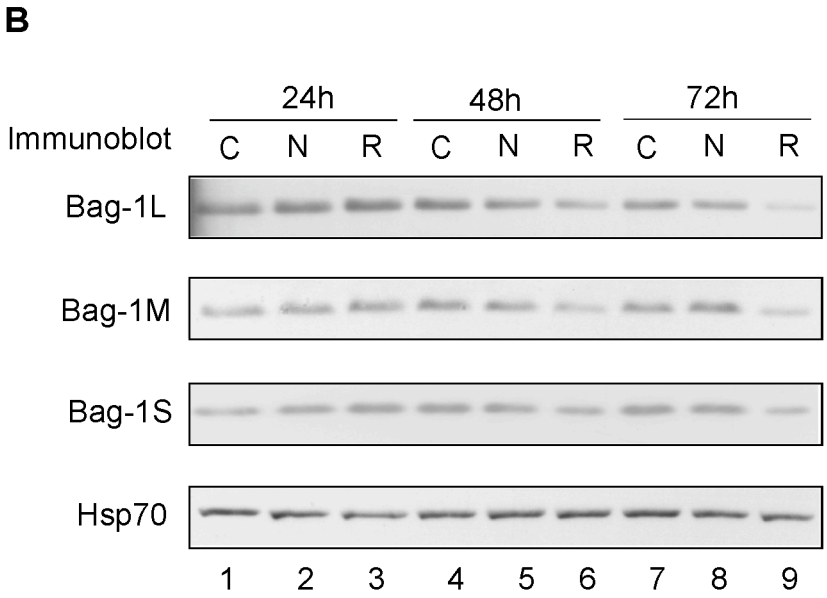
Fig 3.10. In vivo occupancy of GREs by GR and Bag-1M. **Rat hepatoma FTO and human HeLa cells were treated either with vehicle (0.1% ethanol) or with vehicle containing dexamethasone (10^{-7}M) for 30 min. The binding of the GR, Bag-1M, molecular chaperones, coactivators and corepressors to two functional GREs that mediate the glucocorticoid induced expression of tyrosine aminotransferase (TAT) in FTO cells** (B) **or metallothionein IIa (MT IIa) in HeLa cells** (A) **was assessed by chromatin immunoprecipitation. Two non-GRE-containing fragments, a fragment 2.5 kb upstream of the GRE of the TAT gene in FTO cells and a fragment at the promoter region of the snRNA gene in HeLa cells were monitored as controls. Presented as rIgG and mIgG are lanes containing chromatin material treated with rabbit and mouse immunoglobulin G respectively.**

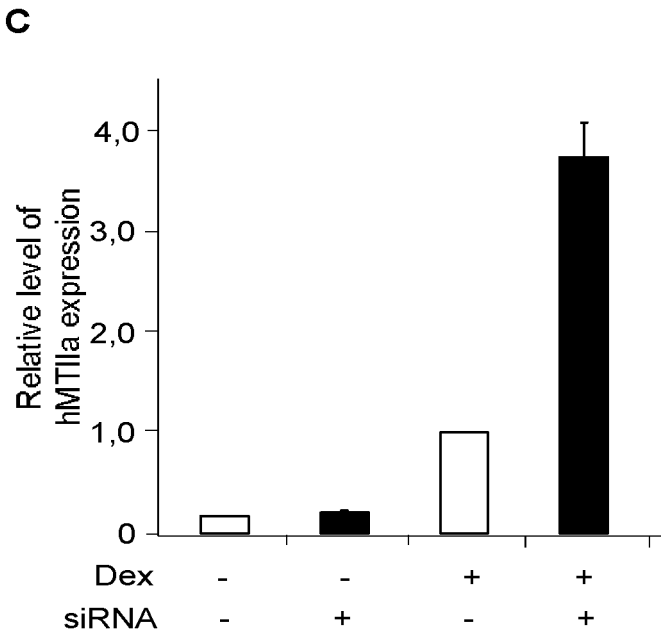
3. 5 Bag-1 downregulates DNA binding by the GR

To determine the effect Bag-1 exerts at the response element, siRNA was used to alter the level of this protein in HeLa cells and to examine its effect on GR action. Bag-1 siRNA and control siRNA were transfected into HeLa cells and the level of Bag-1 RNA was examined after 24 h, 48 h and 72 h in an RT-PCR reaction. Although the RNA from the non transfected and control siRNA transfected cells (Fig 3.11. A lanes 1, 2, 4, 5, 7 and 8) did not show any change in the Bag-1 RNA level up to 72 h, the siRNA transfected cells (Fig 3.11 lanes 3, 6 and 9) showed a clear down regulation 48 and 72 h following transfection (Fig 3.11.A compare lanes 6 and 9 with 3). Consistent with this finding, immunoblottting showed that the three Bag-1 proteins (Bag-1L, -1M and –1S) synthesized from the same mRNA in HeLa cells were downregulated following 48 and 72 h of transfection of the siRNA (Fig 3.11.B lanes 6 and 9). Under conditions of the reduced level of the Bag-1 proteins, glucocorticoid response at the hMTIIa promoter in HeLa cells was determined by real-time PCR analysis. In this study the transcripts of the hMTIIa gene was found upregulated nearly 4-fold (Fig. 3.11.C). The factor occupancy at the HRE of the hMTIIa gene under conditions of reduced and normal levels of Bag-1 was then compared in the ChIP assay.

In conditions of reduced Bag-1M, which is evident by the weak but reproducible PCR product (Fig. 3.11.D compare lanes 7 with 6), an increase in the PCR products indicative of increased binding by the GR, Hsp70, Hsp90 and the coactivators CBP/p300 and SRC-1 was observed (Fig. 3.11.D lanes 5, 9, 11, 21 and 23). These results confirm that in the absence of the siRNA, GR binding to the HRE is under negative control by the Bag-1 proteins. Furthermore, the results show that GR, the molecular chaperones Hsp70 and Hsp90 as well as the coactivators CBP/p300 and SRC-1 are all associated with the promoter in a complex. A change in the binding properties of GR to the promoter also affected the level of the other proteins at the promoter that bind to the GR.


A
24h
48h
72h
RT-PCR
M
C N R C N R C N R
Bag-1
Hsp70
1 2 3 4 5 6 7 8 9


B
24h
48h
72h
Immunoblot
C N R C N R C N R
Bag-1L
Bag-1M
Bag-1S
Hsp70
1 2 3 4 5 6 7 8 9


C
Relative level of hMTIIa expression
4,0
3,0
2,0
1,0
0
Dex - - + +
siRNA - + - +

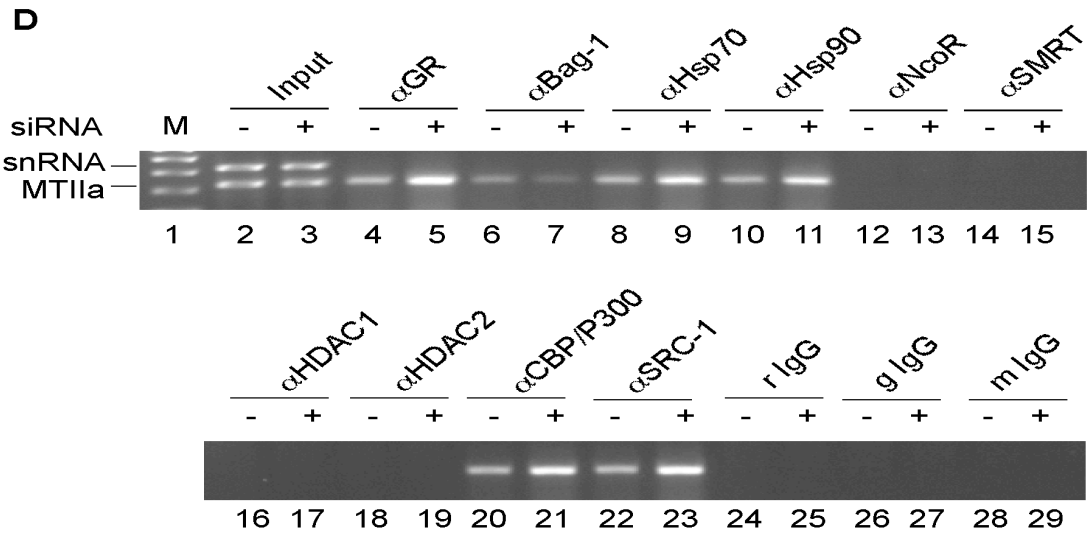
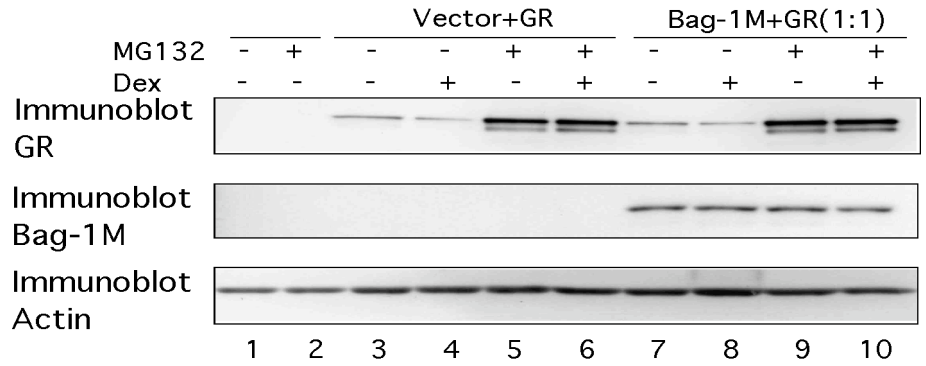


Fig 3.11. siRNA gene silencing abolishes the Bag-1-mediated repression of glucocorticoid activity. A, Bag-1 siRNA downregulates Bag-1 gene transription. HeLa cells were transfected with Bag-1 siRNA (R), an irrelevant siRNA luciferase GL3 (N) or left untreated (C) for the indicated period of time. Total RNA was isolated and used for RT-PCR to determine the remaining amount of Bag-1 RNA. Shown are the products of the RT-PCR reaction after agarose gel electrophoresis. **B,** Bag-1 gene silencing downregulates Bag-1 protein levels. Aliquots of the cells transfected with the siRNA were used for an immunoblot assay with anti-Bag-1 and anti-Hsp70 antibodies. Shown are the immunoblots of the Bag-1 level in the transfected cells. **C,** Bag-1 siRNA transfection enhances the expression of hMTIIa in HeLa cells. Cells transfected with the control and Bag-1 siRNA for 48 h were additionally treated with or without dexamethasone (10^{-7}M) for 16 h. Total RNA was isolated and used for analysis of hMTIIa expression by Real-time PCR. **D,** DNA binding by the GR is enhanced following siRNA gene silencing of Bag-1 gene expression. Promoter occupancy on the hMTIIa gene by the GR, Bag-1, molecular chaperones, coactivators and corepressors was determined by ChIP assay with cells transfected with the Bag-1 siRNA.

