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Modulation der Androgenrezeptorwirkung durch das Kochaperon BAG-1L

Zusammenfassung

Beim Androgenrezeptor (AR) handelt es sich um ein zytoplasmatisches Protein, welches nach Hormonbindung in den Kern wandert und die Expression seiner Zielgene induzieren oder reprimieren kann. Es gibt mehrere Faktoren, welche als Interaktionspartner und Modulatoren der AR-Wirkung bekannt sind. Einer dieser Faktoren ist BAG-1L, ein Mitglied einer Genfamilie von Kochaperonen, welche als Nukleotid-Austausch-Faktoren für das molekulare Chaperone Hsp70 wirken. BAG-1L verstärkt die Hormon-abhängige Transaktivierung des AR in Prostatakarzinomzellen.

In der vorliegenden Arbeit wurde der Mechanismus untersucht, welcher der durch BAG-1L vermittelten Regulation des AR zugrunde liegt. Deletionsstudien im AR-Protein zeigten, daß die □5-Region im N-Terminus des AR die entscheidende Stelle für die Wirkung von BAG-1L ist, auch wenn der C-Terminus des AR ebenfalls eine Rolle bei der über BAG-1L vermittelten Verstärkung seiner Transaktivierungsfunktion spielt.

In Bezug auf BAG-1L zeigten Experimente mit Deletionsmutanten, daß sowohl die letzten 47 C-terminalen, als auch die ersten 70 N-terminalen Aminosäuren dieses Proteins wichtig für den über BAG-1L vermittelten Anstieg der Transaktivierung des AR sind. Diese N-terminale Region von BAG-1L enthält ein nukleäres Lokalisationssignal. Darüber hinaus konnte festgestellt werden, daß diese Region von BAG-1L eine wichtige Rolle bei der Wirkung von BAG-1L spielt.

Durch *in-vitro*-Bindungsstudien gelang es nachzuweisen, daß die N-terminalen Sequenzen von BAG-1L mit dem C-Terminus des AR interagieren und daß der C-Terminus von BAG-1L mit dem N-Terminus des AR im Bereich der Aminosäuren 360 und 554 (auch als □5-Domäne bezeichnet) interagiert. Anhand dieser Ergebnisse konnte BAG-1L als Brückenmolekül für die N- und C-terminale Interaktion des AR, welche eine wichtige Rolle für dessen Funktion spielt, identifiziert werden.

Da der C-Terminus von BAG-1L zusätzlich an das molekulare Chaperon Hsp70 bindet, wurde untersucht, ob diese Region von BAG-1L ebenfalls in die Verstärkung der AR-Wirkung involviert ist. Durch Überexpressionsstudien von Hsp70 konnte der Effekt von BAG-1L auf die Transaktivierung des AR verstärkt werden. Zusätzlich zeigten Chromatin-Immunopräzipitationsexperimente die hormonabhängige Rekrutierung von Hsp70, BAG-1L und AR an den PSA (*prostate specific antigen*)-Promotor in 22Rv1-Prostatakarzinomzellen, welche den AR exprimieren.

Mit Hilfe dieser Studien gelang es, ein Modell für die Regulation der Transaktivierungsfunktion des AR durch ein Kochaperon zu entwerfen, bei dem das Kochaperon BAG-1L und das molekulare Chaperon Hsp70 mit dem AR einen ternären Komplex im Promotorbereich eines Androgen-regulierten Genes bilden.

Modulation of Androgen Receptor Action by the Cochaperone BAG-1L

Abstract

Androgen receptor (AR) is a cytoplasmic protein that becomes translocated into the nucleus after hormone binding and induces or represses the expression of its target genes. Several factors have been identified as interacting partners and modulators of the action of the AR. One of them is BAG-1L, a member of a family of co-chaperones, nucleotide exchange factor for the molecular chaperone Hsp70. BAG-1L enhances hormone-dependent transactivation by AR in prostate cancer cells. In the present work the molecular mechanism of the BAG-1L-mediated regulation of the AR has been studied. Deletion studies revealed that the □5 region in the N-terminus of the AR is the target for BAG-1L action, though the C-terminus of the AR also contribute to the BAG-1L-mediated enhancement of the transactivation function of the AR. On the part of the BAG-1L, experiments with deletion mutants showed that both the last 47 C-terminal and the first 70 N-terminal amino acids are important for the BAG-1L-mediated increase in transactivation by the AR. This N-terminal region of BAG-1L contains a nuclear localization signal, but it was determined that in addition to nuclear targeting, this region of BAG-1L is also actively involved in its function. *In vitro* binding studies demonstrated that the N-terminal sequences of BAG-1L interact with the C-terminus of the AR, and the C-terminus of BAG-1L with the N-terminus of the AR to a region between amino acids 360 and 554 (also termed □5 domain). Thus these results identify BAG-1L as a bridging molecule for the N- and C-termini interaction of the AR, which is known to be important in the function of the receptor. As the C-terminus of BAG-1L also binds to the molecular chaperone Hsp70, involvement of this region of BAG-1L in the enhancement of the AR function was also analyzed. Overexpression of the Hsp70 increased the enhancing effect of BAG-1L on transactivation by the AR. Furthermore, chromatin immunoprecipitaion experiment showed the hormone dependent recruitment of Hsp70, BAG-1L and AR to the prostate specific antigene promoter in the AR positive 22Rv1 prostate carcinoma cells. These studies therefore provide a model for the regulation of the transactivation function of the AR by a cochaperone, where the cochaperone BAG-1L and the molecular chaperone Hsp70 form a ternary complex with the AR at an androgen-regulated promoter.

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ABBREVIATIONS

A	Ampere
aa	aminoacid
Ab	Antibody
ACTR	Activator of the thyroid and retinoic receptor
AF	Activation function
AIB1	Ampliffied in breast cancer 1
APS	Ammonium persulfate
AR	Androgen receptor
ARA	Androgen receptor associated protein
ARE	Androgen response element
ARIP	AR-interacting protein
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BAG-1	Bcl-2-associated Athanogene 1 (from gr.: *athanos* for „anti-death“)
BAG-1L	Bcl-2-associated athanogene 1 (large)
BAG-1M	Bcl-2-associated athanogene 1 (medium)
BAG-1S	Bcl-2-associated athanogene 1 (small)
Bcl-2	*B cell lymphoma 2*
bp	Base pairs
Bq	Bequerel
BSA	Bovine serum albumin
β-ME	β-mercaptoethanol
°C	Degrees Celsius
ca.	*circa*, about
CBP	CREB-binding protein
cDNA	Complementary DNA
Ci	Curie

cpm	Counts per minute
CREB	Cyclo-AMP response element binding protein
C-terminal	Carboxyl-terminal
Cy	Cyanine
kDa	kilo-Dalton
DBD	DNA-binding domain
ddH_2O	Double distilled water
DHT	5α-Dihydrotestosterone
DMEM	Dulbecco´s modified eagles medium
DMSO	Dimethylsulfoxide
DNA	Deoxy-ribo nucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylendiamine-N, N-tetracetate
e.g.	*exempli gratia*, for instance
ER	Estrogen receptor
et al	*Et alii,* and others
FBS	Foetal bovine serum
g	Gram
g	Gravity (unit of relative centrifugal force)
GFP	Green fluorescence protein
GR	Glucocorticoid receptor
GRIP1	Glucocorticoid receptor-interacting protein 1
GST	Glutathione-S-transferase
h	Hour
HA	hemagglutinin
HBD	Hormone-binding domain
HEPES	N-2-hydroxyethilpiperasine-N´-2 ethansulfonic acid
Hip	Hsp70 interacting protein
hKLK2	Glandular kallikrein-2

Hop	Hsp70/Hsp90 organizing protein
HRP	Horseradish peroxidase
Hsp	Heat shock protein
IP	Immunoprecipitation
k	Kilo
kb	Kilobases
l	1 Liter
Luc	luciferase
LSM	Laser scanning microscope
m	Meter
m	Milli
M	Molar
mAb	Monoclonal antibody
min	Minute
MMTV	Mouse mammary Tumour Virus
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
MW	Molecular weight
****	Micro
n	Nano
NLS	Nuclear localization signal
NP-40	Nonidet P- 40
nt	Nucleotides
N-terminal	Amino-terminal
OD	Optical density
o/n	Overnight
p	Pico
PAGE	Polyacrylamide gel electrophoresis
p/CAF	p300/CBP associated factor
PBP	prostate binding protein
PBS	Phosphate buffered saline

pCIP	p300/CBP/co-integrator-associated protein
PCR	Polymerase chain reaction
PI	Pre-immune serum
PR	Progesterone receptor
PSA	Prostate specific antigene
RAC3	Receptor-associated co-activator 3
RAR	Retinoic acid receptor
RNA	Rybonucleic acid
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription PCR
RXR	Retinoi X receptor
^{35}S	Sulphur
s	Second
SBD	Substrate binding domain
SD	Standard deviation
SDS	Sodium-Dodecyl- Sulfate
SRC	Steroid receptor coactivator
NCoA-1	Nuclear receptor coactivator-1
T	Testosterone
TAE	Tri/acetate/EDTA electrophoresis buffer
TBE	Tris-borate EDTA
TEMED	N, N, N´N´-tetramethylethylendiamine
TF	Transcription factior
TIF2	Tanscription intermediary factor 2
TRAM1	Thyroid hormone receptor activator molecule 1
TRAP	Thyroid hormone receptor-associated protein
TRIS	Tris-(hydroxymethyl)-aminomethane
TRITC	Tetramethylrhodamine isothiocyanate
U	Units
UV	Ultraviolet

V	Volt
VDR	Vitamin D receptor
v/v	Volume on volume
Vol	Volume
W	Watt
w/o	without
wt	Wild type
w/v	Weight on volume

1. INTRODUCTION

Androgens are sex steroid hormones that are important for expression of male phenotype, playing a crucial role during male sexual differentiation, development and maintenance of secondary male characteristics, and during the initiation and maintenance of spermatogenesis (George and Wilson, 1994). Androgens act on a number of target tissues, including bone, skeletal muscle, brain, liver, kidney, vocal cords, facial hair follicles, testis and prostate (Chen *et al.*, 2001; Sato *et al.*, 2002; Lee, 2002; Ferrando *et al.*, 2002; Genazzani *et al.*, 2002; Canosa *et al.*, 2002; Cleary, 1990; Mooradian *et al.*, 1987; Catterall *et al.*, 1986; Colquhoun-Flannery and Carruth, 1996; Randall *et al.*, 2000; Wilson *et al.*, 2002; Wirth and Hakenberg, 2000). The most important in this respect are testosterone (T) and its metabolite 5α-dihydrotestosterone (DHT). DHT is produced from testosterone in the male urogenital tract and various peripheral tissues by two 5α-reductase enzymes (Russell and Wilson, 1994). 5α-reductase type I is expressed largely in sebaceous glands and liver, while type II enzyme is expressed in the male urogenital tract. DHT is the more potent androgen. The importance of DHT is clearly revealed in patients with 5α-reductase type II deficiency who have ambiguous external genitalia and a highly underdeveloped prostate (Thigpen *et al.*, 1992; Wilson *et al.*, 1993).

At the molecular level, androgen action in target cells is mediated by an intracellular androgen receptor (AR), which belongs to the nuclear receptor superfamily. This family includes receptors for steroid hormones (glucocorticoid, mineralocorticoid, estrogen, progesterone, androgen), retinoids, thyroid hormone, vitamin D, fatty acids and other small hydrophobic molecules (Laudet *et al.*, 1992; Mangelsdorf *et al.,* 1995). In addition, there is a large group of orphan receptors for which the ligands have not been determined (see Enmark and Gustafsson, 1996). Members of the superfamily display a high similarity in their gene and protein structure.

1.1 Functional domains of the human androgen receptor

The complimentary DNA (cDNA) encoding the human AR was cloned in 1988 by different groups (Chang *et al*., 1988; Lubahn *et al*., 1988; Trapman *et al*., 1988). AR is a 919 amino acid protein of molecular mass approximately 110 kDa. As the other members of the family, AR contains hormone binding domain (HBD) at its C-terminal domain, responsible for the binding of ligand and interaction with heat shock proteins (see below). DNA binding domain and hinge region are centrally localized and transcription activation functions 1 and 2 (AF1 and AF2) at the N and C-terminal regions (MacLean *et al*., 1997). In contrast to other steroid hormone receptors, the C-terminal AF2 domain has a very weak transactivation potential. Transcription activation function of the AR is carried out mainly by the N-terminal domain of the receptor (Jenster *et al*., 1995; Poukka *et al*., 2000). AF1 domain, in turn, consists of two independent transactivation functions, □1 and □5, harboring the AR main ligand-dependent and ligand-independant transactivation functions, respectively (Jenster *et al*., 1995). AR also possesses a nuclear localization signal (NLS), responsible for its nuclear import, at the junction of the DBD and hinge region (**Fig.1**).

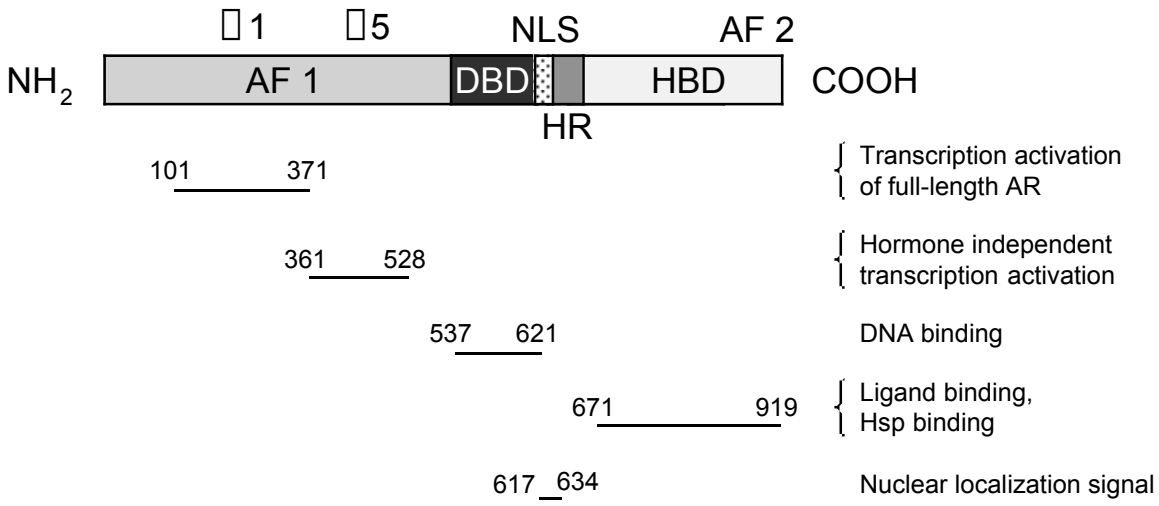

FIGURE 1.1 Functional domain structure of the human androgen receptor

Schematic diagram of the AR indicates the N-terminal AF1 activation domain, containing two independent transactivation functions, τ1 and τ5, DNA binding domain (DBD) in the middle of the molecule, hinge region (HR), hormone binding domain (HBD) and a weak hormone-dependent transactivation function at the C-terminus of the receptor. The signal responsible for nuclear import is located at the junction of the DBD and hinge region.

1.2 Transcriptional regulation by the androgen receptor

AR is a ligand inducible transcription factor. Upon binding to androgen, it becomes translocated into the nucleus and binds as a homodimerto specific DNA sequences termed androgen response elements (AREs). These elements are generally located at the promoter or enhancer regions of AR target genes, like probasin (Rennie *et al.*, 1993), prostate binding protein (PBP) (Claessens *et al.*, 1989 and 1993), glandular kallikrein-2 (hKLK2) (Murtha *et al.*, 1993), prostate specific antigene (PSA) (Riegman *et al.*, 1991) and many others (for review see Chang *et al.*, 1995). The consensus DNA-binding site for the AR is comprised of two imperfect palindromic 6-base-pair (bp) elements (inverted repeats) separated by a 3-bp spacer: 5'-GG(A/T)ACAnnnTGTTCT-3' (Roche *et al.*, 1992). This type of binding site can also be recognized by the progesterone, glucocorticoid and mineralocorticoid receptors. Besides the conventional ARE

consensus, response elements specific for the AR have been identified in androgen-regulated genes (Rennie *et al.*, 1993; Adler *et al.*, 1993; Rundlett and Miesfeld, 1995; Claessens *et al.*, 1996; Verrijdt *et al.*, 2002). These motifs are partial direct repeats of the canonical 5'-TGTTCT-3' hexamer. AR binds this direct repeat possibly by dimerizing on the DNA in a "head-to-tail" conformation, while to the consensus ARE receptor is bound in the dimer conformation "head-to-head".

Binding to the DNA is followed by the receptor interaction with components of the basal transcription machinery, like TFIIH (Lee *et al.*, 2000), TFIIF (Reid *et al.*, 2002), TBP (McEwan and Gustafsson, 1997), sequence specific transcription factors (Ning and Robins, 1999), and different cofactor proteins. This leads to up- or downregulation of transcription of the target genes (Tsai and O'Malley, 1994; Quigley *et al.*, 1995; Yuan *et al.*, 2001).

1.3 Androgen receptor co-regulators

Activation of transcription by AR is regulated by a number of proteins. The best characterized are co-activators of the p160 SRC (steroid receptor co-activator) family members: SRC-1/NCoA-1 (Bevan *et al.*, 1999; Shang *et al.*, 2002), SRC2/GRIP1(glucocorticoid receptor-interacting protein 1)/TIF2 (transcription intermediary factor 2) (Berrevoets *et al.*, 1998; Kotaja *et al.*, 2002;), and SRC3/ACTR (activator of the thyroid and retinoic receptor)/AIB1(amplified in breast cancer)/pCIP (p300/CBP/co-integrator-associated protein)/RAC3 (reseptor-associated co-activator 3)/TRAM 1(thyroid hormone receptor activator molecule 1) (Chen *et al.*, 1997; Li *et al.*, 1997; Takeshita *et al.*, 1997; Torchia *et al.*, 1997; Anzick *et al.*, 1997), CREB-binding protein (CBP/p300) (Fu *et al.*, 2000; Fronsdal *et al.*, 1998; Kim *et al.*, 2001; McKay and Cidlowski, 2000; Smith *et al.*, 1996), and P/CAF (CBP/p300 associated factor) (Reutens *et al.*, 2001; McMahon *et al.*, 1999). AR recruits p160 proteins, CBP/p300 and P/CAF to the promoter of target genes (Berrevoets *et al.*, 1998; Shang *et al.*, 2002). These proteins possess histone acetyltransferase activity and are capable of acetylating both histones and steroid receptors, including AR (McKenna and O'Malley, 2002; Glass and Rosenfeld, 2000; Fu *et al.*, 2000). Acetylation of histone tails results in relaxation of chromatin

packaging and thereby facilitates gene transcription, and acetylation of AR has been shown to be essential for the transcription activation function of the receptor (Fu *et al.*, 2000). Other proteins, reported to modulate transactivation function of AR, are factors such as ARA70 (Alen *et al.*, 1999), FHL2 (Müller *et al.*, 2000), ARA54 (Kang *et al.*, 1999), Tip60 (Brady *et al.*, 1999), Ubc9 (Poukka *et al.*, 1999), ARIP3 (AR-interacting protein 3) (Kotaja *et al.*, 2002), cyclin E (Yamamoto *et al.*, 2000), TRAP220, TRAP170 and TRAP100 subunits of the thyroid hormone receptor-associated protein (TRAP)-Mediator complex (Wang *et al.*, 2002), histone methyltransferase CARM1 and β-Catenin (Koh *et al.*, 2002; Yang *et al.*, 2002; Truica *et al.*, 2000). Some of these factors, e.g. ARIP3, acting in a synergistic fashion with one of the p160 family members GRIP1 on steroid-dependent transactivation, most likely act as bridging molecules between AR and HAT activity possessing proteins (Kotaja *et al.*, 2002). Nevertheless for the most of the co-activator proteins the mechanism of action is not yet clear.

Interaction with co-activators is mediated through the transcriptional activation domains in steroid receptors. This includes AF1 in the NH_2-terminal domain and AF2 in the HBD. Co-activators interact with the AF2 hydrophobic surface in the HBD through conserved amphipathic alpha helical LXXLL motifs, where L is leucine and X is any amino acid (Heery *et al.*, 1997; Le Douarin *et al.*, 1996). However, it was shown that SRC1 mutant with disrupted LXXLL motif, deficient in binding to the HBD of a nuclear receptor, is still capable of potentiating the transactivation by the AR (Bevan *et al.*, 1999), indicating that in case of the AR regulation of the transactivation function by SRC1 must occur through another domain of the receptor. Recently p160 co-activators SRC1 and TIF2 have been shown to interact directly with the AF1 function in the AR (Bevan *et al.*, 1999; Berrevoets *et al.*, 1998). This interaction is mediated by the δ5 domain in the AR AF1 transactivation unit. AF2 of the AR, on the contrary, demonstrated a reduced ability to recruit p160 coactivators (He *et al.*, 1999). Thus, in contrast to other nuclear receptors, recruitment of the co-activators by the AR is occurs through the N-terminal AF1 transactivation function.

1.4 NH_2- and carboxyl-terminal interaction of the androgen receptor

A unique property of the AR is the androgen-induced interaction between the NH_2-terminal domain and the carboxyl-terminal HBD (Langley *et al.*, 1995; Langley *et al.*, 1998). This interaction is mediated through FXXLF motif at amino acids 23-27 and WXXLF motif located between amino acid residues 429 and 439 in the N-terminus of the AR, which bind to distinct regions in the AR HBD (He *et al.*, 2000). Interaction between N- and C-termini of the AR was shown to be required for the receptor activity *in vivo*, playing a role in the testosterone-induced AR stabilization and an antiparallel arrangement of AR monomers in the AR dimmer (Berrevoets *et al.*, 1998; Ikonen *et al.*, 1998; Langley *et al.*, 1995 and 1998). Recently it was demonstrated that interaction of the N- and C-termini of the AR is essential for the recruitment of coactivator proteins SRC1, TIF2 and CBP (Saitoh *et al.*, 2002). The importance of the N/C terminal interaction was demonstrated in the mutation analysis of the AF2 domain of the AR, which showed that disruption of the functional interaction between N- and carboxyl-termini of the AR is linked to androgen insensitivity syndrome (Thompson *et al.*, 2001).

1.5 Steroid receptors and the molecularchaperone system

Studies on the steroid hormone receptors revealed that they exist in cells in two forms: a cytoplasmic ~9S and a nuclear ~4S form (Sherman, 1970; Milgrom, 1970; McGuire, 1971; Reel, 1971; Rao, 1973; Buller, 1975; Munck, 1967; Munck, 1968; Schaumburg, 1968; Baxter, 1971; Swaneck, 1970; Marver, 1972; Pasqualini, 1972; Mainwaring, 1969; Baulieu, 1970; Jung, 1972; Fang, 1969). This observation led to the two-step theory of the mechanism of steroid hormone receptor action, in which the hormone enters the cell by passive diffusion and interacts with the large 9S receptor. This is followed by a conformational change in the receptor molecule and subsequent migration of the complex to the nucleus, resulting in the appearance of the 4S nuclear hormone receptor. Though first suggested for the ER (Jensen *et al.*, 1967; Gorski *et al.,* 1968; Jensen *et al.*, 1968), this theory proved to be a general mechanistic pattern for steroid receptors. Further studies demonstrated that, indeed, in the absence of ligand, steroid hormone receptors exist in inactive complexes with molecular chaperones (hsp90, hsp70, hsp56 and immunophilins). Association with molecular chaperone complex is required for the

nuclear receptors to reach and maintain the conformation necessary for the efficient ligand binding (Pratt and Toft, 1997).

Molecular chaperones are defined as a group of proteins that mediate the correct assembly of other proteins but are not themselves components of the final functional structure (Anfinsen, 1973). One of molecular chaperones shown to be important for ligand binding by steroid receptors is Hsp90. This protein was described to interact with unliganded AR, glucocorticoid receptor (GR), mineralocorticoid receptor (MR), estrogen receptor (ER) and progesterone receptor (PR) (Pratt and Toft, 1997). However, the pathway leading to Hsp90 association involves the action of several other chaperones and co-chaperones (or helpers), including Hsp70, p23, Hop (Hsp70/Hsp90 organizing protein), Hip (Hsp70 interacting protein) and BAG-1.

Hsp70 along with Hsp90 are the most important chaperones in nuclear receptor function (Rajapandi *et al.*, 2000). Hsp70 is a conserved family of proteins, acting as chaperones of protein folding and translocation (reviewed by Welch, 1992; Gething and Sambrook, 1992; Hendrick and Hartl, 1993; Hartl, 1996). Two forms exist in a cell: a 73 kDa form that is constitutively expressed (Hsc70) and a 72 kDa stress inducible form (Hsp70). Both members of the Hsp70 family possess ATPase activity and bind in an ATP-dependent manner to unfolded regions of proteins or to hydrophobic peptides.

Protein folding has common requirements among several receptors. The minimal complement of proteins necessary for efficient folding includes molecular chaperones Hsp90, Hsp70, p23 and co-chaperones Hsp40 and Hop (Dittmar *et al.*, 1998; Kosano *et al.*, 1998). Hsp70 is thought to be the first to bind the receptor (Morishima *et al.*, 2000) and its function is positively regulated by the cochaperones Hsp40 (Dittmar *et al.*, 1998). Hop, binds to both Hsp90 and Hsp70 and functions as an adaptor protein, which brings a dimer of Hsp90 to the complex (Johnson *et al.*, 1998; Dittmar and Pratt, 1997; Chen and Smith, 1998). The function of p23 stays unclear, though this chaperone has been shown to be important for hormone binding of GR and PR, where p23 stabilizes the aporeceptor complex at a late step of the receptor folding (Dittmar and Pratt, 1997; Kosano *et al.*, 1998).

The chaperone activity of Hsp70, however, is regulated by two other co-chaperones Hip and BAG-1 (Fig. 2). Hip (Höhfeld *et al.*, 1995) positively regulates Hsp70 function,

while BAG-1 competes with Hip for binding to Hsp70 ATPase domain and is a negative regulator of Hsp70 (Bimston *et al.*, 1998; Höhfeld and Jentsch, 1997; Takayama *et al.*, 1997; Zeiner *et al.*, 1997; Nollen *et al.*, 2000; Sondermann *et al.*, 2001). In addition BAG-1 indirectly interferes with the binding of Hop to Hsp70 (Gebauer *et al*, 1998; Kanelakis *et al.*, 1999). BAG-1, therefore, together with other chaperones and cochaperones may be involved at different steps of protein folding.

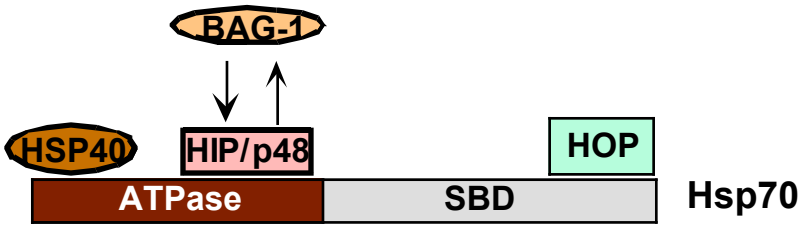

FIGURE 1.2 Hsp70 and its co-chaperones

Schematic diagram of the Hsp70 indicates the N-terminal ATPase domain of the protein and C-terminal substrate binding domain (SBD), responsible for interaction with unfolded proteins. Co-chaperones Hsp40, Hip and BAG-1 interact with the ATPase domain of Hsp70. Hop binds to the C-terminus of Hsp70 and serves as a bridging factor for interaction with Hsp90.

1.6 The BAG-1 proteins

BAG-1 (Bcl-2-associated athanogene 1) was first identified in the mouse as a Bcl-2-binding protein (Takayama *et al.*, 1995). It appeared to be highly homologous to a 46 kDa receptor associating protein (RAP46) identified in an interaction screaning experiment with the activated GR (Zeiner and Gehring, 1995). Meanwhile the name "BAG-1" refers to a family of proteins and RAP46 has been established as an isoform of this family and is termed BAG-1M (Takayama *et al.*, 1998). In humans, four isoforms of BAG-1 have been described, all encoded by the same mRNA through the use of alternative translation-initiation sites (**Fig.1.3**). The largest isoform (BAG-1L) starts at a noncanonical CUG codon followed by an in-frame downstream AUG start sites giving rise to BAG-1M, BAG-1S and p29 (Takayama *et al.*, 1998; Yang *et al.*, 1998). All the BAG-1 proteins contain Hsp70 binding domain and an ubiquitin-like domain near N-

terminus and a characteristic $[EEX_4]_n$ motif of so far unclear function at the N-terminus. The longest isoform, BAG-1L, has a nuclear localization signal, which is not shared by the other members of the family.

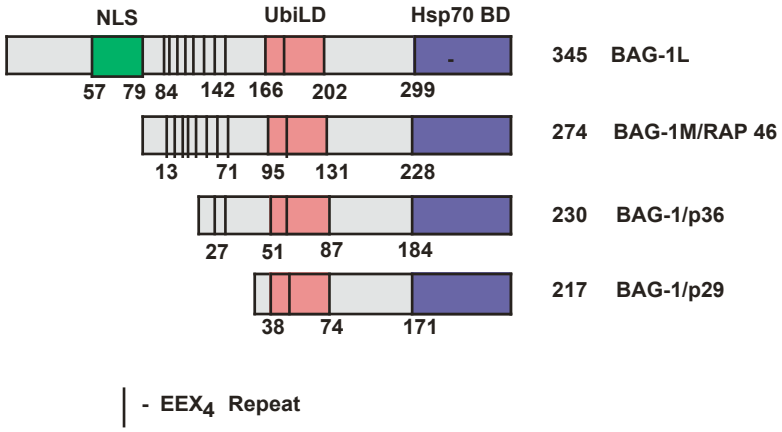

FIGURE 1.3 BAG-1 family of proteins in human

Schematic diagram of BAG-1 proteins indicates the conserved Hsp70 binding domain at the C-terminus, the ubiquitin-like domain near the N-terminus, the $[EEX_4]_n$ motif and a nuclear localization signal in BAG-1L.

BAG-1 proteins are characterised by a strong binding to Hsp70/Hsc70 molecular chaperone through their carboxyl-terminal sequences. They act as nucleotide exchange factors of Hsp70 and are negative regulators of the refolding activity of this chaperone (Bimston *et al.*, 1998; Höhfeld and Jentsch, 1997; Takayama *et al.*, 1997; Zeiner *et al.*, 1997; Nollen *et al.*, 2000; Sondermann *et al.*, 2001). However, BAG-1 co-chaperones do not only regulate the function of Hsp70/Hsc70. BAG-1 has been also shown to associate with the 26S proteasome (Luders *et al.*, 2000), indicating its possible role in protein degradation. Members of BAG-1 family were demonstrated also to interact with and regulate the activities of the anti-apoptotic factor Bcl-2 (Takayama *et al.*, 1995), the RAF-1 serine/threonine kinase (Wang *et al.*, 1996) and steroid hormone receptors (Kanelakis *et al.*, 1999; Zeiner and Gehring, 1995; Kullman *et al.*, 1998; Froesch *et al.*, 1998), thus indicating that BAG-1 proteins play a role in different signaling pathways.

1.7 Chaperones and steroid receptor nuclear function

So far the effect of molecular chaperones and co-chaperones on the steroid hormone receptor function are believed to be exclusively cytoplasmic (Pratt and Toft, 1997). However, Hsp70, Hsp90 and Hsp40 forms have been shown to exist in the nuclear compartment (Velazquez and Lindquist, 1984; Gasc *et al.,* 1990; Hattori *et al.*, 1993; Csermely *et al.*, 1998), and Hsp70 and Hsp90 were reported to modulate the DNA binding of ER (Landel *et al.*, 1994) and GR (Kang *et al.*, 1999), respectively. Chaperone p23 was shown to have an effect on the transactivation function of several steroid receptors, which varied from enhancement of transcriptional activation by GR, PR, MR or ER to repression of the activity of the AR (Freeman *et al.*, 2000). p23 was also demonstrated to play a role in disassembly of transcriptional regulatory complexes in case of TR and GR (Freeman and Yamamoto, 2002). p23 allows the receptor to dissociate from its response element and to be released into the cytoplasm, thus being able to sense and respond efficiently to fluctuations in hormone levels. Another molecular chaperone, Cdc37, has been shown to specifically interact with the AR HBD *in vitro* in a manner that is at least partially dependent on Hsp90 and to enhance hormone-dependent activity of AR in both yeast and mammalian systems (Fliss *et al.*, 1997; Rao *et al.*, 2001). The groups of A.Cato and J.Reed demonstrated negative regulation of GR, VDR and retinoic acid receptor transactivation function by BAG-1 co-chaperones (Kullman *et al.*, 1998; Witcher *et al.*, 2001; Liu *et al.*, 1998). However, the longest BAG-1 isoform, BAG-1L, has been reported to have a positive effect on the transactivation function of the AR (Froesch *et al.*, 1998).

Thus molecular chaperones and co-chaperones regulate the activity of steroid receptors not only at the level of the receptor folding, but also downstream the binding of hormone. However, it is not clear, why the same proteins have different effect on the transactivation function of different members of the nuclear receptor superfamily, e.g. BAG-1L downregulates the transactivation function of the GR and VDR, but enhances the transactivation by the AR. To address this question, it is important to find out the mechanism of the regulation of various nuclear receptors by the members of the chaperone-co-chaperone machinery. In addition, nuclear receptors play a crucial role in

regulation of many physiological and pathological processes mediating the signal transduction pathways of steroid hormones (reviewed in Burris and McCabe, 2001). Understanding of the mechanisms of the nuclear receptor regulation might therefore find therapeutic applications in handling various health disorders.

AIM

BAG-1 is a family of proteins that regulate the action of the molecular chaperone Hsp70. The longest isoform of this family, BAG-1L, has been shown to enhance transcription activation by the AR. However, the molecular mechanism of regulation of the AR function by BAG-1L is not yet clear.

The aim of this work is to elucidate the mechanism, by which BAG-1L enhances the transcription activation function of the AR. One of the questions to be answered is which domains of the AR and BAG-1L are involved in the modulation of the transactivation function of the AR by BAG-1L and whether direct binding of AR and BAG-1L is involved. The second question is whether other chaperones take part in the regulation of action of the AR by BAG-1L. An interesting candidate that was analyzed was Hsp70, the protein known to interact with both BAG-1L and the AR.

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
Aprotinin	Sigma, Deisenhofen
Bacterial Petri dishes	Greiner Labortechnik, Nürtingen
Bacto-Agar	Roth, Karlsruhe
Bacto-Yeast Extract	Roth, Karlsruhe
Bacto-Pepton	Roth, Karlsruhe
2-β Mercaptoethanol	Roth, Karlsruhe
Bromophenol blue	Roth, Karlsruhe
Chloroform	Merck, Darmstadt
Coomassie Brilliant Blue R250	Serva, Heidelberg
Dithiothreitol	Sigma, Taufkirchen
DMEM (synthetic culture medium)	Gibco BRL Life Tech., 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
GuGene transfection kit	Roche Diagnostics, Mannheim
Glycerol	Merck, Darmstadt
D-Glucose	Roche Diagnostics, Mannheim
HCl	Merck, Darmstadt
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

RPMI medium 1640	Gibco BRL Life Tech., Eggenstein
RPMI medium 1640, *without Phenol red*	Gibco BRL Life Tech., Eggenstein
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

All the oligonucleotides were made by *MWG Biotech GmbH (Germany)* and were all of an high-pressure liquid chromatography (HPLC)-purified grade.