3.6 Bag-1M is not involved in Ubiquitin-Proteasome –mediated GR degradation

Bag-1M mediated downregulation of the DNA binding of the GR can result from Bag-1 mediated degradation of the GR since this protein is reported to degrade the receptor in the presence of another cochaperone of Hsp70 termed CHIP. CHIP has been found binds to the GR and GR can be ubiquitinated by CHIP in vitro and can be degraded in a proteasome-depended manner in vivo by this molecule (Connell et al, 2001). It is worthwhile to investigate whether Bag-1M-mediated downregualtion on the action of GR is due to its involvement in the ubiquitin-proteasome pathway for receptor degradation. Thus, COS-7 cells were cotransfected with GR and Bag-1M expression vectors and then immunoblotting was performed to determine the level of the receptor following treatment with glucocorticoid. In addition, the transfected cells were also treated with the proteasome inhibitor, MG132, to identify the involvement of the proteasome in the degradation of the GR. The data in this study showed that when MG132 was used to inhibit the proteasome activity the receptor level was increased, indicating that under basal conditions, the GR was degraded through the proteasome (Fig 3.12 A compare lanes 5 and 6 with 3 and 4). However, in the presence of Bag-1M, the receptor levels were not altered in the absence or presence of MG132 (Figure 3.12 A compare lanes 7 and 8 with 3 and 4; lanes 9 and 10 with 5 and 6). Furthermore, when the amount of transfected Bag-1M was increased to 3 folds, the result achieved was identical to the former one (Figure 3.12 B). Taken together, the results of this study demonstrated that Bag-1M is not involved in the GR degradation via the ubiquitin-proteasome

A



B

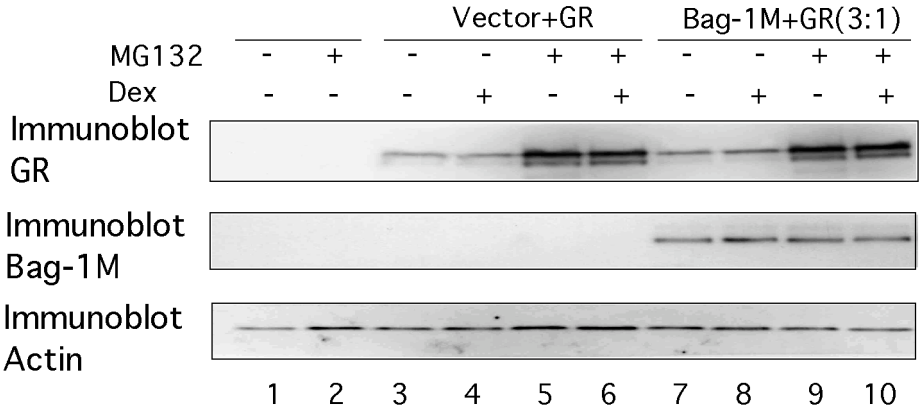


Figure 3. 12 BAG-1M is not involved in proteasome-mediated GR degradation. A. COS-7 cells were either non-transfected or transfected with 0.2□g of expression vector encoding GR. In addtion, the cells were cotransfected with either 0.2□g of empty vector or 0.2□g of expression vector encoding BAG-1M. 50ng internal control plasmid encoding Renilla luciferase was also trasnfected. The cells were treated with either vehicle (Dimethyl sulfoxide,DMSO) alone or 10^{-6}M MG132 for 1h and then treated with either vehicle(ethanol) or dexamethasone (10^{-7}M) for 16h. Whole cell lysates were generated and used to measure the Renilla luciferase activities from the Renilla construct which was used as controls for transfection efficiencies. Immunoblots for GR, BAG-1M or actin were carried out with respective antibodies. **B.** The same conditions were applied except cotransfecting 0.6□g of empty vector or 0.6□g of expression vector encoding BAG-1M.

3.7 Bag-1M-mediated recruitment of histone deacetylases

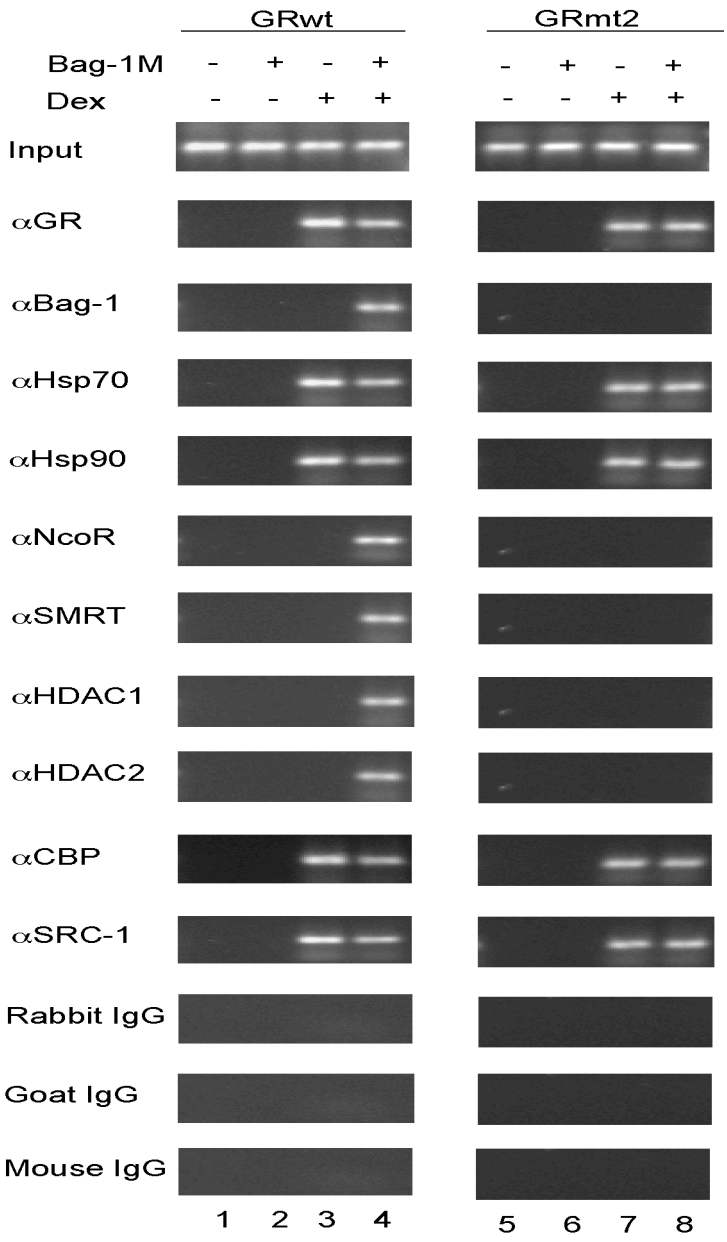
As shown previously, Bag-1M inhibits DNA binding by the GR and Bag-1M is not involved in ubiquitin-proteasome –mediated receptor degradation which may influences the DNA binding property of the GR. The next question that needs to be answered in how Bag-1M alters the action of the GR complex at regulatable promoters. To answer this question, ChIP assays with transfected cells expressing the wild-type and mutant (GRmt2) GR together with or without Bag-1M were carried out. Twenty-four hours after transfection, the cells were treated with vehicle ethanol or vehicle containing dexamethasone for additional 16 hours. The cells were then cross-linked by formaldehyde and were sonicated. The lysate was used to immunoprecipitate the DNA-protein complexes with specific antibodies and the immunoprecipitated complexes were decross-linked afterwards. The DNA was purified and amplified by PCR reaction and monitored on the agarose gel.

In the presence of Bag-1M but in the absence of hormone, no factors were bound to the HRE of transfected hMTIIa reporter gene as shown by the absence of PCR products (Fig. 3.13 A lanes 1,2 and 5, 6). In the presence of dexamethasone, the GR was bound to the HRE together with the coactivators CBP/p300 and SRC-1 as well as molecular chaperones as we had shown in HeLa cells (Fig. 3.13 A lanes 3 and 7). In the presence of dexamethasone and Bag-1M, this cochaperone was present at the HRE in cells expressing the wild-type receptor but not the GRmt2 (Fig. 3.13 A lanes 4 and 8) since Bag-1M does not bind the mutant GR. As expected the amount of wild-type GR, molecular chaperones and coactivators bound to the HRE was decreased by the presence of Bag-1M but no inhibition was seen in the case of cells expressing GRmt2 where Bag-1M was not present at the promoter (Fig. 3.13 A lanes 4 and 8). Intriguingly, the presence of Bag-1M and the wild type GR at the HRE led to the recruitment of the corepressors SMRT and NcoR as well as the histone deacetylases HDAC-1 and -2 but this was not the case when the GRmt2 was present but Bag-1M was absent (Fig. 3.13 A lanes 4 and 8). This would indicate that in addition to downregulating GR, cochaperone and

coactivator occupancy at the HRE, Bag-1M recruited corepressors and histone deacetylases to the HRE.

To demonstrate that the HDACs, Bag-1M, GR and the molecular chaperones are all present at the same promoter, a re-ChIP analysis was performed where the chromatin was immunoprecipited with Bag-1 antibody and re-immunoprecipitated with GR, Hsp70, Hsp90, HDAC-1 and HDAC-2 antibodies. Control experiments with the eluate of the first immunoprecipitation and protein A sepharose beads alone showed that the first complexes were completely disrupted and inactivated before the use of the second set of antibodies (Fig. 3.13 B). As the HRE was amplified following immunoprecipitation with all the second sets of antibodies, these experiments show that the same promoter is bound by GR, Hsp70, Hsp90, Bag-1M, HDAC-1 and HDAC-2 (Fig. 3.13 B).