2.1.2.1 Oligonucleotides for cloning into pCR 2.1 vector

Specific forward (for) and reverse (rev) oligonucleotides were used to amplify portions of genes of interest by PCR, plasmid DNA as a template, in order to clone the PCR products in a pCR 2.1 vector (TOPO cloning kit, *Invitrogen, The Nederlands)* as described in paragraph 2.1.13. In brackets, the names of the genes and the length of the PCR products are indicated.

Bag-1L (N-terminal sequence (a.a. 3-115), 350 bp)
5’ primer, LSBAL1: 5′ TCGGATCCCTCAGCGCGGGGGGGCG 3′
3’ primer, SMBA3: 5′ TCTCTAGACTCTTCGCCCTGGGTCGC 3′;

NLS-Bag-1M (fusion of SV40 nuclear localization signal and the N-terminal sequence of Bag-1M with addetional BamHI site, 190 bp)
5’ primer, NLS5p: 5′ TCGGATCCACCATGGTCCCCAAGAAGAAGCGGAAG GTCCGAAAGAAGAAAACCCGGC 3′
3’ primer, NLS3p: 5′ TCCGGGTCGACTCCTCGTCCCGGG 3′;

HAfr-NLS-Bag-1MN (fusion of SV40 nuclear localization signal and the N-terminal sequence of Bag-1M with 'GAT' frame and addetional BamHI site, 190 bp)
5’ primer, NLS5pT2: 5′ TCGGATCCGCCCAAGAAGAAGCGGAAGGTC 3′
3’ primer, NLS3p: 5′ TCCGGGTCGACTCCTCGTCCCGGG 3′;

2.1.2.2 Oligonucleotides for chromatin immunoprecipitation (ChIP)

PSA promoter

Region –459-121

5’ primer, PSA1*459d: 5′ GCCAAGACATCTATTTCAGGAGC 3′
3’ primer, PSA1*121r: 5′ CCCACACCCAGAGCTGTGGAAGG 3

Region –4288-3922

5’ primer, PSA3*4288d: 5′ GGGGTTTGTGCCACTGGTGAG 3′
3’ primer, PSA3*3922r: 5′ GGGAGGCAATTCTCCATGGTT 3′

Region –1997-1846

5’ primer, intern_for: 5′ CTGTGCTTGGAGTTTACCTGA 3′
3’ primer, intern_rev: 5′ GCAGAGGTTGCAGTGAGCC 3′

Region –7694-7484

5’ primer, distant_for: 5′ GATGGTGTTTCACCGTGTTG 3′
3’ primer, distant_rev: 5′ AGAGTGCAGTGAGCCGAGAT 3

ßactin promoter

5’ primer, for: 5′ TCCTCCTCTTCCTCAATCTCG 3′
3’ primer, rev: 5′ AAGGCAACTTTCGGAACGG 3

2.1.3 Plasmids and constructs

pCR 2.1 TA vector (TOPO cloning kit) was purchased from *Invitrogen, (Munich, Germany);*

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 comprehending 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;

pG5E4-38Luc is a firefly luciferase reporter construct and was previously described by Mink *et al.* (1996);

Renillatkluc was obtained from *Promega (Mannheim, Germany);*

pT2Rap46 has been described by Schneikert *et al.* (1999);

pT2-NLS-Bag1M was created by ligation of SV40 nuclear localisation signal and Bag1M N-teminus fusion *BamHI-SmaI* fragment from pTOPO-HAfr-NLS-Bag1MN construct and *SmaI-XbaI* Bag1M C-terminal fragment from pT2-Rap46 into pT2 vector linearized with *BamHI* and *XbaI* restriction digest;

pcDNA3-Bag1L construct was a gift from J.Reed;

pcDNA3-HA-Bag1L, pcDNA3-HA-AR1 and **pcDNA3-HA-AR5** plasmids were created by introducing a cDNA fragment encoding the HA immunotag into BamHI-XhoI sites of pcDNA3 (Invitrogen) with subsequent in frame

insertion of the coding sequence for BAG-1L, AR-1 and AR-5, respectively, into the resulting vector;

pcDNA3-HA-BAG-1L-C the construct encoding the HA tagged BAG-1L-C was created by replacing of SacII-XbaI fragment of pcDNA3-HA-Bag1L with the SacII-XbaI fragment of pGex-3x-Bag1L-C;

pZEM-Hsp70-tag construct has already been described by Bellman *et al.* (1996);

pZEM-Hsp70-ATPase-tag and **pZEM-Hsp70-SBD-tag** constructs encoding LDH-tagged substrate binding and ATPase domains of human Hsp70, respectively, are freandly gifts of M.Jäättelä, Kopenhagen;

pSG5-AR, pSG5-AR-188, pSG5-AR-280, pSG5-AR-440, pSG5-AR-488 and **pSG5-AR-HBD** constructs were described previously (Gast *et al.,* 1998);

pEGFP-AR construct belongs to the plasmid collection of the Lab114;

pSG5-AR-HR and **pSG5-AR633stop** constructs encoding AR lacking the hinge region and hinge region together with hormone binding domain, respectively, are from the plasmid collection of the Lab 114;

pM-C/EBP- plasmid has been described by Mink *et al.* (1997);

pM-AR-1 and **pM-AR-5** constructs encoding the fusions of Gal4 DBD and amino acids 101-370 and 360-548 of human AR, respectively are from the plasmid collection of the Lab 114;

GST constructs

pGex-2T-Rap46 and **pGex-2T-Rap46-C47** were described by Schneikert *et al.* (2000);

pGex-4T-1-BAG1L construct was a gift from J.Reed;

pGex-3x-BAG1L-C plasmid is from the plasmid collection of the Lab 114;

pGex-4T-1-BAG1LNterm, encoding the first 127 amino acids of BAG-1L, fused to the GST, was generated by cuting out of the SalI-XhoI fragment of pGex-4T-1-BAG1L with subsequent religation of the vector;

2.1.4 Antibodies

anti-AR

F39.4.1 (*BioGenex)*

Mouse monoclonal antibody against aa 301-320 of the human androgen receptor;

AR (c-19) (*Santa Cruz Biotechnology Inc.)*

Rabbit polyclonal antibody against the last 19 aa of the human androgen receptor;

AR PG-21 *(Upstate Biotecnology (Biomol))*

Rabbit polyclonal antibody against aa 1-21 of the human androgen receptor*;*

anti-Bag1

Bag-1 (C-16**) (***Santa Cruz Biotechnology Inc.)*

rabbit polyclonal antibody against the C-terminus of Bag-1 protein;

Bag-1L-specific antiserum (*Eurogentec*)

rabbit poyclonal antiserum against N-terminal sequences of BAG-1L;

anti-Hsp70 (K-20) (*Santa Cruz Biotechnology Inc.);*

goat polyclonal IgGs agains sequences of human heat shock inducible isoform of Hsp/Hsc70 family proteins;

anti-LDH (*Eurogentec*)

rabbit polyclonal IgG against the sequences of human testis lactate dehydrogenase;

anti-HA

clone 12CA5 *(Boehringer, Mannheim)*

mouse monoclonal IgG against influenza Haemagglutinine peptide;

All secondary antibodies HRP conjugated were purchased from *DAKO Diagnostika GmbH, (Hamburg, Germany).*

All secondary fluorescently labled antibodies were purchased from *Dianova, Hamburg.*

Rabbit and goat pre-immune sera were obtained from *VECTOR Laboratories Inc., CA, U.S.A.*.

2.1.5 Enzymes

Unless otherwise indicated, all restriction endonucleases and other enzymes were purchased from *New England Biolabs (Frankfurt am Main, Germany); Gibco (Karlsruhe, Germany); Takara (Taufkirchen, Germany); Roche (Mannheim, Germany).*

2.1.6 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 Nederlands).*

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.

2.1.7 Cell lines and media

Tissue culture media for mammalian cells were, unless otherwise stated, purchased from *Life Technologies GmbH (Karlsruhe, Germany).* FBS of New Sealand origin was purchased from *GIBCO (Taufkirchen, Germany)*. 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.

The human prostate carcinoma PC3 cell line belonges to the cell bank of ITG.

The PC3#104 TO cell line was created by stable transfection of PC3 cells with Tetracycline responcive regulator by Dr.Mink (Lab.114)

The human prostate carcinoma cell line 22Rv1 is a kind gift of Prof. Dr. Schalken, Netherlands.

2.1.8 Other materials

Protein A Agarose (packed beads);

kit were purchased from *Upstate Biotecnology (Biomol), (Hamburg, Germany).*

2.2 METHODS

The majority of protocols and recipes for commonly used buffers and routine procedures used 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 CELL CULTUERE AND TRANSFECTION METHODS

2.2.1.1 Cell culture

All mammalian cells were maintained at 37°C in an incubator (*Forma Scientific, Labortect GmbH, Göttingen, Germany)* in 5% CO_2 and 95% air humidity. All cells were grown in Petri dishes or flasks *(Greiner, Frickenhausen, Germany)* of varying sizes depending on the application. 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 1X PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4). After removal of PBS, 0.25% trypsin was applied to the cells and the cells incubated at 37°C until they became detached. Fresh medium was then directly applied and the cells re-plated at the desired density in new Petri dishes. The human prostate carcinoma PC3 cell line was cultured in DMEM, 10% FBS and LNCaP and 22Rv1 prostate cancer cells were cultured in RPMI-1640 medimum, 10% FBS.

2.2.1.2 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.

2.2.1.3 Transfection of cells by FuGene6^{TM} reagent

FuGene6^{TM} transfection reagent was used according to the manufacturer's instructions. Briefly, proliferating cells were sub-cultured the day before transfection so that they could reach 50-80% confluency on the day of the experiment. For a 35 mm culture dish, 3-6 µl of FuGene6^{TM} reagent were diluted in serum-free medium to a total volume of 100 µl and incubated at RT for 5 min. The diluted FuGene6^{TM} solution was then added dropwise to a second sterile tube containing 1-2 µg of DNA and incubated at RT for 15 min. The mixture was finally transferred to the cells dropwise and by gentle swirling to ensure an even distribution. For the AR transactivation studies typically 0,6µg of desired reporter plasmid, 0,1µg of renilla luciferase construct as an internal control together with 0,2µg of an AR expression vector in presence of 0,5µg of the control vector or BAG-1L expressing plasmid were transfected per dish. 24h after transfection the cells were treated either with vehicle alone or vehicle containing 10^{-7}M dihydrotestosterone (DHT) for another 20h. Then the cells were harvested for the luciferase mesurements. For the protein localization expreiments the cells were transfected with 1,5-3µg of the expression vector(s) for the protein(s) of interest. The cells were treated with hormone 36h after transfection for 3h. For all transfection experiments the cells were kept in the medium containing 3% charcoal treated FCS (CCS) to remove resedual steroids.

2.2.1.4 Transfection of cells with calcium phosphate method

20h before transfection the cells were trypsinized and plated onto the 6-well plates in the medium supplemented with 3% CCS at the concentration of $2x10^5$ cells in 2ml per well. For the transfection the plasmid DNA mix (2-3µg) was dissolved in 250mM $CaCl_2$ and drop after drop mixed with the equal volume of 2xHBS (274 mM NaCl, 10 mM Kcl, 1,4 mM Na_2HPO_4, 12 mM D-Glucose and 40 mM Hepes, pH 7,1). After 20-40 min of incubation at room temperature the calcium phosphate/DNA precipitate was added to the cell medium. The cells were then incubated for 3-5h at 37°C and 5% CO_2. Later the medium was removed and the cells were treated with 15% Glycerol in PBS for 2 min,

followed by 2x washing with PBS. After that the fresh medium containing 3% CCS was added. 24h later the cells were treated either with vehicle alone or vehicle containing 10^{-7}M dihydrotestosterone (DHT) for another 20h. After that the cells were harvested for the luciferase mesurements.

2.2.2 NUCLEIC ACIDS METHODS

2.2.2.1 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 a ratio of 1:1 *(v/v)* was added and the mixture was vortexed. The two phases were separated by centrifugation at 12000 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 *(*1:1, *v/v)*.

2.2.2.2 Ethanol precipitation of nucleic acids

In order to recover nucleic acids from solution, the salt concentration was brought to 200 mM with 3 M Na-acetate (pH 4.8-5.0) and 2.5 volumes of cold ethanol was added. After 30 min 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 re-suspended in either H_2O or TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

2.2.2.3 Determination of nucleic acid concentration

The concentration of nucleic acids was determined by measuring their optical density (OD) at 260 and 280 nm. 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. A ratio of OD_{260}/OD_{280} ranging from 1.8 to 2.0 indicates an acceptable purity of the nucleic acid.

2.2.2.4 Plasmid DNA preparation

2.2.2.4.1 Large scale plasmid preparation

Plasmid DNA was prepared on a large scale using the Qiagen Plasmid Maxi Kit *(Qiagen)* following the manufacturer's instructions. Briefly, a volume of 200-250 ml of LB medium (10 g Tryptone; 5 g Yeast extract and 10 g NaCl for 1 l, autoclaved before use) supplemented with the appropriate antibiotic was inoculated with a single bacterial colony and incubated with shaking (220 rpm) at 37°C overnight until the bacteria had reached a stationary growth phase. The bacteria were pelleted by centrifugation in a fixed angle rotor at 6000 x g for 10 min and the pellet resuspended in 10 ml solution P1 (10 mM EDTA, 50 mM Tris-HCl pH 8.0 and 400 µg/ml RNAse A). Following 5-10 min incubation at RT, the cells were lysed by addition of 10 ml solution P2 (200 mM NaOH and 1% SDS). Once the solution had taken an opaque appearance, the mixture was neutralised with 10 ml of solution P3 (3 M Na acetate pH 4.8), the entire content inverted gently to aid mixing of the solutions. After additional 10-20 min on ice, the cell wall fragments and the bacterial chromosomal DNA were sedimented by centrifugation at 9000 x g for 20 min at 4°C in a fixed angle rotor. The retained supernatant was then added directly to a pre-equilibrated Qiagen-tip 500 column and the plasmid DNA was recovered in the provided elution buffer according to the manufacturer's instructions. The purified DNA was then precipitated using 0.8-1 volumes of isopropanol, washed twice in 70% ethanol before being re-suspended to a final concentration of 1-3 mg/ml in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -20°C.

2.2.2.4.2 Small scale plasmid preparation

Single bacterial colonies were picked, inoculated in 5 ml of LB medium (10 g Tryptone; 5 g Yeast extract and 10 g NaCl for 1 l, autoclaved before use) containing the appropriate antibiotic and grown overnight under constant shaking at 37°C. 1.5 ml of these cultures were transferred to a fresh vial and the bacteria were collected as a pellet by centrifugation at 6000 rpm for 10 min at RT. For isolation of plasmid DNA, a modified "large scale plasmid preparation" protocol was used. Briefly, the bacterial pellet was resuspended in 150 µl of solution P1 containing RNase A, from the MaxiPrep Kit *(Qiagen)* and let 5 min at RT. Then 300 µl of solution P2 were added and the mixture

was left 5 min on ice. After addition of 225 µl of solution P3 followed by vortexing, the lysate was again left for 5 min on ice. After centrifugation at 12000 rpm for 10 min at 4°C, 400 µl of the supernatant were collected and transferred to a fresh tube containing 1 ml of ice-cold ethanol. Incubation for 30 min at -80°C would allow the nucleic acid to precipitate. The plasmid DNA pellet was collect by centrifugation at 12000 rpm for 10 min at 4°C and was washed once with 70% ethanol. The resulting pellet was air-dried and resuspended in 50 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

2.2.2.5 Isolation/Purification of genomic DNA from eukaryotic cells

In order to isolate DNA, fresh tissue (approximately 1 g) was lysed by overnight incubation in 0.6 ml of lysis buffer (10 mM Tris pH 8.0, 10 mM EDTA, 150 mM NaCl, 0.2% SDS, 500 µg/mL Proteinase K) at 55°C with shaking. Tissue culture cells instead were grown in a 6-well plate until sub-confluency, washed once in PBS (137 mM NaCl, 2.7 mM KCl, 0,7 mM $CaCl_2$, 0,6 mM $MgCl_2$, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and lysed by incubation with 0.9 ml of lysis buffer at 37°C overnight. Lysates were then transferred into a fresh vial and after addition of 1/3 total volume of saturated NaCl (approximately 6-7 M NaCl) the tubes were shaken for another 15 min. The cell debris were removed by mild centrifugation at 5000 rpm for 10 min. The DNA-containing supernatant was transferred into a fresh vial and DNA was precipitated by addition of 2 volumes of ethanol and centrifugation at 14000 rpm for 10 min at room temperature. The DNA pellet was washed with 70% ethanol and re-suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -20°C until used.

2.2.2.6 Restriction endonuclease digestion of DNA

Typically 2-3 units of a restriction enzyme were used per µg DNA. DNA was digested at a concentration of 1 µg/µl in the buffer recommended by the supplier. The reaction was carried out for 1 hour to overnight at 37°C (unless otherwise recommended by the supplier). The quality of the digest was controlled by gel electrophoresis.

2.2.2.7 Size separation of nucleic acid by agarose gel electrophoresis

Given amounts of agarose *(Peqlab, Erlangen, Germany)* final concentration between 0.7 and 2%) were dissolved in TAE buffer (0.04 M Tris pH 7.2, 0.02 M Sodium acetate, 1 mM 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 (50-100 V) at RT for the required time. Samples were loaded onto the gel in loading buffer (0.5 M 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.

2.2.2.8 Isolation/purification of DNA from agarose gels

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 (provided) and the DNA-binding resin (provided) was added. After two subsequent washing steps the resin with bound DNA was air-dried and the DNA eluted by addition of bi-distilled H_2O. The presence of the DNA fragment in solution was confirmed by agarose gel electrophoresis.

2.2.2.9 DNA ligation

All ligation reactions, except for the TOPO cloning (see 2.2.2.13) were performed using T4 ligase and appropriate buffer supplied by the manufacturer *(Life Technologies, Karlsruhe, Germany)* and incubated either overnight at 16°C or 2-3 h at RT. When a fragment was intended to be inserted in a vector, usually the reaction mixture would contain a 10:1 ratio insert:vector. The reaction was stopped by inactivation of the enzyme for 10 min at 70°C.

2.2.2.10 Sub-cloning

Typically, the cloned fragment of DNA was released from the vector using appropriate restriction endonucleases, resolved on an agarose gel by electrophoresis, isolated from the gel and subsequently cloned into the new vector using compatible sites or through blunt end ligation.

2.2.2.11 Polymerase Chain Reaction (PCR)

All PCR reactions were carried out in a Thermal Cycler machine *(Perkin Elmer 9600/2400)*. Usually the PCR reaction in a total volume 20 µl contained 10 ng of plasmid or 100 ng of genomic template DNA, 200 µM dNTP, 10 pmol of primers, 0.25 U to 1 U of Taq Polymerase and 1× supplier's buffer. Addition of 5-10% DMSO was sometimes necessary to optimise the reaction. Depending on the application specific cycling parameters were used for each individual PCR reaction. Usually 1 cycle (94°C, 1 min) followed by 30 cycles (94°C, 1 min; 45°C, 1 min; 72°C, 1 min) were carried out. The annealing temperature was changed according to the different primers' T_m. In case of TOPO TA cloning in pCR2.1 vector, an additional cycle of 72°C for 10 minutes was performed to ensure the generation of A-overhangs by Taq Polymerase in DNA templates for cloning.

2.2.2.12 Cloning into pCR2.1-TOPO TA vector

The TOPO (TA) cloning Kit *(Invitrogen, Groningen, The Nederlands)* was used according to the manufacturer's instructions. 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 deoxyadenosine (A) to the 3´end of PCR products. This allows PCR inserts to efficiently ligate with the vector´s overhanging 3´-deoxythymidine residues. The Topoisomerase I from *Vaccinia* virus functions like both a restriction enzyme and a ligase. It binds to duplex DNA at specific sites and forms a covalent bond with the phosphate group of the 3´thymidine. It cleaves one DNA strand, enabling the DNA to unwind. The enzyme then religates the ends of the cleaved strand and releases

itself from the DNA. The reaction mixture consists of 0.5 to 4 µl of fresh PCR product and 1 µl of pCR2.1 TOPO TA vector; sterile water is added to a total volume of 6 µl. The reaction mixture is mixed gently and incubated for 5 min at RT. The ligation is thus complete and 2 µl are used for transformation into chemically competent *E.Coli* TOP10F´.

2.2.2.13 Transformation of chemically competent bacteria

This type of transformation was used for propagation of different plasmids. The DNA sample, usually 0.01-1 µg, dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) or water, was added to 100 µl of the ice-thawed chemically competent cells. After brief vortexing and incubation on ice for 5-30 min the cells were heat-shocked for 30-60 s at 42°C and immediately transferred on ice. The transformed cells were finally mixed with 250 µl room temperature SOC medium (2% tryptone; 0.5% yeast extract; 10mM NaCl; 2.5mM KCl; 10mM $MgCl_2$; 10mM $MgSO_4$; 20mM glucose), incubated at 37°C with shaking (200 rpm) for 1 hour, spread onto pre-warmed selective agar plates (typically, supplemented with ampicillin (100 µg/ml) and grown overnight at 37°C.

2.2.2.14 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.

2.2.1.15 Isolation of total RNA from cultured cells

Cultured cells were grown to sub-confluency in 10 cm petri dishes (*Greiner*). After removing the medium, cells were lysed by addition of 3 ml of the TRIFAST Reagent (*Peqlab*). 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. After addition of chloroform (0.2 ml per 1 ml of TRIFAST reagent used) and vigorous shaking by hand the samples were allowed to stand at room temperature for another 5-10 min and centrifuged at 12000 rpm for 15 min at RT. Centrifugation resulted in phase separation. 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, RNA was re-suspended in bi-distilled water at a final concentration of 1 mg/mL and stored at -80°C.

2.2.3 PROTEIN METHODS

2.2.3.1 Determination of protein concentration

Protein concentration was determined using 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 mentioned order. To 495 µl of this mixture, called Lowry IV reagent, 5 µl 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 taken and transferred to a 96 wells plate. Absorbance at 600 nm was measured. To establish the exact amount of protein present in the probe a standard

curve was obtained by measuring the absorbance of known amounts of BSA (typically from 0 to 50 µg).

2.2.3.2 Preparation of protein lysates from mammalian cells

Cells at 80-90% confluency, typically grown in a 6 wells plate, were washed twice with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 0,7 mM $CaCl_2$, 0,6 mM $MgCl_2$, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4), then collected in 300-400 µl of appropriate lysis buffer.

According to experimental need, different lysis buffers were used:

2X Laemmli buffer (160 mM Tris HCl pH 6.8, 4% SDS, 16% glycerol and 0.005% bromophenol blue with addition of 10% β-mercaptoethanol);

Passive Lysis buffer (*Promega*).

2.2.3.3 Separation of proteins by SDS polyacrylamide gel electrophoresis

Usually 10% SDS-Polyacrilamide gels were used: 9.9 ml acrylamide/*bis*-acrylamide 30:0.8, *(Carl Roth GmbH & Co, Karlsruhe, Germany)*; 11.7 ml 1 M Tris-HCl pH 8.8; 8.4 ml bi-distilled H_2O; 150 µl 20% *(w/v)* SDS 20%; 300 µl APS; 15 µl TEMED. The Penguin Doppelgelsystem P9DS apparatus *(Peqlab, Erlangen, Germany)* was used to cast the gel. Twenty ml of resolving gel were poured and overlaid with Rotisol. After polymerisation Rotisol was washed off with deionised water. Ten ml of stacking gel were then added (7.3 ml bi-distilled H_2O; 1.68 ml acrylamide/bis-acrylamide; 0.69 ml 1 M Tris-HCl, pH 6.8; 56 µl 20% SDS; 45 µl 10% APS and 15 µl TEMED). After polymerisation, the gel was fixed within the gel tank. 1X Laemmli buffer was used as running buffer (25 mM Tris, 200 mM glycine, 0.1% (w/v) SDS). Typically 40-60 µl of cell lysate were loaded per lane onto a SDS-PAGE. The run was performed at 45 mA.

2.2.3.4 Preparation of GST fusion proteins

GST fusion protein expression was induced in logarithmically groing cultures of transformed bucteria (E.coli BL21). 500ml of 2xLB (20 g Tryptone; 10 g Yeast extract and 20 g NaCl for 1 l) were inoculated with 5ml of over night culter and incubated at 37°C with vigorous shaking for 3-4h till the culture reached the logarithmic fase

(OD_{600}=0,6-0,8). Then expression of the fusion protein was induced by adding isopropyl-ß-D-thiogalactopyranoside (IPTG) to a final concentration of 0,5 mM, and groing the bacteria for an additional 4,5 h at room temperature. Bacteria were resuspended in the Sonication buffer (PBSSon) (137 mM NaCl, 2.7 mM KCl, 0,7 mM $CaCl_2$, 0,6 mM $MgCl_2$, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM DTT, 2 mM EDTA, ß-Mercaptoethanol (1:1000), 1% protease inhibitor cocktail (*Sigma*)), sonified and an extract of the soluble protein was prepared by centrifugation for 10min at 14,000rpm. An extruct was aliqouted and stored at -80°C.

2.2.3.5 Staining the SDS-PAGE gels *(Coomassie staining)*

Gels were incubated with Coomassie stain solution (0.5 g/l Coomassie brilliant blue, 45% methanol and 10% acetic acid) for 1 hour. To de-stain, the gels were incubated in 10% acetic acid, 10% methanol for 24 hours or longer with several changes of the de-stain solution until the background appeared clear.

2.2.3.6 Western blotting

After proteins were separated by SDS-PAGE, they were electrically transferred onto Immobilon membrane (*Millipore*, type PVDF, pre-soaked in methanol) at 250-300 mA for at least 6 hours in transfer buffer (25 mM Tris, 190 mM glycine, 20% (*v/v*) methanol and 0.05% *(w/v)* SDS). Immunoblotting was performed according to individual instructions for each antibody. Typically, in order to reduce unspecific binding of the antibodies to the membrane, the blot was incubated in blocking solution: PBST (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.05% Tween 20) supplemented with either 3-5% *(w/v)* non-fat dried milk at RT for 1 h with shaking. For detection of proteins of interest the membrane was incubated in blocking solution containing the appropriate primary antibody (optimal working dilution was determined empirically) at room temperature for 2h or 4°C overnight. After 3 washes of 10 min each in PBST, the membrane was incubated 1 hour in blocking solution containing a 1:2000 dilution of HRP-conjugated secondary antibody. Once the membrane had been washed 3 times 10 min each, detection of specific proteins was achieved by enhanced chemiluminescence using ECL Western blotting detection reagents as recommended by the manufacturer *(Amersham, Braunschweig, Germany)* and exposure to ECL Hyperfilm *(Amersham, Braunschweig, Germany)* .

2.2.3.7 Stripping of the Western blot membranes

To allow more than a single use of Western blot membranes, the membranes were incubated with a stripping solution (62.5 mM Tris, pH 6.8, 2% SDS, 0.75% 2-mercaptoethanol) at 50°C for 30 min with shaking. The membranes were then washed three times in the 1X PBS without Ca- and Mg- (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4) for 15 min each time.

2.2.3.8 Analysis of protein-protein interaction in vitro using GST pull-down assay

In vitro translation

To get the radioactively labeled proteins for GST pull-down assay, the template cDNAs were *in vitro* transcribed and sequentially translated in presence of L-[^{35}S]-Methionin. This reaction was carried out by using *TNT® Quick Coupled Transcription/Translation System* (Promega, Madison, WI, USA). The following components were combined:

40 µl TNT® T7 Quick Master Mix
4 µl L-[^{35}S]-Methionin
0,5 µg plasmid DNA template
H_2O to a final volume of 50 µl.

The reaction mixture was incubated for 60-90 min at 30°C. After the TNT-reaction *in vitro* translated AR proteins were incubated with vehicle or DHT (10^{-7} M) at room temperature for addetional 15 min.

Preparation of protein lysates from mammalian cells

Cells at 80-90% confluency, typically grown in a 6 wells plate, were washed twice with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 0,7 mM $CaCl_2$, 0,6 mM $MgCl_2$,6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4), then collected in 1ml PBS, spinned down for 3 min at 4,000 rpm at 4°C, resuspended in 100 µl of a special lysis buffer LB (10 mM Na_2HPO_4, pH 7.4, 1 mM EDTA, 400 mM KCl, 15% glycerol) and lysed with 3 freezing-thawing cycles. Then the lysates were sentrifuged for 10 min at 12,000 rpm at 4°C, the supernatants were taken and stored at -80°C.

In vitro protein binding assay

This assay is based on the high affinity of GST to the immobilized Glutathione and was used to study the interaction between GST fusion and *in vitro* translated or mammalian cell lysate proteins.

An aliquat of a soluble protein extract, prepared as described in 2.2.3.5, containing 2-5 µg of GST fusion protein, was incubated with 60 µl of 50% slurry of *GlutathioneSepharose 4B* (Pharmacia Biotech) in PBS (137 mM NaCl, 2.7 mM KCl, 0,7 mM $CaCl_2$, 0,6 mM $MgCl_2$,6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4) for 30 min at 4°C in a volume of 1 ml of PBSSon (137 mM NaCl, 2.7 mM KCl, 0,7 mM $CaCl_2$, 0,6 mM $MgCl_2$,6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM DTT, 2 mM EDTA, ß-Mercaptoethanol (1:1000), 1% protease inhibitor cocktail (*Sigma*)). After the incubation the Sepharose beads bound proteins were washed 3x with PBSSon, supplemented with 0,2% NP-40 (PBSSonN). The in vitro translated proteins or cell lysates were diluted 10-fold with PBSSonN and incubated with the GST fusion proteins under constant agitation for 2,5 h at 4°C. The Sepharose beads were then washed 3x with PBSSonN. Finally, the proteins were eluted with 2X Laemmli buffer (160 mM Tris HCl pH 6.8, 4% SDS, 16% glycerol and 0.005% bromophenol blue with addition of 10% µ-mercaptoethanol) and analysed by SDS-polyacrylamide gel electrophoresis (PAGE) and either autoradiography or Western blott.

2.2.3.9 Detection of radioactive signal

The dried gel was exposed to Amersham hyperfilm or exposed to a Phosphorimager screen *(Fujifilm, Japan)* and subsequently analysed with the AIDA 200.1 software *(Raytest GmbH, Straubenhardt, Germany).*

2.2.3.10 Immunofluorescent staining of the intracellular proteins

For the immunofluorescence experiments were used transientely transfected PC3#104 TO cells. $2x10^5$ cells per well were seeded onto the 6-well plates, with a sterile coverslip put into every well. The next day the cells were transfected with the FuGene6™ transfection reagent and 24 h after transfection treated with either the vehicle or 10^{-7} M DHT for 3 h. After that the cells were washed 2 times with PBS (137 mM NaCl, 2.7 mM

KCl, 0,7 mM $CaCl_2$, 0,6 mM $MgCl_2$,6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and fixed for 20 min with 3% Paraformaldehyde (in PBS). To remove the paraformaldehyde ions the cells were then incubated for 15 min in 50 mM NH_4Cl (in PBS) and finally pemeabilized in 0,1% Triton-100 (in PBS) for 5 min. After that the cells were washed 2 times with PBS and blocked for 1 h with PBS supplemented with 3% FCS (PBS-3%FCS).

After the blocking PBS-3%FCS was removed and 150 µl of the primery antibody solution in PBS-3%FCS was pipeted directly onto the coverslip and incubated for 1h. Then the cells were washed 3 times with PBS-3%FCS and covered with 150 µl of the secondary antibody for another 30-60 min. After that the cells were washed 3 times with PBS.

Finally the coverslips were taken from the 6-well plate, rinsed in bidest. water and the water drops were removed and the coverslip was placed onto the glass slide with a drop of Fluorescence mounting medium (*Dako*) with the cell covered side. Then the slides were dried for 30-60 min at room temperature and stored in a dark place at 4°C.

Preparation of the Paraformaldehyde solution

To make a 10% stock solution 20 g of paraformaldehyde cristalls were dissolved in 80 ml of water and heated up to 60°C. To facilitate the dissolving of paraformaldehyde several drops of NaOH was added. After the cristalls were dissolved the solution was cooled down, mixed with 100 ml of 2x PBS, aliquated and stored at -20°C.

2.2.4 OTHER METHODS

2.2.4.1 Measurement of firefly luciferase activity

After transfection, cells were let grow for at least 24-48 h to allow expression of the transfected reporter gene. Typically they were kept in 6-wells plates. Cells were then washed twice in PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4), strictly without Ca^{2+} and Mg^{2+}. PBS was then removed and the cells harvested in 300 µl 1X lysis buffer (Passive Lysis Buffer, *Promega, Mannheim, Germany)*. The cells were kept on ice for ca. 10 min and occasionally rocked to distribute the buffer

evenly and favour the detachment of the cells from the plates. The cell lysates were pipetted up and down several times, collected with a rubber policeman and finally transferred to pre-cooled vials. 100 µl of cell lysate were transferred to a reading tube with autoinjection of 350 µl of assay buffer (1mM DTT, 1 mM ATP in glycylglycine buffer (25 mM glycylglycine, 15 mM $MgSO_4$ and 4 mM EGTA) and 100 µl of luciferin assay solution (1mM luciferin; stock solution: 0.28 mg/ml) were then added. Luciferase activity was measured by use of a luminometer *(Berthold, Wildbad, Germany).*

2.2.4.2 Measurement of Renilla luciferase activity

100 µl of cell lysates obtained as described in the paragraph 2. were mixed with 500 µl of coelenterasin buffer (0.1 M KPi-buffer (0.2 M KH_2PO_4 and 0.2 M K_2HPO_4, pH 7,6), 0.5 NaCl, 1 mM EDTA, pH 7.6) together with substrate solution (25 nM end concentration coelenterazine, *Byosinth AG, Gstaad, Switzerland)*. Luminescence was measured by use of a luminometer *(Berthold, Wildbad, Germany).*

2.2.4.3 Cromatin immunoprecipitation (ChIP) assay

For the ChIP experiments human prostate cancer 22Rv1 cells were grown in phenol red-free RPMI-medium 1640 (GIBCO-BRL.) supplemented with 5% charcoal-tripped fetal bovine serum (CCS). After 3-4 days of cultivation the cells were treated either with vehicle alone or with vehicle containing 10^{-7}M DHT for 2,5 h, washed with PBS and cross-linked with 1% formaldehyde (in PBS) for 20 min at room temperature. Cells were then rinsed twice with ice-cold PBS, collected into PBS and centrifuged for 5 min at 4,000 rpm at 4°C. The pellets were then resuspended in 0,3 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8,0, and 1% protease inhibitor cocktail (*Sigma*)) and sonicated 12 times 20 pulses at the output 1-2, followed by the centrifugation for 10 min at maximal speed at 4°C. Supernatants were collected and diluted 10-fold in dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl and 20 mM Tris-HCl, pH 8,0) followed by immunoclearing with 2 µg sheared fish sperm DNA, 1:100 diluted preimmune serum and protein A-agarose (*UpState*) (6o µl of 50% slurry in 10 mM Tris-HCl, pH 8,0, and 1 mM EDTA) for 2h at 4°C. Immunoprecipitation was performed for overnight at 4°C with specific antibodies. After immunoprecipitation, 60 µl protein A-

agarose and 2 µg fish sperm DNA were added and incubation was continued for another 1 h. Agarose beads were washed sequentially for 10 min each in TSE I (0,1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8,0, and 150 mM NaCl), TSE II (0,1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8,0, and 500 mM NaCl) and buffer III (0,25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA and 10 mM Tris-HCl, pH 8,0). Beads were then washed 3 times with TE buffer (10 mM Tris-HCl, pH 8,0, and 1 mM EDTA) and extracted 3 times with 60 µl of 1% SDS, 0,1 M $NaHCO_3$). Eluates were poolled and heated at 65°C for overnight to reverse the cross-linking. DNA fragments were purified with a DNA purification kit (QIAquick PCR purification Kit, *Qiagen GmbH*). For PCR, 1-5 µl out of 50 µl DNA extraction was used PCR was carred out essentially as described in paragraph 2.2.2.12, but the cycle parametres:

30-35 cycles:

94°C	30s
65°C	1 min
72°C	1 min .