A


GRwt
GRmt2
Bag-1M - + - + - + - +
Dex - - + + - - + +
Input
αGR
αBag-1
αHsp70
αHsp90
αNcoR
αSMRT
αHDAC1
αHDAC2
αCBP
αSRC-1
Rabbit IgG
Goat IgG
Mouse IgG
1 2 3 4 5 6 7 8

B

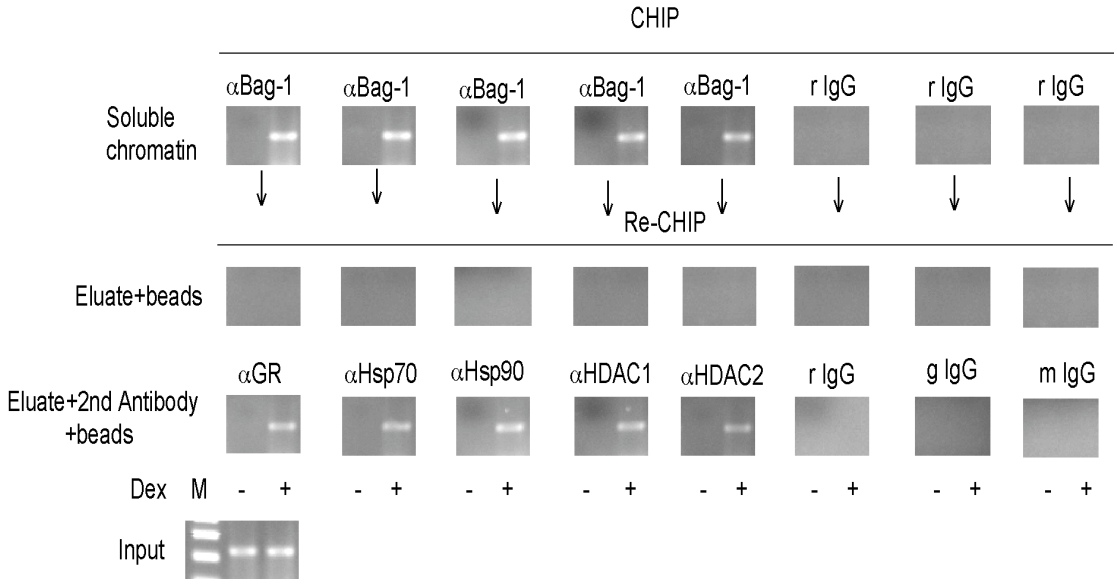
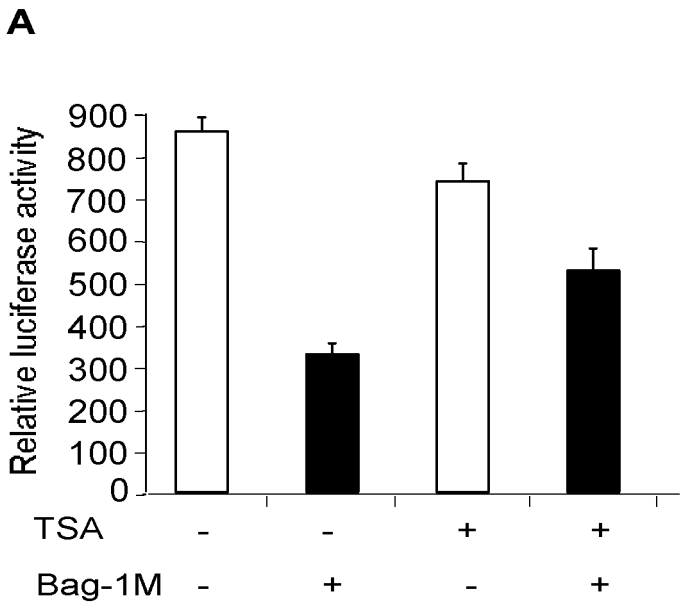


Fig 3.13 Bag-1M recruits corepressors to the GRE of hMTIIa in transfected COS-7 cells. A, Corepressors are bound to the GRE of hMTIIa in the presence of Bag-1M and the wild-type GR but not the mutant GR (GRmt2). COS-7 cells were transiently cotransfected with a vector harbouring an hMTIIa indicator gene, expression vectors encoding either the wild type GR or GRmt2, together with or without an expression vector for Bag-1M. The cells were treated with or without dexamethasone for 16 h and were analysed for factor occupancy at the hMTIIa gene promoter by the ChIP assay. **B,** Bag-1 and molecular chaperones as well as corepressors are assembled in the GR containing complexes bound to the GRE. Aliquots of the chromatin extract immunoprecipitated by anti-Bag-1 antibody and rabbit IgG (r IgG) as control were eluted and divided into two portions. One portion was incubated with protein A sepharose beads and the other portion with the indicated second set of antibodies and beads. The precipitated material with the second set of antibodies was then subjected to the PCR amplification procedure with hMTIIa promoter specific primers. Immunoprecipitation with the second set of antibodies was carried out along with rabbit, mouse and goat IgG (rIgG, mIgG and gIgG) as controls.

3.8 Effect of trichostatin A on Bag-1M–mediated repression of glucocorticoid receptor activity.

The binding of HDACs to the promoter leads to the repression of the target gene and the negative regulatory effect of HDACs can be released by trichostatin A (TSA) (Nagy et al, 1997). COS-7 cells transiently transfected with the hMTIIa reporter gene, the renilla control plasmid, the wild-tpye GR and Bag-1M for luciferase assay were therefore treated with dexamethasone and TSA. The cells were then lysed and the lysates were used to measure the luciferase activity. In figure 14, it is shown that the Bag-1M mediated inhibition of transactivation by the GR is partially reversed by TSA (Fig. 14 A). This proves that recruitment of histone deacetylases by Bag-1M in the negative regulation of glucocorticoid action in COS-7 cells involves histone deacetylation. In contrast, no significant effect of TSA was found on the glucocorticoid regulated expression of hMTIIa and TAT in HeLa cells and in FTO cells (Fig. 14 B) supporting the finding that histone deacetylases are not recruited to the HREs of these genes by Bag-1M.



B

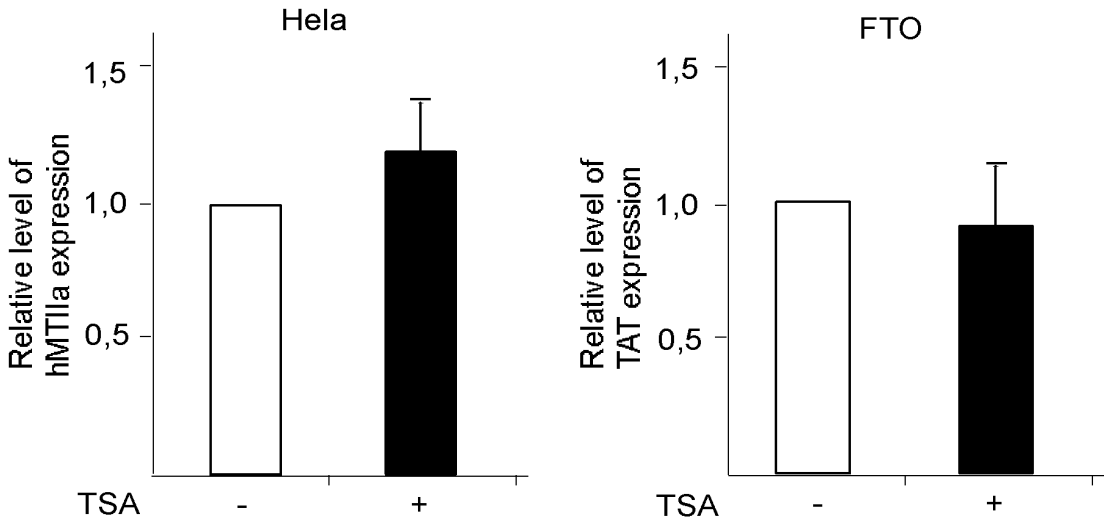


Fig 14. Effect of trichostatin A on Bag-1M–mediated repression of glucocorticoid receptor activity. A, **TSA partially relieves Bag-1M-mediated repression of transactivation by the GR. COS-7 cells were transiently transfected with a GR responsive MMTV-luciferase indicator gene and an internal control renilla luciferase gene. In addition, GR expression vector was cotransfected with or without Bag-1M expression vector. The cells were treated with dexamethasone along with or wilthout TSA (100 nM) for 16 h. Thereafter the cells were harvested and disrupted and the lysates were used for luciferase activity measurements. The results of the relative activity of the MMTV reporter gene in the presence of hormone are expressed as bar charts. This represents the mean value and the standard deviation of three independent experiments.** B, **Expression of endogenous hMTIIa and TAT gene is not affected by TSA. Approximately 90% confluent HeLa and FTO cells were treated with dexamethasone together with or without TSA for 16 h. Total RNA was isolated and was used to examine the transcripts of hMTIIa gene and TAT gene by the real-time PCR technique.**

3.9 Corepressors and histone deacetylase 1 and 2 are not assembled with the Bag-1 complex targeted to the GRE of hMTIIa gene in FTO cells.

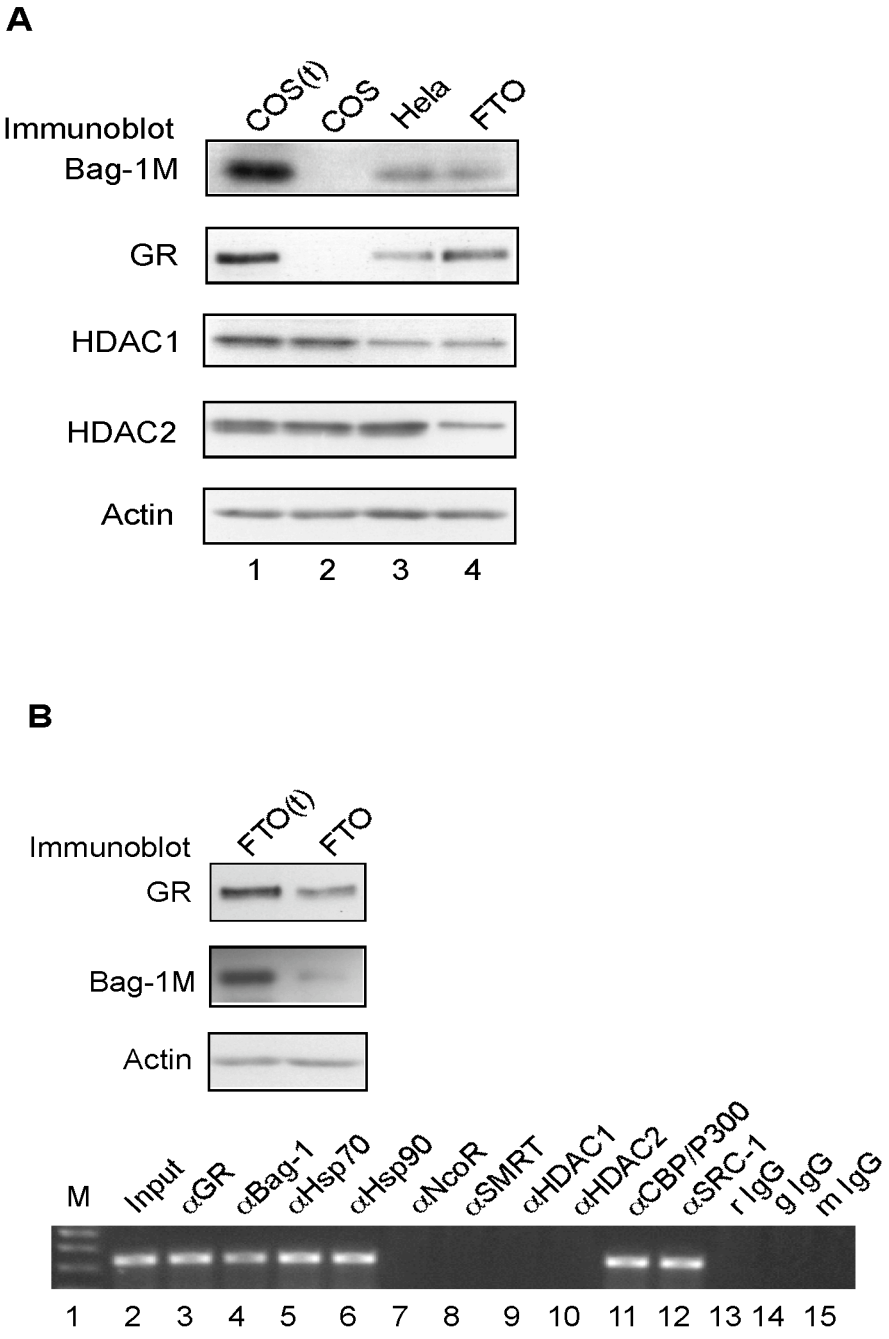
The experiments presented here show a dual mechanism of action of Bag-1M. First, Bag-1M downregulates the DNA binding of the GR and second, Bag-1M recruits corepressors and HDACs to the promoter of glucocorticoid target gene to inhibit the transactivation function of the GR.