The PCR products were analysed by 2% agarose gel electrophoresis.

3. RESULTS

3.1 Establishment of an experimental system

To find an experimental system to study the effect of BAG-1L on ligand dependent transactivation function of the AR, the effect of BAG-1L on the AR activity was examined in AR-negative PC3 human prostate cancer and Cos7 simian kidney fibroblast cell lines. These cells were transiently transfected with a MMTV firefly luciferase indicator gene and a renilla luciferase construct as an internal control for the transfection efficiency together with an AR expression vector in the presence of BAG-1L expressing plasmid or empty vector. Transactivation by the AR was analyzed in the absence and presence of the androgen dehydrotestosterone (DHT). Luciferase assays were conducted and the result are presented as bar charts.

In these studies in both PC3 (**Fig.3.1A**) and Cos7 (**Fig.3.1B**) cell lines, BAG-1L did not affect the expression of the MMTV driven reporter gene in the absence of DHT. Androgen-dependent transactivation by the AR was enhanced in the presence of BAG-1L. BAG-1L-mediated effect on the transactivation function of the AR was much stronger in PC3 (about 7 fold) than in Cos7 (less than 2.5 fold) cells, probably due to cell-type specific differences. PC3 cell line was therefore chosen for subsequent transfection experiments to analyse the mechanism of the AR regulation by BAG-1L.

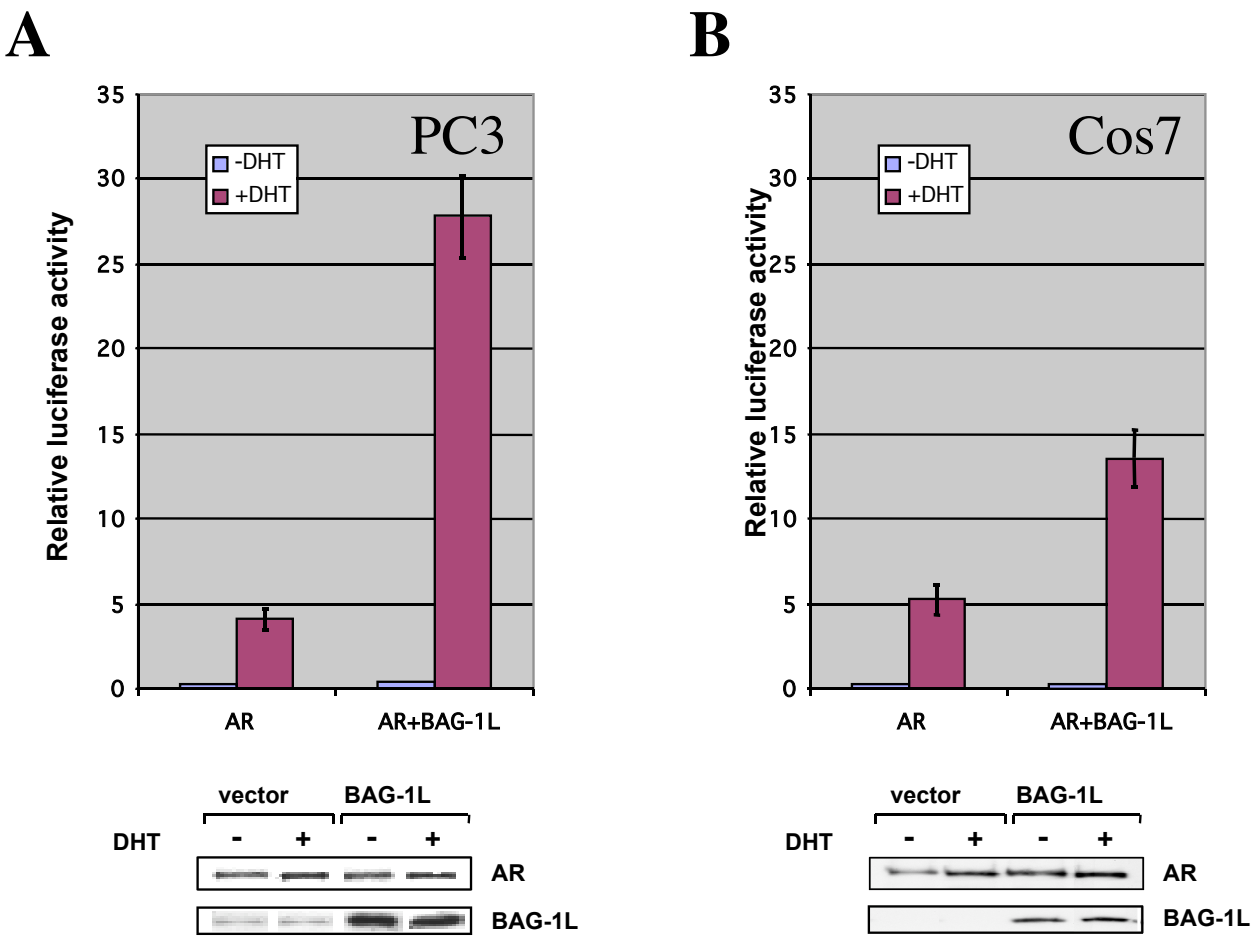

FIGURE 3.1 The effect of BAG-1L on AR transactivation function in PC3 and Cos 7 cells

PC3 (**A**) and Cos 7 (**B**) cells were transiently cotransfected with a MMTV firefly luciferase indicator gene and a renilla luciferase construct as an internal control together with the AR expression vector in the presence of BAG-1L expressing plasmid or control vector. Hormone treatment (0.1 μM of 5α-Dihydrotestosterone (DHT)) was performed in twenty-four hours after transfection and the cells were harvested twenty hours later for luciferase activity measurements. The results are expressed as the level of hormone induced expression of the MMTV construct after correcting for the transfection efficiency by renilla luciferase measurements and normalising to that of AR in presence of control vector. For the control of the protein levels the lysates were subjected to polyacrylamide gel electrophoresis (PAGE) and analysed by Western blot. The input was corrected for the transfection efficiency by renilla luciferase measurements.

3.2 Sequence requirements of androgen receptor for BAG-1L mediated enhancement of transactivation by the androgen receptor

AR is composed of several functional domains: DNA binding and the hinge region domains in the central part of the molecule, the hormone binding domain (HBD) at its C-terminus and two transactivation domains, the weak one at the C- and a fairly strong, responsible for up to 75% of the transactivation by the AR, at the N-terminal part of the receptor (Jenster *et al*., 1995; Poukka *et al*., 2000).

To determine how the transactivation function of the AR regulated by BAG-1L, it is necessary to find out the domains of the AR which function is required for the BAG-1L.mediated enhancement of the transactivation by AR. A panel of AR deletion mutants was usedin a transient transfection assay in PC3 cells, where the effect of BAG-1L on AR-mediated activation of the MMTV promoter activity was measured.

3.2.1 BAG-1L acts predominantly through the N-terminus of the androgen receptor

First, the AR deletion mutants lacking the hinge region (AR□HR), HBD (AR□HBD), or both of these domains (AR633stop), was examined.

The results show that the DHT-induced transactivation by the wild type AR was enhanced by 6.3 fold in the presence of BAG-1L (**Fig.3.2A**). Transactivation by the AR mutant lacking the hinge-region was enhanced by BAG-1L to almost the same extant as the wild type AR (6.2 fold) (**Fig.3.2A**), indicating that the hinge-region of the AR is not required for the enhancement of transactivation by the AR. Deletion of the hormone-binding domain (HBD) of the receptor or both hinge region and HBD turned the mutants to constitutively active proteins in a way that they can enhance the MMTV promoter activity in the absence of hormone. Nevertheless this ligand independent transactivation by the AR was still enhanced by BAG-1L, though to a slightly lower extent. The level of AR expression in the BAG-1L transfected cells determined by Western blot show that the effect of the latter was not due to an increase in the AR expression. The level of the

AR in the AR□HBD and AR633stop transfected cells is even reduced suggesting that the effect of BAG-1L may have been underestimated (**Fig.3.2B**). Thus, the result of this experiment demonstrates that the N-terminus of the AR is reguired for the enhancing action of BAG-1L.

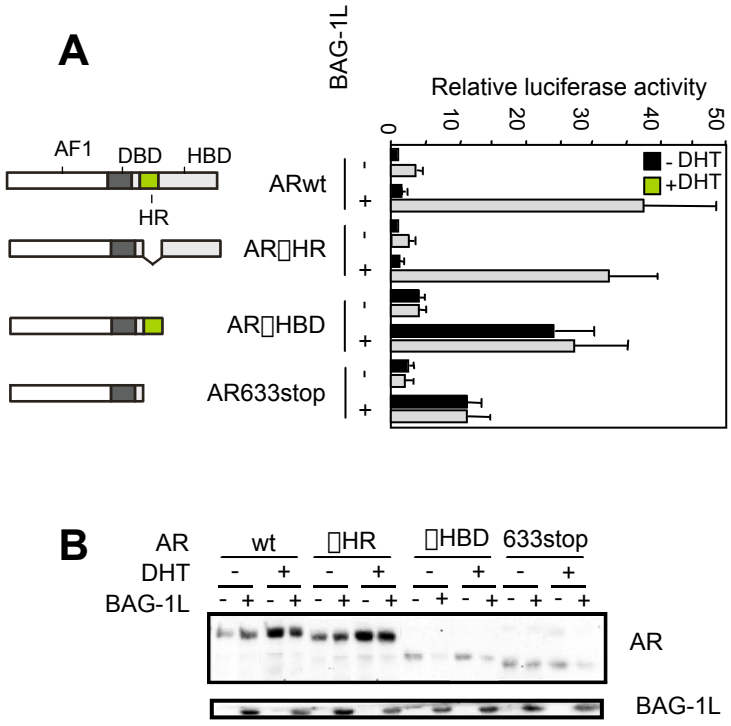

FIGURE 3.2 Effect of BAG-1L on the ARwt or AR C-terminus deletion mutants

A. PC3 cells were transiently cotransfected with a MMTV firefly luciferase indicator gene and a renilla luciferase construct as an internal control together with ARwt ot AR C-terminus deletion mutant expression vector in presence of the control vector or BAG-1L expressing plasmid. Treatment with 10^{-7}M DHT or control treatment with 80% ethanol was performed in twenty-four hours after transfection and the cells were harvested twenty houers later for luciferase activity measurements. **B**. For the control of the protein level the lysates were subjected to polyacrylamide gel electrophoresis (PAGE) and analysed by Western blot. The input was corrected for the transfection efficiency by renilla luciferase measurements.

To delineate the sequences in the N-terminus of the AR responcible for the action of BAG-1L, a panel of AR N-terminal progressive deletion mutants were examined for the ability of BAG-1L to modulate their transcription activation function. These mutants were employed in the transient transfection experiments as used in the analysis of the other AR mutants.

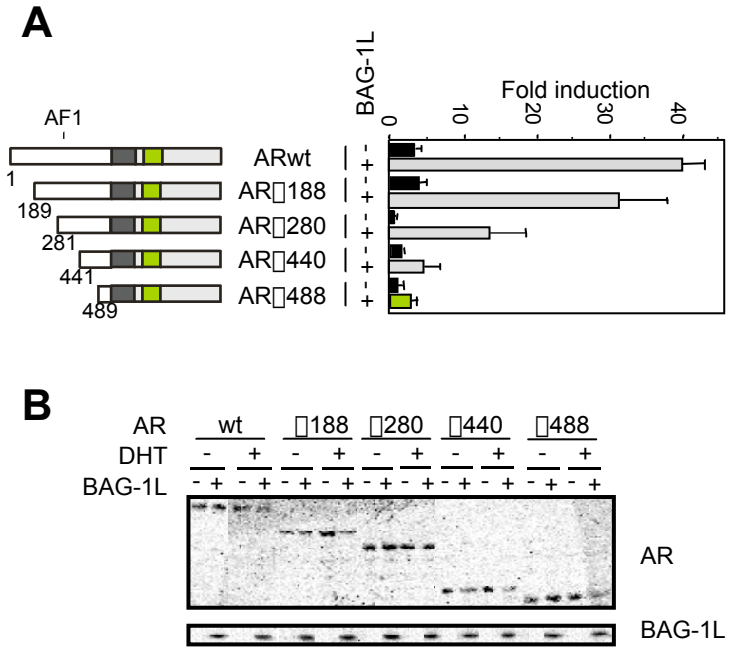

FIGURE 3.3 Effect of BAG-1L on the ARwt and AR N-terminus deletion mutants

A. PC3 cells were transiently cotransfected with a MMTV firefly luciferase indicator gene and a renilla luciferase construct as an internal control together with ARwt ot AR N-terminus deletion mutant expression vector in presence of the control vector or BAG-1L expressing plasmid. Treatment with 10^{-7}M DHT or control treatment with 80% ethanol was performed in 24 h after transfection and the cells were harvested 20 h later for luciferase activity measurements. **B**. For the control of the protein level the lysates were subjected to polyacrylamide gel electrophoresis (PAGE) and analysed by Western blot. The input was corrected for the transfection efficiency by renilla luciferase measurements and controlled by Coomassie staining

The experimental data showed, that deletions of the first 280 amino acids of the androgen receptor abrogated transactivation by the construct, most likely due to the deletion of a

large part of the □1 domain of the AR, responsible for the transactivation function of the receptor (**Fig. 3.3A**). Nevertheless, this deletion did not abolish the BAG-1L-mediated enhancement of transactivation function of the mutant AR (~8 fold effect for the AR□280 against ~10 fold for the ARwt). However deletion of further 200 amino acids led to the reduction of the effect of BAG-1L on the receptor function (2.2 and 1.9 fold for the AR□440 and AR□488, respectively, against ~10 fold for the ARwt) (**Fig. 3.3A**). This identified the region between positions 280 and 488 as an important region for the modulation of the receptor activity by BAG-1L. The examination of the AR and BAG-1L protein levels by Western blotting showed no effect of BAG-1L on the level of AR (**Fig. 3.3B**), demonstrating that the reduction in the activity of the AR□440 and AR□488 constructs was not due to the differences in the amount of the protein. This result confirmed that BAG-1L enhances the transactivation function of the AR mainly through the N-terminus of the AR.

3.2.2 □5 transactivation domain of the androgen receptor is required for the BAG-1L –mediated enhancement of transactivation by the receptor

The fragment between positions 280 and 488 partially overlaps the □5 domain (amino acids 360-528) of the AF1 transcription activation function of the androgen receptor, harbouring the hormone independent activity of the AR (Jenster *et al*., 1995; Fig. 3.4). The finding that BAG-1L enhances the activity of both the hormone inducible and constitutively active AR mutants (**Fig. 3.2**), lead to the question whether BAG-1L acts through the □5 domain of the receptor.

To address this question, PC3 cells were transiently transfected with GAL4 firefly luciferase reporter plasmid together with renilla luciferase construct as an internal control for the transfection efficiency and GAL4 DNA-binding domain (GAL4-DBD) alone or fused to the AR □5 domain expressing construct in the presence of the control vector or BAG-1L expressing plasmid. Thirty-six hours after transfection the cells were harvested and luciferase activity measurements were carried out.

GAL4-DBD had no transactivation function but GAL4DBD-□5 showed significant transactivation of the UAS luciferase reporter gene (**Fig.3.4A**). BAG-1L strongly enhanced the activity of AR □5 domain fused to the GAL4-DBD (compare the grey and the black bars), but had no effect on the GAL4-DBD activity alone, indicating that □5 domain of the receptor is important for the BAG-1L activity.