To determine whether the differences in action of Bag-1M is due to different cellular levels of the factors involved in the regulation of GR action, immunoblot assays were carried out and the levels of GR, Bag-1M, HDAC-1 and HDAC-2 in transfected and nontransfected COS-7 as well as in HeLa and FTO cells were compared. This study showed a lower level of most of these factors in HeLa and FTO cells compared to the transfected COS-7 cells (Fig. 15 A).

Transfection of the FTO cells with the hMTIIa reporter construct and expression vectors encoding the associating factors was therefore undertaken. Twenty-four hours after transfection, the cells were treated with hormone for additional 16 hours and the cells were cross-linked and performed for ChIP assay. Aliquots of the transfected cells and the normal cells were also used for immunoblots to examine the expressions of transfected constructs.

An increase in the level of the GR and Bag-1M (Fig. 15 B) or the level of the histone deacetylases (results not shown) or all four proteins (Fig. 15 C) had no effect on the recruitment of histone deacetylases to the HRE of the hMTIIa as determined in the ChIP assay. While the GR, Bag-1 and the coactivators could be identified by immunoprecipitation, the corepressors and histone deacetylases could not be found at the response element (Figs. 15 B and C). These results indicate that cell-type specific factors or the level of corepressors necessary for the assembly of the histone deacetylase complex may be limiting in HeLa and FTO cells.

Thus, the Bag-1 proteins use different mechanisms to inhibit GR activity and depending on the cell-type, more than one mechanism may be used to efficiently block the action of the GR.



C

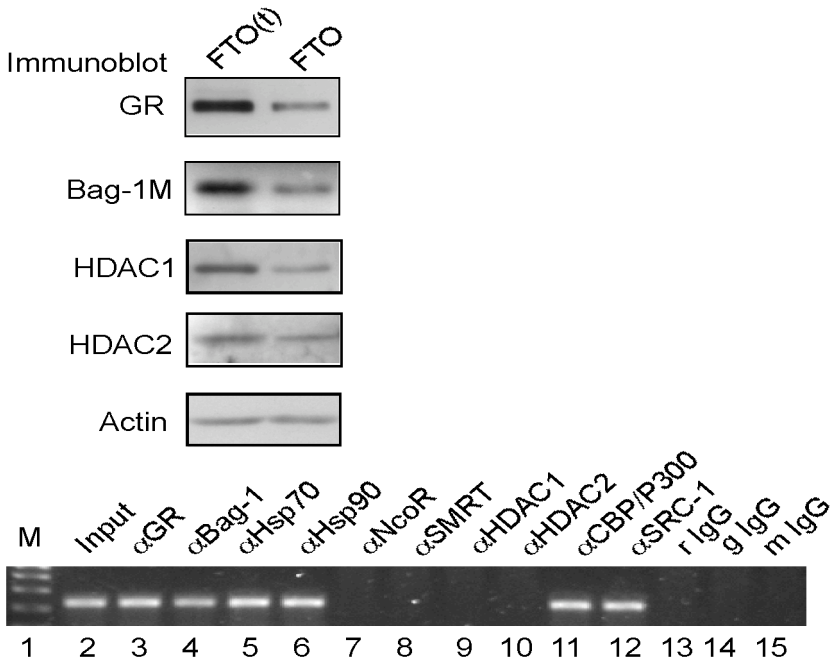


Fig 15. Corepressors are not assembled with the Bag-1 complex targeted to the GRE of hMTIIa gene in FTO cells. A, **Expression of GR, Bag-1M, HDAC1 and HDAC2 in different cells. COS-7 cells were transiently cotransfected with Bag-1M expression vector along with an expression vector for GR. The transfected cells together with normal COS-7, HeLa and FTO cells were lysed and the extracts were used for an immunoblot assay with anti-GR, -Hsp70, -HDAC-1 and –HDAC-2 specific antibodies.** B, **and** C, **FTO cells were transiently cotransfected with the hMTIIa indicator gene and expression vectors encoding GR, Bag-1M** (B) **or with the indicator gene and expression vectors for GR, Bag-1M, HDAC1 and HDAC2** (C). **The cells were treated with dexamethasone for 1 h, harvested and divided into aliquots. One aliquot was used to monitor the expression of the transfected plasmids by immunoblotting and the other was used to examine the promoter occupancy on the transfected hMTIIa indicator gene by the ChIP assay.**

4. Discussion

4.1 Bag-1M downregulates the transactivation activity of the GR

Nuclear hormone receptors function as transcription factors that are activated in response to their specific hormones. Multiple studies have shown that interactions between Hsp70 and Hsp90 and their respective cochaperones are essential for activation of the nuclear receptors (Picard et al, 1990; Pratt and Toft, 1997; Arbeitman and Hogness, 2000; Rajapandi et al, 2000). The receptor in the absence of hormone forms a multiprotein complex consisting chaperones and cochaperones. Upon hormone binding, the receptor is thought to be dissociated from the multiprotein chaperone complex and is translocated into the nucleus where it binds specific DNA sequences. Chaperones and cochaperones are therefore assumed to play an exclusive role in the cytoplasm in maintaining the receptor in an inactive state.

However, recent findings divert from this notion and demonstrate that the activity of nuclear receptors is regulated by chaperones both before and after ligand binding and some chaperones and cochaperones are recruited to the nucleus and may have a nuclear function downstream of ligand binding, for instance, Hsp70 and Hsp90 have been found colocalized with nuclear receptors and regulated the activities of the receptors in the nucleus (Landel et al, 1994; Meng, et al, 1996; DeFranco, et al, 1998). Cochaperone Bag-1M has also been found to downregulate the transactivation activity by the GR (Kullmann et al, 1998; Schneikert et al, 1999). Bag-1M negatively regulates the transactivation activity of the GR through the hinge region of the receptor (Kullmann et al, 1998; Schneikert et al, 1999). However, the amino acids within the hinge region that may be involved in the interaction with Bag-1M were not identified. The work presented here clearly demonstrated that the 496 lysine and 497 isoleucine within the hinge region are essential for Bag-1M-mediated negative effect, since Bag-1M neither inhibit the DNA binding

nor repress the transactivation function by the K496L/I497G double GR mutant.

The mechanism of how Bag-1M inhibits the transactivation by the GR is so far not understood. It was postulated that an $[EEX_4]_8$ sequence, located in the first NH_2-terminal 70 amino acids of Bag-1M, which is highly enriched in threonine and serine residues might contribute to the downregulation of transactivation by the GR (Schneikert et al, 1999). Bag-1M is a phosphoprotein with almost all the phosphorylated residues localized at the $[EEX_4]_8$ sequence (Schneikert et al, 2000), in addition, Bag-1S, the smallest isoform of Bag-1 proteins, which harbors only two rather than eight $[EEX_4]$ motives, has no effect on the transactivation function of the GR, suggesting a role of phosphorylation in the action of Bag-1M (Schneikert et al, 1999).

Diverse evidence from the finding of Schneikert showed that the first NH_2-terminal 8 amino acids of Bag-1M containing a stretch of three lysines and a stretch of three arginines are necessary for inhibition of GR action (Schmidt et al, 2003). This is a small DNA binding sequence which is uncommon for transcriptional regulatory proteins, because DNA binding appears to be nonspecific (Zeiner et al, 1999). The nonspecificity of DNA binding of Bag-1M is in line with the observation that spacing of the two positively charged stretches of three lysines and three arginines is not important for DNA binding indicating an eletrostatic interaction of these positive charged amino acids with the negatively charged phosphate backbone of DNA. The relation between nonspecific DNA binding of Bag-1M and its repression on GR transactivation is however still ambiguous.

Evidence provided in this research demonstrated that Bag-1 in the presence of GR binds to specific GRE in different cell types and binding of Bag-1 to GRE recruits corepressors in specific cell type. Against the evidence of nonspecific DNA binding of Bag-1M, the data presented here were achieved not only by cotransfecting Bag-1M and a reporter gene, but also by examining the occupancy of endogenous genomic DNA by Bag-1M.

It has been demonstrated that administration of dexamethasone results in a significant downregulation of the expression of the GR (Sapolsky et al, 1984; Tornello et al, 1982; Schlechte et al, 1982) and pretreatment of COS-1 cells

expressing the mouse GR with the proteasome inhibitor MG132 inhibited dexamethasone-induced downregulation of the receptor, and result in enhanced transactivation activity of the receptor (Wallace and Cidlowski, 2001), indicating that the proteasome is involved in hormone induced GR degradation. Bag-1M contains a ubiquitin-like domain (UBL), suggesting a link to the ubiquitin-proteasome system. Bag-1S and Bag-1M have been demonstrated to co-immunoprecipitate with the 20S core and the 19S subunit of the proteasome (Lüders et al, 2000). Bag-1S has also been shown to cooperate with the C-terminus of Hsc70-interacting protein (CHIP), a newly identified ubiquitin E3 ligase, in the degradation of the GR via the proteasome (Connell et al, 2001). However, no function for the interaction of Bag-1M with the proteasome has been demonstrated. Thus, COS-7 cells overexpressing Bag-1M and GR were treated with MG132 in this study and demonstrated that Bag-1M was not involved in ubiquitin-proteasome-mediated degradation of the GR, in agreement with the finding that Bag-1M has no effect on GR ubiquitylation (Connell et al, 2001). It can therefore be ruled out that Bag-1M downregulates the transactivation function of the GR by targeting the receptor to the proteasome for degradation.

4.2 Hsp70 is involved in Bag-1M-mediated inhibition of GR activity

Hsp70 has been identified as an adaptor protein for the interaction of Bag-1M with the GR (Schneikert et al, 2000). In surface plasmon resonance (SPR) measurements, Bag-1M was demonstrated not to interact with the GR unless Hsp70 was bound to the receptor. Furthermore in cofocal immunofluorescence assays a nuclear transport of Hsp70 by the liganded GR was observed (Schneikert et al, 2000). In addition, Schneikert et al have found as well that Hsp70 interacts the unliganded GR, indicating that Hsp70 bound both the non-activated as well as the ligand-activated receptor. The chromatin immunoprecipitation (ChIP) and Re-ChIP assays of this present work confirm those findings in that Hsp70 is indeed in the same GR

containing complex bound to the promoter of the glucocorticoid target genes, suggesting a nuclear role of Hsp70.

The Bag domain of Bag-1 mediates direct interaction with the Hsp70 ATPase domain (Takayama et al, 1997; Zeiner et al, 1997) and stimulates the ATPase rate of Hsp70 in an Hsp40-dependent manner to promote substrate release from the chaperone (Höhfeld and Jentsch 1997). In this case, Bag-1M binds to Hsp70-GR complex (Schneikert et al, 2000) and can release the bound GR in an ATP-dependent process (Brehmer et al, 2001; Sondermann et al, 2001), it was therefore postulated that this might form the basis of its action. The GR released from the complex with the molecular chaperones, is presumably in an unfavorable conformation for DNA binding and this may be the reason for the reduced DNA binding by the receptor. ATPase activity plays an important role not only in the action of Hsp70 but also in the chaperone activity of Hsp90 and ATP hydrolysis is reported to be important for the release of bound substrate from Hsp90 (Young and Hartl, 2000). It is therefore likely that during ATPase hydrolysis, GR-Hsp70-Bag-1M complex is released from Hsp90 and may be one of the steps leading to the release of the GR from the heterocomplex with the molecular chaperones. In any case, the downregulation of DNA binding by Bag-1M seen during ATP hydrolysis is most likely due to a conformation alteration of the receptor and not Bag-1M-mediated degradation of the receptor. The whole regulatory action of Bag-1M takes place on DNA since the decrease of bound GR is also associated with a decrease of Hsp70, Hsp90 and coactivators that associate with the GR on DNA.

One of the most important findings of this study is that molecular chaperones and cochaperones are present on glucocorticoid response elements and play an important role in controlling binding of the receptor to DNA. This finding is believed important in view of many reports on the contribution of molecular chaperones to ligand binding of the nuclear receptors (Pratt 1993; Dittmar et al, 1997; Morishima et al, 2000; Picard et al, 1990; Bohen et al, 1995), that are so far thought to be their main function in nuclear receptor action. This result agrees with the finding that Bag-1L and Hsp70 are present on the response element of an androgen target gene (Shatkina et al, 2003). Furthermore, it has been reported that the cochaperone p23 is present on

response element and release the bound GR to allow its recycling (Freeman and Yamamoto, 2001; Freeman and Yamamoto, 2002). This process may also require ATP hydrolysis as p23 couples the ATPase activity of Hsp90 to polypeptide dissociation and thus functions as a substrate release factor of Hsp90 (Young and Hartl, 2000).

It is not so easy to determine at this stage which GR molecule are released by p23 and which ones by Bag-1M on a glucocorticoid target gene, since both processes require ATP hydrolysis and both Hsp70 and Hsp90 are involved. However, the two cochaperones, Bag-1M and p23 seem to be functionally opposed to each other. While Bag-1M functions to attenuate GR signaling, p23 functions to restore the original status of the receptor for DNA binding by allowing for recycling of the receptor. This positive and negative regulation of GR action may be important control process in glucocorticoid physiology.

4.3 Bag-1M recruits histone deactylases in COS-7 cells

Transcriptional repression by a sequence specific DNA-binding factor, such as nuclear hormone receptor, can be mediated by the recruitment of histone deacetylases to the promoter region. It has been shown that antagonists of nuclear receptors repress the expression of target genes via altering the conformation of the receptor which results in recruitment of corepressors to the target gene. In this study, histone deacetylases (HDAC) 1 and 2 have been indicated as being recruited to the promoter of a glucocorticoid responsive gene in the presence of Bag-1M in transfected COS-7 cells. This suggests that apart from reducing the DNA binding of the GR Bag-1M also recruits HDACs to repress the expression of the GR target gene. However, in HeLa and FTO cells which contain endogenous Bag-1M, HDACs were not recruited to the glucocorticoid response elements. The reason for this could be due to variations in the level of HDACs, Bag-1M or the receptor in these cell types compared with the transfected COS cells. This possibility however was ruled out because HDACs still do not bind the GREs in transfected HeLa and FTO cells, indicating that the recruitment of HDACs by Bag-1M is cell

type specific. This is most likely due to other factors in COS-7 cells that function as limiting factors for the recruitment of HDACs.

4.4 Dual mechanism for modulation of GR activity by Bag-1M

One of the key questions that remain to be answered is why different mechanisms have been developed to ensure the downregulation of GR action by Bag-1M. To answer this question requires a good knowledge of the physiology of Bag-1. The expression pattern of the Bag-1 family proteins during different stages of mouse embryogenesis has been carried out to determine whether it correlated with that of the GR (Crocoll et al, 2000). Bag-1M has been detected as early as 10.5 embryronic days postcoitum (E) with high level expression in the kidney and after E 17.5 it goes down and can not be detected before birth, although it is again detected in the adult kidney. As kidney is a classic mineralocorticoid target that expresses both the GR and MR, the previous data and the result of this study together showed that Bag-1M has no effect on the transactivation function of MR although both receptors can be functionally activated by glucocorticoids, suggesting that Bag-1M-mediated downregulation of GR action may also be relevant in the control of GR activity during development. Glucocorcorticoid levels and GR action need to be controlled during embryogenesis because excess glucocorticoid exposure and action of this hormone are thought to be harmful to the developing fetus (Edwards et al, 1993). The level of glucocorticoid available to the fetus is believed to be controlled by placental 11□-hydroxylsteroid-dehydrogenase 2, an enzyme that inactivates glucocorticoid and protects the developing fetus from high maternal glucocorticoid levels (Brown et al, 1996), Another way whereby glucocorticoid action can be controlled is through downregulation of the action of the GR during development. This regulation by Bag-1M is so crucial that different mechanisms might have been developed to make sure that GR action at those development stages is completely blocked.

4.5 Summary

The mechanisms of how Bag-1M downregulates the transactivation function by the GR in different cell types were analyzed. The presented data showed that the lysine 496 and isoleucine 497 within the hinge region of the GR are essential for Bag-1M-mediated inhibition on the transactivation by the receptor. Both the amino and carboxyl termini of Bag-1M are involved in its negative regulation on the GR transactivation activity. Molecular chaperones Hsp70 and Hsp90 have also been found to bind to the glucocorticoid responsive genes. Bag-1M represses the DNA binding of the GR in an ATP hydrolysis dependent manner instead of targeting the receptor to proteasome for degradation to downregulate the transactivation function of the GR. In addition to this mechanism, Bag-1M recruits histone deacetylase1 and 2 to the promoter of glucocorticoid target gene and inhibits the transcriptional activity of the receptor in a trichostatin A sensitive and cell type specific way. However, Bag-1M does neither downregulate the DNA binding by the MR nor recruit histone deacetylases to the MR containing complex bound to DNA. Therefore, by using two regulatory mechanisms, Bag-1M probably ensures an effective attenuation of glucocorticoid action.

Abbreviation

A	adenine
aa	amino acid
ATP	adenosine tri-phosphate
bp	base pair
BSA	bovine serum albumin
C	cytosine
CBP	CREB-binding protein
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
cpm	count per minute
Da	Dalton
DBD	DNA-binding domain
DMEM	Dulbecco´s modified Eagle´s medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ECL	enhanced chemiluminescence
EDTA	ethylenediamine-N, N-tetracetate
g	gram
G	guannosine
GR	glucocorticoid receptor
GRE	glucocorticoid response element
h	hour
HAT	histone acetyltransferase
HBD	hormone binding domain
HDAC	histone deactylase
Hepes	**4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid**
HRP	horseradish peroxidase
Hsp	heat shock protein

Ig	immunoglobulin
KDa	kilodalton
kb	kilo-base
L	liter
LBD	ligand binding domain
Luc	luciferase
M	molar
min	minute
mM	milimolar
MMTV	mouse mammary tumor virus
mRNA	messenger RNA
□	micro
n	nano
nGRE	negative glucocorticoid response element
OD	optical density
p	pico
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain rection
PMSF	phenylmethanesulfonyl fluoride
RIPA	radioimmunoprecipitation assay (buffer)
RNA	ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription PCR
sec	second
siRNA	small interfering RNA
SMRT	silencing mediator of retinoic acid and thyroid hormone receptor
SRC	steroid receptor coactivator
SD	standard deviation
SDS	sodium sodecyl sulfate
T	thymine

TEMED	N,N,N´N´-tetramethylethylendiamine
TSA	trichostatin A
U	uridine
V	volt
v/v	volume on volume
wt	wild type

REFERENCES

Abma, E. M., Blanken, R., and De Heide, L. J. (2002). Cushing's syndrome caused by topical steroid therapy for psoriasis. Neth J Med *60*, 148-150.

Adachi, M., Sekiya, M., Torigoe, T., Takayama, S., Reed, J. C., Miyazaki, T., Minami, Y., Taniguchi, T., and Imai, K. (1996). Interleukin-2 (IL-2) upregulates BAG-1 gene expression through serine-rich region within IL-2 receptor beta c chain. Blood *88*, 4118-4123.