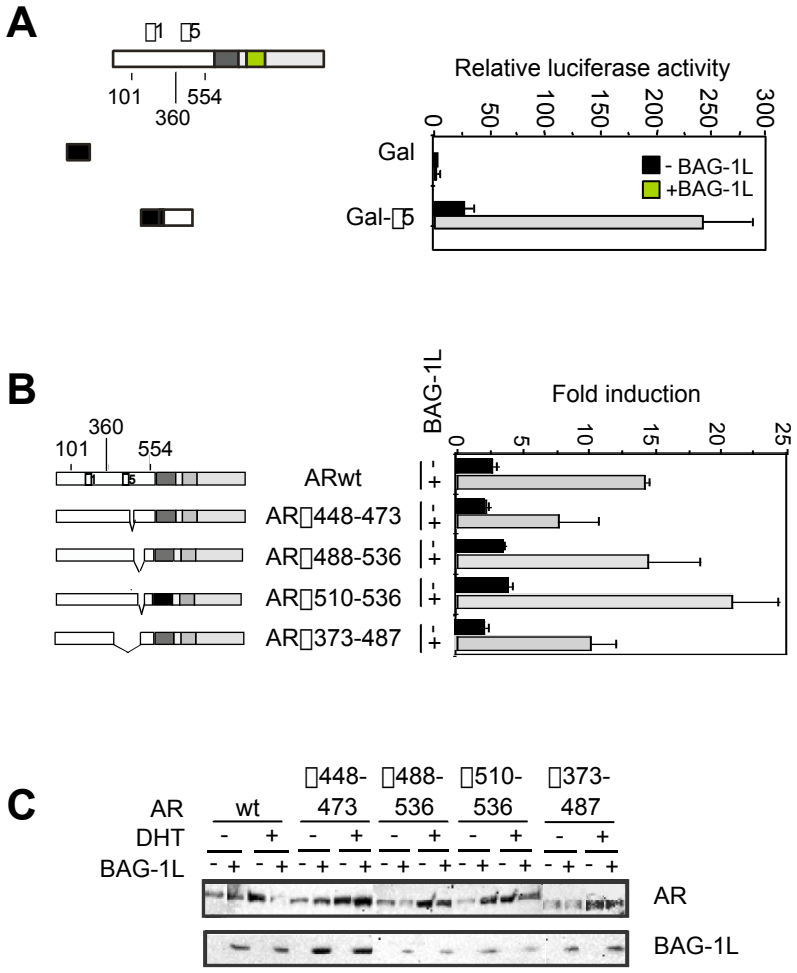

FIGURE 3.4 Effect of BAG-1L on the activity of AR □5 transactivation domain and AR N-terminus internal deletion mutants

A. PC3 cells were transiently cotransfected with a pG5□E4-38Luc firefly luciferase reporter plasmid, renilla luciferase construct as an internal control and pM or pM-AR□5 expression vector in presence of the control vector (black bars) or BAG-1L expressing plasmid (grey bars). Thirty-six hours after transfection the cells were harvested for luciferase activity measurements. The bar chat represents relative luciferase values (Firefly/Renilla). The scheme diagram presents the full-length AR with depicted □1 and □5 domains and the GAL4DBD and GAL4DBD-□5 constructs. **B.** PC3 cells were transiently cotransfected with

MMTV firefly luciferase indicator gene and a renilla luciferase construct as an internal control together with presented on the schematic diagram ARwt ot AR □5 deletion mutant expression vector in presence of the control vector (black bars) or BAG-1L expressing plasmid (grey bars). Treatment with 10^{-7}M DHT or control treatment with 80% ethanol was performed in twenty-four hours after transfection and the cells were harvested twenty hours later for luciferase activity measurements. The bar chat represents the fold induction of the receptor activity in presence of DHT (ratio of the relative luciferase activity in presence of DHT to that in absence of DHT). **C**. For the control of the protein level the lysates were subjected to polyacrylamide gel electrophoresis (PAGE) and analysed by Western blot. The input was corrected for the transfection efficiency by renilla luciferase measurements and controlled by Coomassie staining

Deletion of the sequences at the C-terminus of the AR□5 fragment (aa 488-536 and 510-536) in the context of the full-length AR showed no influence on the BAG-1L-mediated effect on AR-mediated transactivation of the MMTV promoter (**Fig.3.4B**). However, deletion of amino acids 448-473 or 373-487 reduced the effect of BAG-1L on the transactivation of the mutant (**Fig.3.4B**). Western blot analysis of the levels of the transfected proteins showed that this decline in the BAG-1L activity was not due to the differences in the protein amount (**Fig.3.4C**). Thus, although AR□448-473 and AR□373-487 have not completely lost the ability to be regulated by BAG-1L, the experimental result indicated that sequences in the □5 domain of the AR contribute to BAG-1L-mediated modulation of the transactivation function of the AR. The partial effect of BAG-1L on transactivation by the mutant AR may be due to the fact that only a part of the □5 domain was deleted.

Taken together, the analysis of the effect of BAG-1L on the activity of different AR deletion mutant demonstrated that BAG-1L acts predominantly through the N-terminus of the androgen receptor, and the □5 domain is most likely the region of the AR required for the regulation by BAG-1L.

3.3 Determination of the domains of BAG-1L involved in the enhancement of the transactivation function of the androgen receptor

To determine which domains of BAG-1L are essential for the regulation of the transactivation function of the AR, we examined the effect of BAG-1L and its N- and C-terminal deletion mutants on the transactivation function of full-length AR or AR□5. The BAG-1L deletion mutant analized are BAG-1L□C where the C-terminal Hsp70 binding site has been deleted and BAG-1M, another member of BAG-1 family, lacking the first

70 N-terminal amino acids of BAG-1L (**Fig.3.5A**). PC3 cells were transiently transfected with the expression vectors containing the full length AR or □5 together with the reporter luciferase gene driven by MMTV or GAL4 promoter, respectively, in the absence or presence of BAG-1L full length or its deletion mutants.

The result demonstrated that deletion of carboxy-terminal Hsp70 binding domain of BAG-1L (BAG-1L□C) resulted in complete abrogation of the enhancement of the AR activity by this protein (**Fig. 3.5B**). Truncation of BAG-1L from the N-terminus (BAG-1M) led to the significant decrease of the effect on the AR transactivation function (2.4 against ~11 fold for the BAG-1M and BAG-1L, respectively) (**Fig. 3.5B**). The reduction in the ability of the BAG-1L deletion mutants to enhance the transactivation by the AR was not due to differences in the protein levels, which was verified by the Western blot analysis (**Fig. 3.5C**). Analysis of the effect of BAG-1L mutants on the transactivation function of the □5 domain of the AR showed the pattern similar to that for the AR full length (**Fig. 3.5D**). Thus the functional analysis revealed that both the N- and C-termini of BAG-1L are important for enhancing the transactivation function of the AR.

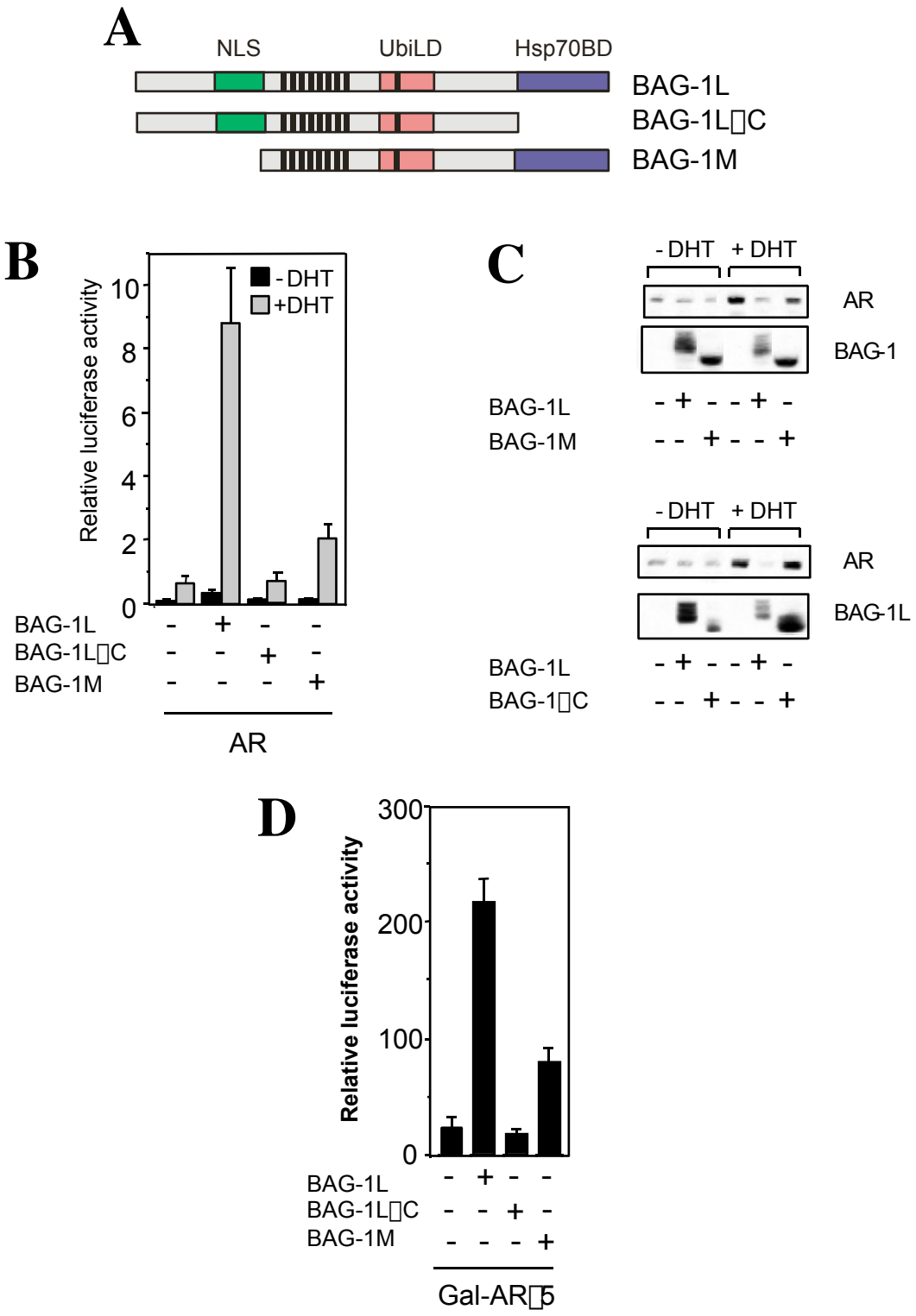

FIGURE 3.5 Effect of BAG-1L and its deletion mutants on the activity of AR

A. Schematic diagram of BAG-1L and its deletion mutants. Depicted are Hsp70-binding domain (Hsp70BD), ubiquitin-like domain (UbiLD), nuclear localization signal (NLS) and the $[EEX_4]_n$ motif. **B.** PC3 cells were transiently cotransfected with MMTV firefly luciferase indicator gene and a renilla luciferase construct as an internal control together with ARwt expression vector in presence of the control

vector or either BAG-1L or BAG1L deletion mutant (BAG-1LΔC and BAG-1M) expressing plasmid. Treatment with 10^{-7}M DHT (grey bars) or control treatment with ethanol (black bars) was performed in twenty-four hours after transfection and the cells were harvested twenty hours later for luciferase activity measurements. **C.** For the control of the protein level the lysates were subjected to polyacrylamide gel electrophoresis (PAGE) and analysed by Western blot. The input was corrected for the transfection efficiency by renilla luciferase measurements and controlled by Coomassie staining
D. PC3 cells were transiently cotransfected with a pG5ΔE4-38Luc firefly luciferase reporter plasmid, renilla luciferase construct as an internal control and pM or pM-ARΔ5 expression vector in presence of the control vector (black bars) or BAG-1L expressing plasmid (grey bars). Thirty-six hours after transfection the cells were harvested for luciferase activity measurements. The bar chats represent the average and standard deviation of 3 independent experiments showing the relative luciferase values (Firefly/Renilla).

3.4 Co-localisation of BAG-1L and BAG-1M with androgen receptor

BAG-1L contains a nuclear localization signal (NLS) at the N-terminus (**Fig.3.5A**). In BAG-1M, which lacks the first 70 amino acids of BAG-1L, this NLS is missing. It is therefore possible that that the reduced ability of BAG-1M to enhance the transactivation function of the AR is due to the inability of BAG-1M to get into the nucleus.

To determine the possibility that the impaired nuclear localization of BAG-1M is responsible for its reduced action in regulation of transactivation by the AR, PC3 cells were co-transfected with BAG-1L or BAG-1M expression vectors together with the expression construct for the AR fused to the green fluorescent protein (GFP-AR). Thirty-six hours after transfection, the cells were treated either with vehicle alone (ethanol) or with vehicle containing 10^{-7} M DHT for 3 hours and immunofluorescece analysis of the co-localisation of the BAG-1L and its deletion mutant BAG-1M with the AR was carried out.

BAG-1L resided in the nuclear compartment in both the absence (**Fig.3.6**, panel A2) and presence (**Fig.3.6**, panel B2) of DHT, showing mostly perinuclear localization. In contrast, in the absence of DHT, most of the AR resided in the cytoplasm (**Fig.3.6,** panels A1 and C1) with only a slight co-localization with BAG-1L (see yellow staining in the nucleus of the cell, **Fig.3.6,** panel A3). However in the presence of the hormone, BAG-1L demonstrated a clear co-localization with AR, translocated into the cell nucleus upon DHT treatment (see yellow staining in the nuclear compartment of the cells, **Fig. 3.6,** panel B3).

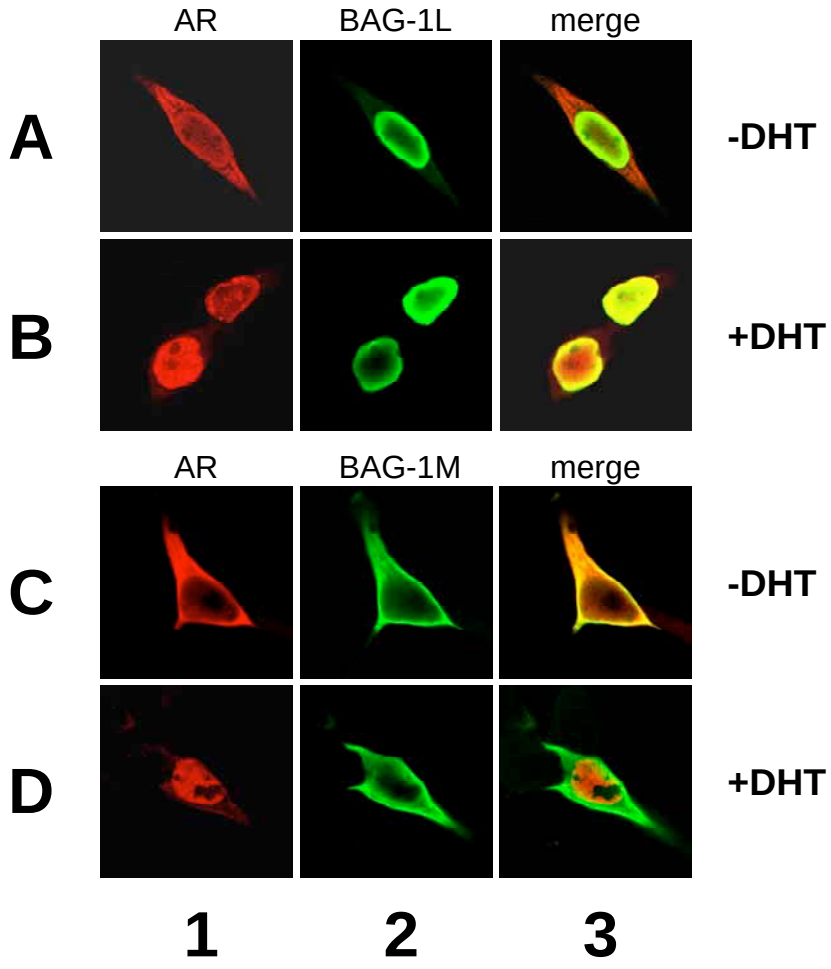

FIGURE 3.6 Confocal immunofluorescence analysis of intracellular localization of AR and BAG-1 proteins

PC3 cells were transiently transfected with expression vectors encoding GFP tagged ARwt together with a plasmid expressing HA-tagged either BAG-1L or BAG-1M. Thirty-six hours after transfection the cells were treated for 3 h with vehicle (0,1% ethanol) alone (-DHT) or vehicle, containing 10^{-7}M DHT (+DHT), before harvesting, processing and visualization with an LSM 510 invert Zeiss confocal microscope. The channel colors were inverted, therefore red fluorescence arises from the GFP-tagged AR, whereas the green fluorescence comes from staining of BAG-1L or BAG-1M with anti-HA mAb 12CA5, followed by an anti-mouse antibody labled with Cy3. The yellow colour indicates areas of co-localization of the two proteins.

However, BAG-1M, lacking the first 71 amino acids, showed cytoplasmic localization, both in absence (**Fig.3.6,** panel C2) and presence (**Fig.3.6,** panel D2) of DHT, which was well consistent with the previously reported observation that BAG-1L is a predominantly nuclear protein, while BAG-1M is cytoplasmic (Brimmell *et al.*, 1999). BAG-1M co-localized with the AR in the cytoplasmic compartment of the cell in

absence of hormone (see yellow staining, **Fig.3.6,** panel C3), but demonstrated very slight association with the receptor in the nucleus after DHT treatment (see yellow colour, **Fig.3.6,** panel D3), suggesting that reduction of the modulating activity of BAG-1M compared to BAG-1L is due to the predominantly cytoplasmic localization of the protein.

3.5 Function of the N-terminus of BAG-1L is not restricted to the nuclear translocation of the BAG-1L protein

To check whether it is solely the subcellular localisation, that prevents BAG-1M from interfering with the transactivation function of the AR, an expression vector, encoding BAG-1M with the SV40 nuclear localisation signal (SV40 NLS) fused to its N-terminus (NLS-BAG-1M) (**Fig.3.7A**) was generated. The effect of this fusion protein on the AR transactivation function was studied and compared with that of BAG-1L and BAG-1M.

For this purpose, PC3 cells were transiently co-transfected with a MMTV firefly luciferase indicator gene and a renilla luciferase construct as an internal control for the transfection efficiency together with AR expression vector in the presence of the empty vector or the vector containing BAG-1L, BAG-1M or NLS-BAG-1M encoding sequence. Twenty-four hours after transfection the cells were treated either with ethanol as a vehicle or with DHT and 20 hours later harvested for the luciferase measurements. The result of this experiment is presented on **Fig.3.7B**. It shows, that in the presence of DHT (grey bars) BAG-1L and BAG-1M lead to the about 11 and 2.5 fold increase in transactivation compared with the activity of the AR alone. Fusion of the SV40 NLS to BAG-1M (NLS-BAG-1M) results in the enhancement of the effect of the protein on the transactivation function of the AR up to about 9 fold, demonstrating that NLS-BAG-1M is a considerably more potent modulator of the AR-mediated transcription. Nevertheless it is not able to enhance the transactivation function of the AR to the same extent as BAG-1L. This could not be explained by the differences in the levels of the transfected proteins, as the Western blot analysis showed almost equal amounts of transfected BAG-1 proteins. Furthermore there are no increases in the levels of AR in the presence of

BAG-1L compared to BAG-1M and NLS-BAG-1M that could explain the different modulating effect of these proteins (**Fig.3.7C**).