Bacallao, R., Garfinkel, A., Monke, S., Zampighi, G., and Mandel, L. J. (1994). ATP depletion: a novel method to study junctional properties in epithelial tissues. I. Rearrangement of the actin cytoskeleton. J Cell Sci *107 (Pt 12)*, 3301-3313.

Bamberger, C. M., Schulte, H. M., and Chrousos, G. P. (1996). Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. Endocr Rev *17*, 245-261.

Bannister, A. J., and Kouzarides, T. (1996). The CBP co-activator is a histone acetyltransferase. Nature *384*, 641-643.

Barnes, P. J., and Adcock, I. M. (1998). Transcription factors and asthma. Eur Respir J *12*, 221-234.

Beato, M. (1989). Gene regulation by steroid hormones. Cell *56*, 335-344.

Beato, M., Herrlich, P., and Schutz, G. (1995). Steroid hormone receptors: many actors in search of a plot. Cell *83*, 851-857.

Beato, M., and Sanchez-Pacheco, A. (1996). Interaction of steroid hormone receptors with the transcription initiation complex. Endocr Rev *17*, 587-609.

Bohen, S. P., Kralli, A., and Yamamoto, K. R. (1995). Hold 'em and fold 'em: chaperones and signal transduction. Science *268*, 1303-1304.

Brehmer, D., Rudiger, S., Gassler, C. S., Klostermeier, D., Packschies, L., Reinstein, J., Mayer, M. P., and Bukau, B. (2001). Tuning of chaperone activity of Hsp70 proteins by modulation of nucleotide exchange. Nat Struct Biol *8*, 427-432.

Briknarova, K., Takayama, S., Brive, L., Havert, M. L., Knee, D. A., Velasco, J., Homma, S., Cabezas, E., Stuart, J., Hoyt, D. W.*, et al.* (2001). Structural analysis of BAG1 cochaperone and its interactions with Hsc70 heat shock protein. Nat Struct Biol *8*, 349-352.

Brimmell, M., Burns, J. S., Munson, P., McDonald, L., O'Hare, M. J., Lakhani, S. R., and Packham, G. (1999). High level expression of differentially localized BAG-1 isoforms in some oestrogen receptor-positive human breast cancers. Br J Cancer *81*, 1042-1051.

Bronnegard, M., Stierna, P., and Marcus, C. (1996). Glucocorticoid resistant syndromes--molecular basis and clinical presentations. J Neuroendocrinol *8*, 405-415.

Buchner, J. (1999). Hsp90 & Co. - a holding for folding. Trends Biochem Sci *24*, 136-141.

Caldenhoven, E., Liden, J., Wissink, S., Van de Stolpe, A., Raaijmakers, J., Koenderman, L., Okret, S., Gustafsson, J. A., and Van der Saag, P. T. (1995). Negative cross-talk between RelA and the glucocorticoid receptor: a possible mechanism for the antiinflammatory action of glucocorticoids. Mol Endocrinol *9*, 401-412.

Carlstedt-Duke, J., Okret, S., Wrange, O., and Gustafsson, J. A. (1982). Immunochemical analysis of the glucocorticoid receptor: identification of a third domain separate from the steroid-binding and DNA-binding domains. Proc Natl Acad Sci U S A *79*, 4260-4264.

Catelli, M. G., Binart, N., Jung-Testas, I., Renoir, J. M., Baulieu, E. E., Feramisco, J. R., and Welch, W. J. (1985). The common 90-kd protein component of non-transformed '8S' steroid receptors is a heat-shock protein. Embo J *4*, 3131-3135.

Cato, A. C., and Wade, E. (1996). Molecular mechanisms of anti-inflammatory action of glucocorticoids. Bioessays *18*, 371-378.
Cato, A. C. and Mink, S. (2001). BAG-1 family of cochaperones in the modulation of nuclear receptor action. J Steroid Biochem Mol Biol 78. 379-388.

Cavailles, V., Dauvois, S., Danielian, P. S., and Parker, M. G. (1994). Interaction of proteins with transcriptionally active estrogen receptors. Proc Natl Acad Sci U S A *91*, 10009-10013.

Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997). Nuclear receptor coactivator ACTR is a novel histone

acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. Cell *90*, 569-580.

Clevenger, C. V., Thickman, K., Ngo, W., Chang, W. P., Takayama, S., and Reed, J. C. (1997). Role of Bag-1 in the survival and proliferation of the cytokine-dependent lymphocyte lines, Ba/F3 and Nb2. Mol Endocrinol *11*, 608-618.

Collingwood, T. N., Urnov, F. D., and Wolffe, A. P. (1999). Nuclear receptors: coactivators, corepressors and chromatin remodeling in the control of transcription. J Mol Endocrinol *23*, 255-275.

Connell, P., Ballinger, C. A., Jiang, J., Wu, Y., Thompson, L. J., Hohfeld, J., and Patterson, C. (2001). The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins. Nat Cell Biol *3*, 93-96.

Crocoll, A., Blum, M., and Cato, A. C. (2000). Isoform-specific expression of BAG-1 in mouse development. Mech Dev *91*, 355-359.

Darimont, B. D., Wagner, R. L., Apriletti, J. W., Stallcup, M. R., Kushner, P. J., Baxter, J. D., Fletterick, R. J., and Yamamoto, K. R. (1998). Structure and specificity of nuclear receptor-coactivator interactions. Genes Dev *12*, 3343-3356.

De Bosscher, K., Vanden Berghe, W., and Haegeman, G. (2001). Glucocorticoid repression of AP-1 is not mediated by competition for nuclear coactivators. Mol Endocrinol *15*, 219-227.

DeFranco, D. B., Ramakrishnan, C., and Tang, Y. (1998). Molecular chaperones and subcellular trafficking of steroid receptors. J Steroid Biochem Mol Biol *65*, 51-58.

Demand, J., Alberti, S., Patterson, C., and Hohfeld, J. (2001). Cooperation of a ubiquitin domain protein and an E3 ubiquitin ligase during chaperone/proteasome coupling. Curr Biol *11*, 1569-1577.

Dendukuri, N., Blais, L., and LeLorier, J. (2002). Inhaled corticosteroids and the risk of diabetes among the elderly. Br J Clin Pharmacol *54*, 59-64.

Denis, M., Gustafsson, J. A., and Wikstrom, A. C. (1988). Interaction of the Mr = 90,000 heat shock protein with the steroid-binding domain of the glucocorticoid receptor. J Biol Chem *263*, 18520-18523.

Diamond, M. I., Miner, J. N., Yoshinaga, S. K., and Yamamoto, K. R. (1990). Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. Science *249*, 1266-1272.

Diehl, E. E., and Schmidt, T. J. (1993). Heat shock protein 70 is associated in substoichiometric amounts with the rat hepatic glucocorticoid receptor. Biochemistry *32*, 13510-13515.

Ding, X. F., Anderson, C. M., Ma, H., Hong, H., Uht, R. M., Kushner, P. J., and Stallcup, M. R. (1998). Nuclear receptor-binding sites of coactivators glucocorticoid receptor interacting protein 1 (GRIP1) and steroid receptor coactivator 1 (SRC-1): multiple motifs with different binding specificities. Mol Endocrinol *12*, 302-313.

Dittmar, K. D., Demady, D. R., Stancato, L. F., Krishna, P., and Pratt, W. B. (1997). Folding of the glucocorticoid receptor by the heat shock protein (hsp) 90-based chaperone machinery. The role of p23 is to stabilize receptor.hsp90 heterocomplexes formed by hsp90.p60.hsp70. J Biol Chem *272*, 21213-21220.

Doong, H., Vrailas, A., and Kohn, E. C. (2002). What's in the 'BAG'?--A functional domain analysis of the BAG-family proteins. Cancer Lett *188*, 25-32.

Drouin, J., Sun, Y. L., Chamberland, M., Gauthier, Y., De Lean, A., Nemer, M., and Schmidt, T. J. (1993). Novel glucocorticoid receptor complex with DNA element of the hormone-repressed POMC gene. Embo J *12*, 145-156.

Drouin, J., Trifiro, M. A., Plante, R. K., Nemer, M., Eriksson, P., and Wrange, O. (1989). Glucocorticoid receptor binding to a specific DNA sequence is required for hormone-dependent repression of pro-opiomelanocortin gene transcription. Mol Cell Biol *9*, 5305-5314.

Dutta, A., Parikh, B., Maben, R., and Melnykovych, G. (1989). Increased dolichol content in glucocorticoid-sensitive human T-cell leukemia line grown in the presence of dexamethasone. Biochem Biophys Res Commun *158*, 163-169.

Edwards, C. R., Benediktsson, R., Lindsay, R. S., and Seckl, J. R. (1993). Dysfunction of placental glucocorticoid barrier: link between fetal environment and adult hypertension? Lancet *341*, 355-357.

Eggert, M., Mows, C. C., Tripier, D., Arnold, R., Michel, J., Nickel, J., Schmidt, S., Beato, M., and Renkawitz, R. (1995). A fraction enriched in a novel glucocorticoid receptor-interacting protein stimulates receptor-dependent transcription in vitro. J Biol Chem *270*, 30755-30759.

Freedman, L. P. (1999). Multimeric Coactivator Complexes for Steroid/Nuclear Receptors. Trends Endocrinol Metab *10*, 403-407.

Freeman, B. C., and Yamamoto, K. R. (2001). Continuous recycling: a mechanism for modulatory signal transduction. Trends Biochem Sci *26*, 285-290.

Freeman, B. C., and Yamamoto, K. R. (2002). Disassembly of transcriptional regulatory complexes by molecular chaperones. Science *296*, 2232-2235.

Froesch, B. A., Takayama, S., and Reed, J. C. (1998). BAG-1L protein enhances androgen receptor function. J Biol Chem *273*, 11660-11666.

Glass, C. K., and Rosenfeld, M. G. (2000). The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev *14*, 121-141.

Gottlicher, M., Heck, S., and Herrlich, P. (1998). Transcriptional cross-talk, the second mode of steroid hormone receptor action. J Mol Med *76*, 480-489.

Halachmi, S., Marden, E., Martin, G., MacKay, H., Abbondanza, C., and Brown, M. (1994). Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. Science *264*, 1455-1458.

Harrison, R. J., McNeil, G. P., and Dobner, P. R. (1995). Synergistic activation of neurotensin/neuromedin N gene expression by c-Jun and glucocorticoids: novel effects of Fos family proteins. Mol Endocrinol *9*, 981-993.

Hartl, F. U. (1996). Molecular chaperones in cellular protein folding. Nature *381*, 571-579.

Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. (1997). A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature *387*, 733-736.

Hench, P. S. (1952). The reversibility of certain rheumatic and non-rheumatic conditions by the use of cortisone or o fthe putuitary sdrenocorticotropic hormone. Ann Intern Med *36*, 1-38.