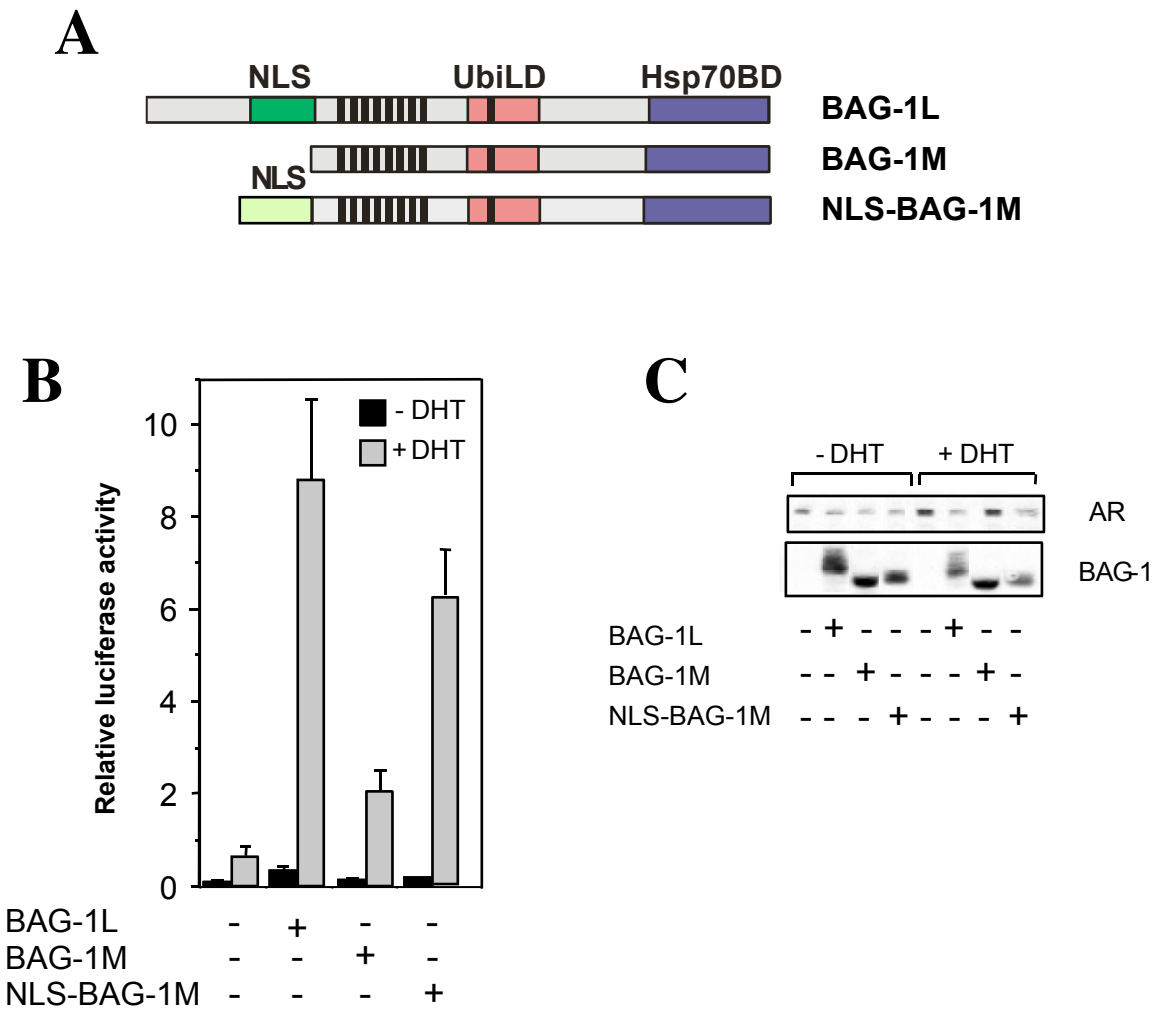

FIGURE 3.7 Effect of BAG-1L, BAG-1M and NLS-BAG-1M on the activity of AR

A. Schematic diagram of BAG-1L, BAG-1M and NLS-BAG-1M. Depicted are Hsp70-binding domain (Hsp70BD), ubiquitin-like domain (UbiLD), the $[EEX_4]_n$ motif and nuclear localization signals (NLS) in BAG-1L (green box) and NLS-BAG-1M (light green box). **B.** PC3 cells were transiently cotransfected with MMTV firefly luciferase indicator gene and a renilla luciferase construct as an internal control together with ARwt expression vector in the presence of the control vector, BAG-1L, BAG-1M or NLS-BAG-1M expressing plasmids. Treatment with 10^{-7}M DHT (grey bars) or control treatment with ethanol (black bars) was performed in 24 h after transfection and the cells were harvested 20 h later for luciferase activity measurements. The bar charts represent relative luciferase values (Firefly/Renilla). **C**. For the control of the protein level the lysates were subjected to polyacrylamide gel electrophoresis (PAGE) and analysed by Western blot..

To rule out the possibility that the difference in the BAG-1L and NLS-BAG-1M activity is due to the variation in the NLS function, the efficiency of the nuclear transport of NLS-BAG-M fusion protein was compared with that of BAG-1L using a fluorescence microscopy technique. PC3 cells were transfected with HA-tagged BAG-1L, NLS-BAG-1M or BAG-1M expressing vector. Thirty-six hours after transfection the cells were treated for 3 hours with ethanol as vehicle or DHT and harvested, processed and visualized with a laser confocal microscope. The cell were stained with a primary HA antibody followed by a secondary antibody labelled with a fluorescent dye. Different patterns of staining were observed which are presented as S1-S4 (S1 corresponds to the nuclear staining, S4 cytoplasmic and S2 and S3 to different gradations of nuclear and cytoplasmic staining). Over three thousand cells were analysed for each of the proteins and scored into the different groups. The result of statistical analysis of cellular distribution of the transfected BAG-1L, BAG-1M and NLS-BAG-1M proteins is presented on **Fig.3.8**.

Antibodies that recognize the HA-tag on transfected BAG-1 proteins clearly showed that about 60% of the cells, transfected with BAG-1M (blue bars), were both cytoplasmic and nuclear and about 25% - almost purely cytoplasmic, while BAG-1L (yellow bars) and NLS-RAP46 (pink bars) resided predominantly in the nucleus in 90% of the cells, revealing no significant difference in the cellular distribution of BAG-1L and NLS-BAG-1M. Thus the NLS-BAG-1M efficiently translocates into the nucleus and ther is no difference in its nuclear localization compared to BAG-1L.

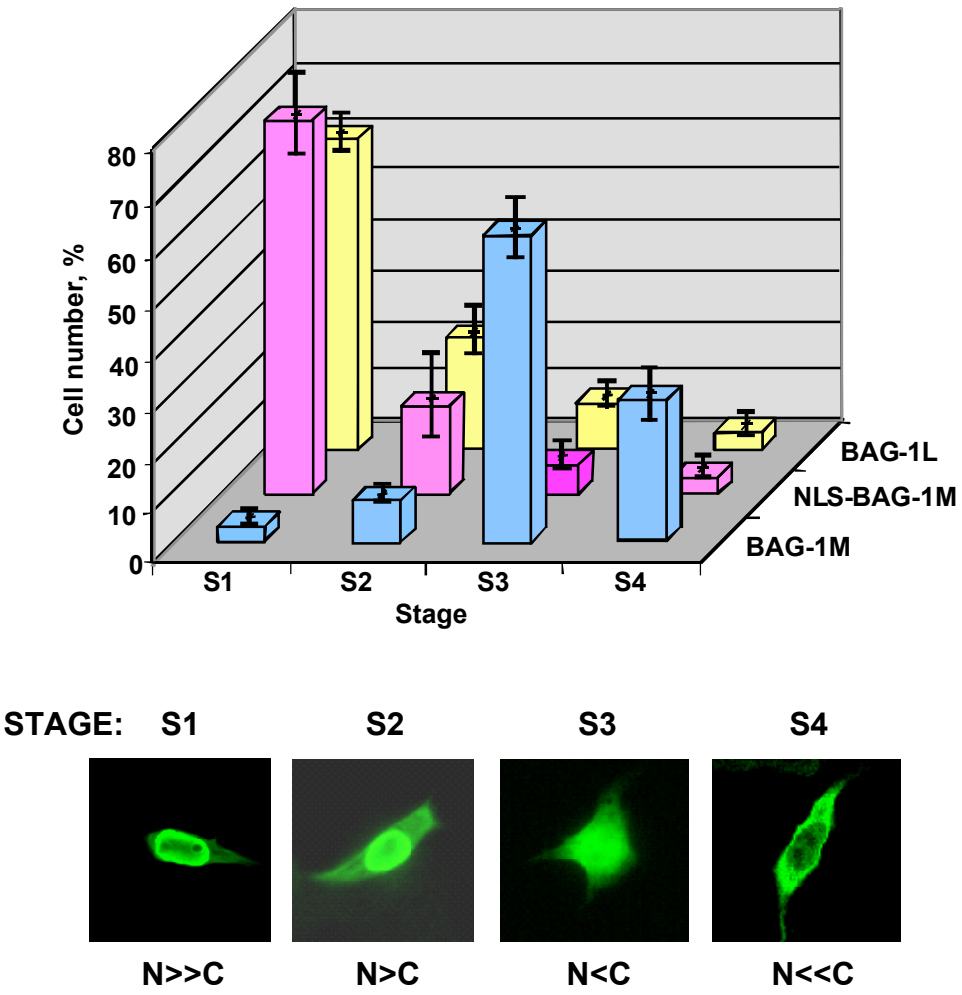

FIGURE 3.8 Subcellular localisation of BAG-1L, BAG-1M and NLS-BAG-1M

PC3 cells were transiently transfected with HA-tagged BAG-1L, BAG-1M or NLS-BAG-1M expression vector. Thirty-six hours after transfection the cells were treated with either ethanol or DHT for 3 hours, then harvested, stained with anti-HA mAb 12CA5, followed by an anti-mouse antibody labelled with Cy2, and visualised with an LSM 510 invert Zeiss confocal microscope. Subcellular protein localisation was empirically divided in 4 stages and number of the cells, representing every stage was counted for each protein. The diagram represents the mean value (-/+SD) of 3 independent experiments.

This result was additionally confirmed by the study of the co-localization of NLS-BAG-1M and AR. PC3 cells were co-transfected with NLS-BAG-1M expression vector, as it was done for BAG-1L and BAG-1M, together with the construct for the GFP-AR expression. Thirty-six hours after transfection the cells were treated either with vehicle (ethanol) or with 10^{-7} M DHT for 3 hours and immunofluorescece analysis of the co-localisation of NLS-BAG-1M with the AR was carried out.

In the absence of hormone, NLS-BAG-1M was predominantly nuclear, showing perinuclear localization pattern, while the AR was mainly cytoplasmic (**Fig.3.9**, panels A2 and A1).

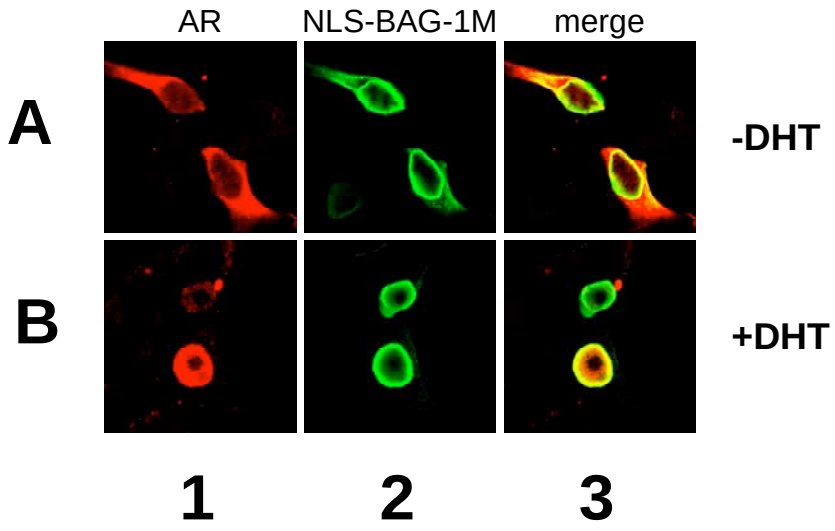

FIGURE 3.9 Confocal immunofluorescence analysis of intracellular localization of AR and NLS-BAG-1M

PC3 cells were transiently transfected with expression vectors encoding GFP tagged ARwt together with a plasmid expressing HA-tagged NLS-BAG-1M. Thirty-six hours after transfection the cells were treated for 3 h with vehicle (0,1% ethanol) alone (-DHT) or vehicle, containing 10^{-7}M DHT (+DHT) before harvesting, processing and visualization with an LSM 510 invert Zeiss confocal microscope. The channel colors were inverted, therefore red fluorescence arises from the GFP-tagged AR, whereas the green fluorescence comes from staining of NLS-BAG-1M with anti-HA mAb 12CA5, followed by an anti-mouse antibody labelled with Cy3. The yellow colour indicates areas of co-localization of the two proteins.

In the presence of DHT, NLS-BAG-1M was completely nuclear and showed strong co-staining with the AR in the nucleus of the transfected cell (**Fig.3.9**, panel B3).

NLS-BAG-1M that is as efficiently transported into the nucleus as BAG-1L still shows a weaker effect on the transactivation than BAG-1L. Thus the fact that BAG-1M does not enhance transactivation by the AR as effectively as BAG-1L may reside in an inherent property of this protein and not only in its inability to get to the nucleus.

3.6 BAG-1L may require Hsp70 for enhancement of the transactivation by androgen receptor

The BAG-1 proteins are known to interact with Hsp/Hsc70 molecular chaperones via their C-terminal BAG domain (Takayama *et al*., 1997; Zeiner *et al*., 1997; Nollen *et al*., 2000; Sondermann *et al*., 2001). Observation, that deletion of the C-terminus of BAG-1L, containing the Hsp70 binding site, abrogates the ability of BAG-1L to regulate the AR function is suggestive of the involvement of Hsp70 in the BAG-1L-mediated enhancement of the transactivation function of the AR. To address the question whether Hsp70 is implicated in the regulation of transactivation by the AR, the ability of Hsp70 to interfere with the BAG-1L-mediated enhancement of the AR transactivation function was tested.

3.6.1 Hsp70 co-localize with BAG-1L and androgen receptor in a transfected cell

Hsp70 is a molecular chaperone thought to reside in the cytoplasmic compartement of the cell (Pratt and Toft, 1997). However, recently Hsp70 has been demonstrated to be present in the nuclear compartment of the cell and to play a role in the regulation of gene expression by the steroid receptors (Schneikert *et al*, 1999 and 2000).

To address the question whether Hsp70 can associate with AR and BAG-1L in the cell, immuno-fluorescence analysis of co-localization of the three proteins was carried out. PC3 cells were co-transfected with LDH-tagged Hsp70 expression construct together with either GFP tagged AR or HA tagged BAG-1L expression vector. Thirty-six hours after transfection, the cells were treated either with vehicle alone (0.1% ethanol) or with

vehicle containing 10^{-7} M DHT for 3 hours and the localization of the transfected proteins was analysed by immunofluorescence staining (**Fig.3.10**).

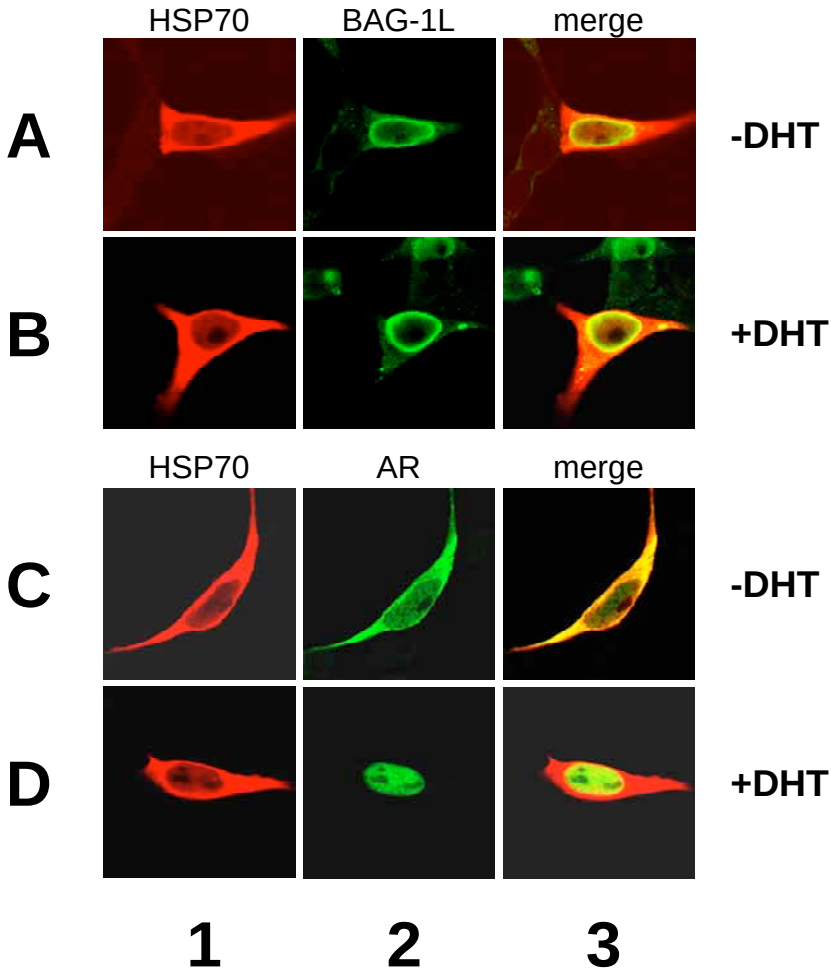

FIGURE 3.10 Confocal immunofluorescence analysis of the cellular localization of Hsp70, AR and BAG-1L

PC3 cells were transiently transfected with expression vectors encoding LDH-tagged Hsp70 together with a plasmid expressing HA-tagged BAG-1L (A, B) or GFP tagged ARwt (C, D). Thirty-six hours after transfection the cells were treated for 3 h with vehicle (0,1% ethanol) alone (-DHT) or vehicle, containing 10^{-7}M DHT (+DHT) before harvesting, processing and visualization with an LSM 510 invert Zeiss confocal microscope. Red fluorescence arises from staining of Hsp70 with anti-LDH Ab, followed by anti-rabbit antibody labled with TRITC, whereas the green fluorescence comes from staining of NLS-BAG-1M with anti-HA mAb 12CA5, followed by an anti-mouse antibody labled with Cy2 (A, B) or GFP-AR (C, D). The yellow colour indicates areas of co-localization of the two proteins.