Hillmann, A. G., Ramdas, J., Multanen, K., Norman, M. R., and Harmon, J. M. (2000). Glucocorticoid receptor gene mutations in leukemic cells acquired in vitro and in vivo. Cancer Res *60*, 2056-2062.

Hohfeld, J. (1998). Regulation of the heat shock conjugate Hsc70 in the mammalian cell: the characterization of the anti-apoptotic protein BAG-1 provides novel insights. Biol Chem *379*, 269-274.

Hohfeld, J., and Jentsch, S. (1997). GrpE-like regulation of the hsc70 chaperone by the anti-apoptotic protein BAG-1. Embo J *16*, 6209-6216.

Horwitz, K. B., Jackson, T. A., Bain, D. L., Richer, J. K., Takimoto, G. S., and Tung, L. (1996). Nuclear receptor coactivators and corepressors. Mol Endocrinol *10*, 1167-1177.

Hsu, S. C., and DeFranco, D. B. (1995). Selectivity of cell cycle regulation of glucocorticoid receptor function. J Biol Chem *270*, 3359-3364.

Joab, I., Radanyi, C., Renoir, M., Buchou, T., Catelli, M. G., Binart, N., Mester, J., and Baulieu, E. E. (1984). Common non-hormone binding component in non-transformed chick oviduct receptors of four steroid hormones. Nature *308*, 850-853.

Jonat, C., Rahmsdorf, H. J., Park, K. K., Cato, A. C., Gebel, S., Ponta, H., and Herrlich, P. (1990). Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. Cell *62*, 1189-1204.

Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S. C., Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1996). A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. Cell *85*, 403-414.

Karin, M., Haslinger, A., Holtgreve, H., Richards, R. I., Krauter, P., Westphal, H. M., and Beato, M. (1984). Characterization of DNA sequences through which cadmium and glucocorticoid hormones induce human metallothionein-IIA gene. Nature *308*, 513-519.

Kino, T., and Chrousos, G. P. (2001). Glucocorticoid and mineralocorticoid resistance/hypersensitivity syndromes. J Endocrinol *169*, 437-445.

Kino, T., Nordeen, S. K., and Chrousos, G. P. (1999). Conditional modulation of glucocorticoid receptor activities by CREB-binding protein (CBP) and p300. J Steroid Biochem Mol Biol *70*, 15-25.

Kucera, T., Waltner-Law, M., Scott, D. K., Prasad, R., and Granner, D. K. (2002). A point mutation of the AF2 transactivation domain of the glucocorticoid receptor disrupts its interaction with steroid receptor coactivator 1. J Biol Chem *277*, 26098-26102.

Kullmann, M., Schneikert, J., Moll, J., Heck, S., Zeiner, M., Gehring, U., and Cato, A. C. (1998). RAP46 is a negative regulator of glucocorticoid receptor action and hormone-induced apoptosis. J Biol Chem *273*, 14620-14625.

Kurihara, I., Shibata, H., Suzuki, T., Ando, T., Kobayashi, S., Hayashi, M., Saito, I., and Saruta, T. (2000). Transcriptional regulation of steroid receptor coactivator-1 (SRC-1) in glucocorticoid action. Endocr Res *26*, 1033-1038.

Kurokawa, R., Soderstrom, M., Horlein, A., Halachmi, S., Brown, M., Rosenfeld, M. G., and Glass, C. K. (1995). Polarity-specific activities of retinoic acid receptors determined by a co-repressor. Nature *377*, 451-454.

Landel, C. C., Kushner, P. J., and Greene, G. L. (1994). The interaction of human estrogen receptor with DNA is modulated by receptor-associated proteins. Mol Endocrinol *8*, 1407-1419.

Le Douarin, B., Zechel, C., Garnier, J. M., Lutz, Y., Tora, L., Pierrat, P., Heery, D., Gronemeyer, H., Chambon, P., and Losson, R. (1995). The N-terminal part of TIF1, a putative mediator of the ligand-dependent activation function (AF-2) of nuclear receptors, is fused to B-raf in the oncogenic protein T18. Embo J *14*, 2020-2033.

Lefstin, J. A., and Yamamoto, K. R. (1998). Allosteric effects of DNA on transcriptional regulators. Nature *392*, 885-888.

Lim-Tio, S. S., Keightley, M. C., and Fuller, P. J. (1997). Determinants of specificity of transactivation by the mineralocorticoid or glucocorticoid receptor. Endocrinology *138*, 2537-2543.

Liu, R., Takayama, S., Zheng, Y., Froesch, B., Chen, G. Q., Zhang, X., Reed, J. C., and Zhang, X. K. (1998). Interaction of BAG-1 with retinoic acid receptor and its inhibition of retinoic acid-induced apoptosis in cancer cells. J Biol Chem *273*, 16985-16992.

Luders, J., Demand, J., and Hohfeld, J. (2000). The ubiquitin-related BAG-1 provides a link between the molecular chaperones Hsc70/Hsp70 and the proteasome. J Biol Chem *275*, 4613-4617.

Luders, J., Demand, J., Schonfelder, S., Frien, M., Zimmermann, R., and Hohfeld, J. (1998). Cofactor-induced modulation of the functional specificity of the molecular chaperone Hsc70. Biol Chem *379*, 1217-1226.

McKay, L. I., and Cidlowski, J. A. (1998). Cross-talk between nuclear factor-kappa B and the steroid hormone receptors: mechanisms of mutual antagonism. Mol Endocrinol *12*, 45-56.

McKenna, N. J., Xu, J., Nawaz, Z., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1999). Nuclear receptor coactivators: multiple enzymes, multiple complexes, multiple functions. J Steroid Biochem Mol Biol *69*, 3-12.

McLaughlin, F., Mackintosh, J., Hayes, B. P., McLaren, A., Uings, I. J., Salmon, P., Humphreys, J., Meldrum, E., and Farrow, S. N. (2002). Glucocorticoid-induced osteopenia in the mouse as assessed by histomorphometry, microcomputed tomography, and biochemical markers. Bone *30*, 924-930.

Meng, X., Devin, J., Sullivan, W. P., Toft, D., Baulieu, E. E., and Catelli, M. G. (1996). Mutational analysis of Hsp90 alpha dimerization and subcellular localization: dimer disruption does not impede "in vivo' interaction with estrogen receptor. J Cell Sci *109 (Pt 7)*, 1677-1687.

Morimoto, R. I. (1998). Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev *12*, 3788-3796.

Morishima, Y., Murphy, P. J., Li, D. P., Sanchez, E. R., and Pratt, W. B. (2000). Stepwise assembly of a glucocorticoid receptor.hsp90 heterocomplex resolves two sequential ATP-dependent events involving first hsp70 and then hsp90 in opening of the steroid binding pocket. J Biol Chem *275*, 18054-18060.

Morrison, N., and Eisman, J. (1993). Role of the negative glucocorticoid regulatory element in glucocorticoid repression of the human osteocalcin promoter. J Bone Miner Res *8*, 969-975.

Nagy, L., Kao, H. Y., Chakravarti, D., Lin, R. J., Hassig, C. A., Ayer, D. E., Schreiber, S. L., and Evans, R. M. (1997). Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. Cell *89*, 373-380.

Naishiro, Y., Adachi, M., Okuda, H., Yawata, A., Mitaka, T., Takayama, S., Reed, J. C., Hinoda, Y., and Imai, K. (1999). BAG-1 accelerates cell motility of human gastric cancer cells. Oncogene *18*, 3244-3251.

Nakai, Y., Usui, T., Tsukada, T., Takahashi, H., Fukata, J., Fukushima, M., Senoo, K., and Imura, H. (1991). Molecular mechanisms of glucocorticoid inhibition of human proopiomelanocortin gene transcription. J Steroid Biochem Mol Biol *40*, 301-306.

Nolte, R. T., Wisely, G. B., Westin, S., Cobb, J. E., Lambert, M. H., Kurokawa, R., Rosenfeld, M. G., Willson, T. M., Glass, C. K., and Milburn, M. V. (1998). Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-gamma. Nature *395*, 137-143.

Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996). The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell *87*, 953-959.

Packham, G., Brimmell, M., and Cleveland, J. L. (1997). Mammalian cells express two differently localized Bag-1 isoforms generated by alternative translation initiation. Biochem J *328 (Pt 3)*, 807-813.

Pearce, D., and Yamamoto, K. R. (1993). Mineralocorticoid and glucocorticoid receptor activities distinguished by nonreceptor factors at a composite response element. Science *259*, 1161-1165.

Picard, D., Khursheed, B., Garabedian, M. J., Fortin, M. G., Lindquist, S., and Yamamoto, K. R. (1990). Reduced levels of hsp90 compromise steroid receptor action in vivo. Nature *348*, 166-168.

Pratt, W. B. (1993). The role of heat shock proteins in regulating the function, folding, and trafficking of the glucocorticoid receptor. J Biol Chem *268*, 21455-21458.

Pratt, W. B., and Toft, D. O. (1997). Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr Rev *18*, 306-360.

Rajapandi, T., Greene, L. E., and Eisenberg, E. (2000). The molecular chaperones Hsp90 and Hsc70 are both necessary and sufficient to activate hormone binding by glucocorticoid receptor. J Biol Chem *275*, 22597-22604.

Ray, A., Prefontaine, K. E., and Ray, P. (1994). Down-modulation of interleukin-6 gene expression by 17 beta-estradiol in the absence of high affinity DNA binding by the estrogen receptor. J Biol Chem *269*, 12940-12946.

Rigaud, G., Roux, J., Pictet, R., and Grange, T. (1991). In vivo footprinting of rat TAT gene: dyna

Robertson, A. M., Bird, C. C., Waddell, A. W., and Currie, A. R. (1978). Morphological aspects of glucocorticoid-induced cell death in human lymphoblastoid cells. J Pathol *126*, 181-187.

Robyr, D., Wolffe, A. P., and Wahli, W. (2000). Nuclear hormone receptor coregulators in action: diversity for shared tasks. Mol Endocrinol *14*, 329-347.

Saatcioglu, F., Claret, F. X., and Karin, M. (1994). Negative transcriptional regulation by nuclear receptors. Semin Cancer Biol *5*, 347-359.

Sakai, D. D., Helms, S., Carlstedt-Duke, J., Gustafsson, J. A., Rottman, F. M., and Yamamoto, K. R. (1988). Hormone-mediated repression: a negative glucocorticoid response element from the bovine prolactin gene. Genes Dev *2*, 1144-1154.

Sapolsky, R. M., Krey, L. C., and McEwen, B. S. (1984). Stress down-regulates corticosterone receptors in a site-specific manner in the brain. Endocrinology *114*, 287-292.

Sarkny, I., Hadgraft, J. W., Caron, G. A., and Barrett, C. W. (1965). The role of vehicles in the prcutaneous absorption of corticosteroids. An wxpermental and clinical study. Br J Dermatlo *77(11)*, 569-575.

Scheidereit, C., Geisse, S., Westphal, H. M., and Beato, M. (1983). The glucocorticoid receptor binds to defined nucleotide sequences near the promoter of mouse mammary tumour virus. Nature *304*, 749-752.

Scheinman, R. I., Gualberto, A., Jewell, C. M., Cidlowski, J. A., and Baldwin, A. S., Jr. (1995). Characterization of mechanisms involved in transrepression of NF-kappa B by activated glucocorticoid receptors. Mol Cell Biol *15*, 943-953.

Schlechte, J. A., Ginsberg, B. H., and Sherman, B. M. (1982). Regulation of the glucocorticoid receptor in human lymphocytes. J Steroid Biochem *16*, 69-74.

Schmidt, U., Wochnik, G. M., Rosenhagen, M. C., Young, J. C., Hartl, F. U., Holsboer, F., and Rein, T. (2003). Essential role of the unusual DNA-binding motif of BAG-1 for inhibition of the glucocorticoid receptor. J Biol Chem *278*, 4926-4931.

Schneikert, J., Hubner, S., Langer, G., Petri, T., Jaattela, M., Reed, J., and Cato, A. C. (2000). Hsp70-RAP46 interaction in downregulation of DNA binding by glucocorticoid receptor. Embo J *19*, 6508-6516.

Schneikert, J., Hubner, S., Martin, E., and Cato, A. C. (1999). A nuclear action of the eukaryotic cochaperone RAP46 in downregulation of glucocorticoid receptor activity. J Cell Biol *146*, 929-940.

Schule, R., Rangarajan, P., Kliewer, S., Ransone, L. J., Bolado, J., Yang, N., Verma, I. M., and Evans, R. M. (1990). Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. Cell *62*, 1217-1226.

Shatkina, L., Mink, S., Rogatsch, H., Klocker, H., Langer, G., Nestl, A., and Cato, A. C. (2003). The Cochaperone Bag-1L Enhances Androgen Receptor Action via Interaction with the NH(2)-Terminal Region of the Receptor. Mol Cell Biol *23*, 7189-7197.

Sheppard, K. A., Phelps, K. M., Williams, A. J., Thanos, D., Glass, C. K., Rosenfeld, M. G., Gerritsen, M. E., and Collins, T. (1998). Nuclear integration of glucocorticoid receptor and nuclear factor-kappaB signaling by CREB-binding protein and steroid receptor coactivator-1. J Biol Chem *273*, 29291-29294.

Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell *95*, 927-937.

Sondermann, H., Scheufler, C., Schneider, C., Hohfeld, J., Hartl, F. U., and Moarefi, I. (2001). Structure of a Bag/Hsc70 complex: convergent functional evolution of Hsp70 nucleotide exchange factors. Science *291*, 1553-1557.

Song, J., Takeda, M., and Morimoto, R. I. (2001). Bag1-Hsp70 mediates a physiological stress signalling pathway that regulates Raf-1/ERK and cell growth. Nat Cell Biol *3*, 276-282.

Spencer, T. E., Jenster, G., Burcin, M. M., Allis, C. D., Zhou, J., Mizzen, C. A., McKenna, N. J., Onate, S. A., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1997). Steroid receptor coactivator-1 is a histone acetyltransferase. Nature *389*, 194-198.

Srinivasan, G., Patel, N. T., and Thompson, E. B. (1994). Heat shock protein is tightly associated with the recombinant human glucocorticoid receptor:glucocorticoid response element complex. Mol Endocrinol *8*, 189-196.

Subramaniam, N., Cairns, W., and Okret, S. (1997). Studies on the mechanism of glucocorticoid-mediated repression from a negative glucocorticoid response element from the bovine prolactin gene. DNA Cell Biol *16*, 153-163.

Takayama, S., Bimston, D. N., Matsuzawa, S., Freeman, B. C., Aime-Sempe, C., Xie, Z., Morimoto, R. I., and Reed, J. C. (1997). BAG-1 modulates the chaperone activity of Hsp70/Hsc70. Embo J *16*, 4887-4896.

Takayama, S., Kochel, K., Irie, S., Inazawa, J., Abe, T., Sato, T., Druck, T., Huebner, K., and Reed, J. C. (1996). Cloning of cDNAs encoding the human BAG1 protein and localization of the human BAG1 gene to chromosome 9p12. Genomics *35*, 494-498.

Takayama, S., Krajewski, S., Krajewska, M., Kitada, S., Zapata, J. M., Kochel, K., Knee, D., Scudiero, D., Tudor, G., Miller, G. J.*, et al.* (1998). Expression and location of Hsp70/Hsc-binding anti-apoptotic protein BAG-1 and its variants in normal tissues and tumor cell lines. Cancer Res *58*, 3116-3131.

Takayama, S., and Reed, J. C. (2001). Molecular chaperone targeting and regulation by BAG family proteins. Nat Cell Biol *3*, E237-241.

Takayama, S., Sato, T., Krajewski, S., Kochel, K., Irie, S., Millan, J. A., and Reed, J. C. (1995). Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity. Cell *80*, 279-284.

Tang, S. C., Shehata, N., Chernenko, G., Khalifa, M., Wang, X., and Shaheta, N. (1999). Expression of BAG-1 in invasive breast carcinomas. J Clin Oncol *17*, 1710-1719.

Torchia, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Glass, C. K., and Rosenfeld, M. G. (1997). The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. Nature *387*, 677-684.

Tornello, S., Orti, E., De Nicola, A. F., Rainbow, T. C., and McEwen, B. S. (1982). Regulation of glucocorticoid receptors in brain by corticosterone treatment of adrenalectomized rats. Neuroendocrinology *35*, 411-417.

Townsend, P. A., Dublin, E., Hart, I. R., Kao, R. H., Hanby, A. M., Cutress, R. I., Poulsom, R., Ryder, K., Barnes, D. M., and Packham, G. (2002). BAG-i expression in human breast cancer: interrelationship between BAG-1 RNA, protein, HSC70 expression and clinico-pathological data. J Pathol *197*, 51-59.

Turner, B. C., Krajewski, S., Krajewska, M., Takayama, S., Gumbs, A. A., Carter, D., Rebbeck, T. R., Haffty, B. G., and Reed, J. C. (2001). BAG-1: a novel biomarker predicting long-term survival in early-stage breast cancer. J Clin Oncol *19*, 992-1000.

Vagner, S., Touriol, C., Galy, B., Audigier, S., Gensac, M. C., Amalric, F., Bayard, F., Prats, H., and Prats, A. C. (1996). Translation of CUG- but not AUG-initiated forms of human fibroblast growth factor 2 is activated in transformed and stressed cells. J Cell Biol *135*, 1391-1402.

Voegel, J. J., Heine, M. J., Tini, M., Vivat, V., Chambon, P., and Gronemeyer, H. (1998). The coactivator TIF2 contains three nuclear receptor-binding motifs and mediates transactivation through CBP binding-dependent and -independent pathways. Embo J *17*, 507-519.

Wallace, A. D., and Cidlowski, J. A. (2001). Proteasome-mediated glucocorticoid receptor degradation restricts transcriptional signaling by glucocorticoids. J Biol Chem *276*, 42714-42721.

Walsh, S. D., and Grant, I. W. (1966). Corticosteroids in treatment of chronic asthma. Br Med J *517*, 796-802.

Weinberger, C., Hollenberg, S. M., Rosenfeld, M. G., and Evans, R. M. (1985). Domain structure of human glucocorticoid receptor and its relationship to the v-erb-A oncogene product. Nature *318*, 670-672.

Werner, S., and Bronnegard, M. (1996). Molecular basis of glucocorticoid-resistant syndromes. Steroids *61*, 216-221.

Witcher, M., Yang, X., Pater, A., and Tang, S. C. (2001). BAG-1 p50 isoform interacts with the vitamin D receptor and its cellular overexpression inhibits the vitamin D pathway. Exp Cell Res *265*, 167-173.

Wrange, O., and Gustafsson, J. A. (1978). Separation of the hormone- and DNA-binding sites of the hepatic glucocorticoid receptor by means of proteolysis. J Biol Chem *253*, 856-865.

Yamauchi, H., Adachi, M., Sakata, K., Hareyama, M., Satoh, M., Himi, T., Takayama, S., Reed, J. C., and Imai, K. (2001). Nuclear BAG-1 localization and the risk of recurrence after radiation therapy in laryngeal carcinomas. Cancer Lett *165*, 103-110.

Yang, X., Chernenko, G., Hao, Y., Ding, Z., Pater, M. M., Pater, A., and Tang, S. C. (1998). Human BAG-1/RAP46 protein is generated as four isoforms by alternative translation initiation and overexpressed in cancer cells. Oncogene *17*, 981-989.

Yang-Yen, H. F., Chambard, J. C., Sun, Y. L., Smeal, T., Schmidt, T. J., Drouin, J., and Karin, M. (1990). Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. Cell *62*, 1205-1215.

Yao, T. P., Ku, G., Zhou, N., Scully, R., and Livingston, D. M. (1996). The nuclear hormone receptor coactivator SRC-1 is a specific target of p300. Proc Natl Acad Sci U S A *93*, 10626-10631.

Yawata, A., Adachi, M., Okuda, H., Naishiro, Y., Takamura, T., Hareyama, M., Takayama, S., Reed, J. C., and Imai, K. (1998). Prolonged cell survival enhances peritoneal dissemination of gastric cancer cells. Oncogene *16*, 2681-2686.

Young, J. C., and Hartl, F. U. (2000). Polypeptide release by Hsp90 involves ATP hydrolysis and is enhanced by the co-chaperone p23. Embo J *19*, 5930-5940.

Zeiner, M., Gebauer, M., and Gehring, U. (1997). Mammalian protein RAP46: an interaction partner and modulator of 70 kDa heat shock proteins. Embo J *16*, 5483-5490.

Zeiner, M., and Gehring, U. (1995). A protein that interacts with members of the nuclear hormone receptor family: identification and cDNA cloning. Proc Natl Acad Sci U S A *92*, 11465-11469.

Zeiner, M., Niyaz, Y., and Gehring, U. (1999). The hsp70-associating protein Hap46 binds to DNA and stimulates transcription. Proc Natl Acad Sci U S A *96*, 10194-10199.