Analysis of protein localization revealed that, though Hsp70 showed predominantly cytoplasmic localization, small amount of this protein resided also in the nucleus of the transfected cells (panels A1, B1, C1, and D1). Hsp70 co-localized with perinuclear residing BAG-1L in both the absence and presence of DHT (see yellow colour in the nucleus of the cell, panel A3 and B3). In immunofluorescence experiment following transfection of the AR and Hsp70, a strong cytoplasmic co-localization of both proteins was observed in the absence of hormone (panel C, see yellow staining in C3). After DHT treatment, the AR was translocated into the nucleus (panel D2) and co-localized with the Hsp70 resident in the nucleus (see yellow staining, D3).

The results of these experiments prove that Hsp70 can localize in the nucleus together with BAG-1L and AR. However, it is not clear how Hsp70 gets into the nucleus. AR can enter the nucleus upon DHT treatment, but it does not apparently bring Hsp70 into the nuclear compartment. BAG-1L contains a NLS and is a nuclear protein. It interacts with Hsp70 through its C-terminal BAG domain (Briknarova *et al*., 2001). Therefore a probability exists that BAG-1L cotransports Hsp70 into the nucleus via the interaction of Hsp70 with the C-terminus of BAG-1L.

To determine whether this is the case, PC3 cells were co-transfected with LDH-tagged Hsp70 expression construct together with HA-tagged BAG-1LΔC expression vector. In BAG-1LΔC the domain responsible for the interaction with Hsp70 has been deleted. Thirty-six hours after transfection the cells were treated either with ethanol or with 10^{-7} M DHT for another 3 hours and then localization of the transfected proteins was analysed by the immuno-fluorescence staining (**Fig.3.11**).

The result of the staining showed the same pattern of protein localization for Hsp70 together with BAG-1LΔC as with BAG-1L full length. The majority of Hsp70 resided in the cytoplasm (panels A1 and B1) but a minor part of the protein did co-localize with BAG-1LΔC in the perinuclear nuclear compartment of the cell (see yellow colour, panels A3 and B3) in both the absence and presence of DHT, indicating that an interaction of Hsp70 and BAG-1L is not needed for the nuclear translocation of Hsp70. Thus Hsp70 most probably enter the nucleus on its own.

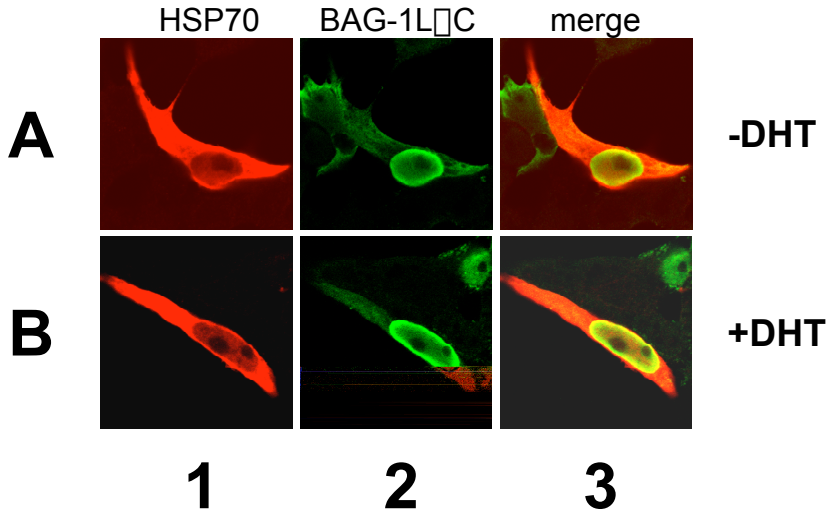

FIGURE 3.11 Confocal immunofluorescence analysis of intracellular localization of Hsp70 and BAG-1LΔC

PC3 cells were transiently transfected with expression vectors encoding LDH-tagged Hsp70 together with a plasmid expressing HA-tagged BAG-1LΔC. Thirty-six hours after transfection the cells were treated for 3 h with vehicle (0.1% ethanol) alone (-DHT) or vehicle, containing 10^{-7}M DHT (+DHT) before harvesting, processing and visualization with an LSM 510 invert Zeiss confocal microscope. Red fluorescence arises from staining of Hsp70 with anti-LDH Ab, followed by anti-rabbit antibody labelled with TRITC, whereas the green fluorescence comes from staining of NLS-BAG-1M with anti-HA mAb 12CA5, followed by an anti-mouse antibody labelled with Cy2. The yellow colour indicates areas of co-localization of the two proteins.

3.6.2 Effect of BAG-1L on the androgen receptor transcription activation function is increased by Hsp70 overexpression

As the immunofluorescence studies demonstrated colocalization of Hsp70 with BAG-1L and AR in the same cellular compartment, it was investigated whether alteration in the level of Hsp70 may alter the level of regulation of AR response by BAG-1L. To determine this, PC3 cells were transfected with the MMTV firefly luciferase indicator gene and a renilla luciferase construct as an internal control for the transfection efficiency together with the AR expression vector in the presence of empty vector or vector containing the BAG-1L cDNA. Different amounts of expression vectors encoding full-length Hsp70, or the ATPase or substrate binding domain (SBD) of Hsp70 (**Fig.3.12A**)

were transfected together with BAG-1L. Twenty-four hours after transfection, the cells were treated either with ethanol as vehicle or with DHT and 20 h later the cells were harvested for luciferase measurements. The amounts of AR, BAG-1L and Hsp70 expressed were determined by Western blot analysis.

Under the conditions of these experiments, in the presence of DHT (grey bars) BAG-1L enhanced AR-mediated transcription about 4 fold and this effect was further increased by overexpression of full-length Hsp70 (up to 5.2 fold) (**Fig.3.12B**). The effect of Hsp70 on BAG-1L-mediated regulation of transactivation by AR was even more pronounced, when instead of the full-length Hsp70, the ATPase domain was expressed (**Fig.3.12B,** ATPase). Ectopic expression of this domain of Hsp70 resulted in a concentration dependent increase of BAG-1L-mediated enhancement of the AR transactivation function up to more than 7 fold compared to 4 fold in the absence of cotransfected Hsp70 expressing plasmid. Interestingly, overexpression of the substrate-binding domain (SBD) of Hsp70 produced the opposite effect. A concentration-dependent reduction of the enhancing action of BAG-1L on the transactivation function of the AR in presence of the overexpressed Hsp70 SBD was observed (**Fig.3.12B,** SBD). Thus the SBD of Hsp70 exerted a dominant negative effect on the regulatory function of BAG-1L. Western blot assays (**Fig.3.12C**) showed that co-transfection of Hsp70 and its mutants did not significantly alter the levels of AR and BAG-1L. The effect of Hsp70 on the BAG-1L-mediated enhancement of the AR transactivation function therefore correlated with level of Hsp70 proteins expressed in the transfected cells.

The Hsp70 proteins exerted a different effect on BAG-1L-mediated enhancement of the transactivation function of the □5 domain of the AR (**Fig.3.12D**). While overexpression of the intact Hsp70 or its ATPase domain enhanced the effect of BAG-1L on the transactivation by □5 as was the case in the regulation of AR response, the SBD did not exerted a dominant negative effect on the BAG-1L-mediated enhancement on the AR□5 transactivation function as in case of the full-length AR (**Fig.3.12D**, SBD). This is a striking difference as the Western blot analysis showed that the expression pattern of the transfected Hsp70 and BAG-1L proteins was similar to that in case of the intact AR (**Fig.3.12E**). This difference in the effects of the Hsp70 SBD on the effect of BAG-1L on

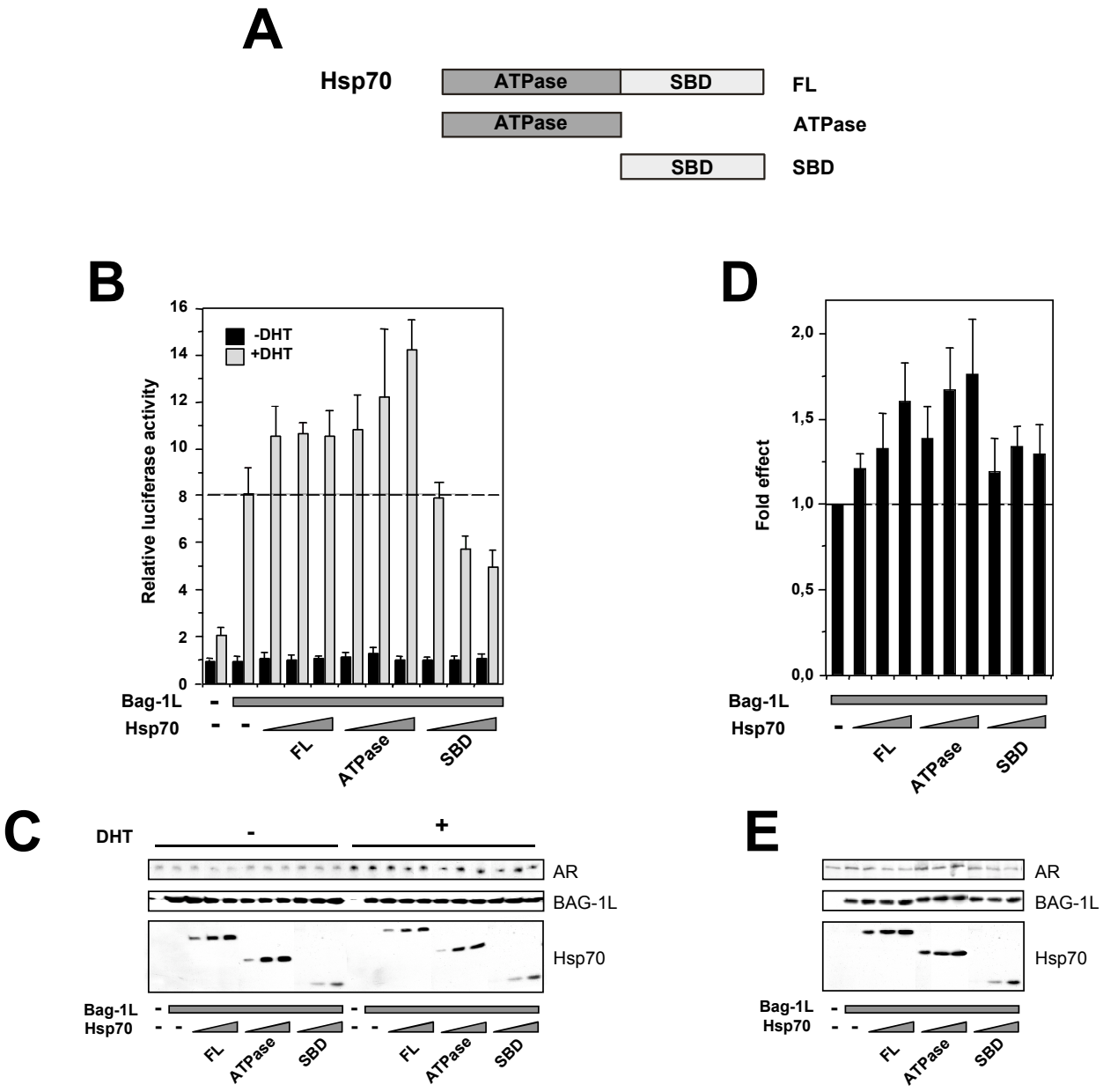

FIGURE 3.12 Effect of Hsp70 overexpression on the BAG-1L-mediated enhancement of transactivation functions of the AR and AR5

A. Schematic diagrams showing the intact Hsp70, ATPase and substrate binding (SBD) domains of the Hsp70 used in the transfection experiments. **B.** PC3 cells were transiently transfected with pGL3-MMTV firefly luciferase indicator gene, renilla luciferase construct as an internal control and Arwt, and pcDNA3 vector or BAG-1L expression plasmid in absence or presence of different amounts (0.25, 0.5 or 1.0μg) of LDH-tagged Hsp70, Hsp70ATPase or Hsp70SBD expression construct. Twenty-four hours after transfection the cells were treated with 10^{-7}M (DHT) or vehicle and harvested 20 h later for luciferase activity measurements. The bar chat represents relative luciferase values (Firefly/Renilla). **C**. For the control of the protein levels the lysates were subjected to polyacrylamide gel electrophoresis (PAGE) and analysed by Western blot. The input was corrected for the transfection efficiency by renilla luciferase measurements. **D.** PC3 cells were transiently transfected with pG5E4-38Luc firefly luciferase reporter

plasmid, renilla luciferase construct and pM-AR□5 and of pcDNA3 vector or BAG-1L expression plasmid in absence or presence of different amounts of LDH-tagged Hsp70, Hsp70□ATPase or Hsp70□SBD expression construct. Thirty-six hours later the cells were harvested for luciferase activity measurements. The result is shown in terms of fold effect of Hsp70 proteins on the AR transactivation function in the presence of BAG-1L (BAG-1L-mediated enhancement of the AR activity is 6-8 fold and is set to 1). **E**. For the control of the protein levels the lysates were subjected to polyacrylamide gel electrophoresis (PAGE) and analysed by Western blot. The input was corrected for the transfection efficiency by renilla luciferase measurements. Diagrams (**B**, **D**) represent the mean value (-/+SD) of 3 independent experiments.

transactivation by the intact AR or AR□5 might be due to the fact that Hsp70 SBD binds to the HBD of steroid receptors (Pratt and Toft, 1997). As AR□5 lacks this domain, SBD of Hsp70 can not interfere with its activity.

Thus Hsp70 may be involved in the action of BAG-1L in modulating the transactivation function of the AR.

3.7 Binding of BAG-1L to androgen receptor

The finding that BAG-1M and BAG-1L□C do not enhance AR transactivation as efficiently as BAG-1L may suggest that the N- and C-terminal domains of BAG-1L are involved in the potentiation of AR response either directly or indirectly. A possibility is that these domains bind directly to the AR.

3.7.1 Both C- and N-terminus of BAG-1L play a role in binding to androgen receptor

To find out whether N- and carboxy-termini of BAG-1L play a role in interaction with the AR, binding of the AR to BAG-1L and its deletion mutants, BAG-1M and BAG-1L□C was analyzed. As control AR binding to BAG-1M□C was also analyzed in GST pull down experiments. BAG-1L and its mutants were expressed in bacteria as glutathion S-transferase (GST) fusion proteins, immobilised onto glutathione-agarose beads and incubated with radioactively labelled full-length AR either in the absence or presence of DHT. After washing off unbound material, bound proteins were eluted and subjected to SDS polyacrylamide gel electrophoresis and the receptor was visualised by autoradiography. As a negative control for the specificity of binding, GST immobilized on the agarose beads was used.

In the absence and presence of hormone the AR bound efficiently to both BAG-1L and its N-terminal deletion mutant (BAG-1M) (**Fig.3.13**). With deletion of the C-terminal domain of BAG-1M (BAG-1M□C) the binding of the AR was completely abrogated, while the AR still showed a weak interaction with BAG-1L, truncated at the C-terminus (BAG-1L□C). These data indicated that interaction of the AR to BAG-1L most likely occurred through the C-terminal region of BAG-1L, although its N-terminus may also play a role in binding to the receptor.

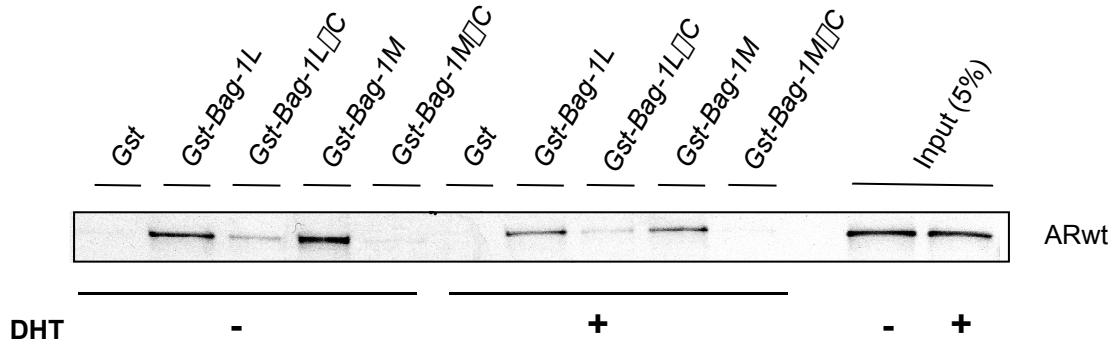

FIGURE 3.13 ***In vitro*** **interaction of BAG-1L and its deletion mutants with AR**

Bacterially expressed GST fusion proteins were bound to glutathione-agarose, incubated with in vitro-translated, radiolabeled ARwtand subjected to the GST pull-down assay, as described in Materials and Methods. Input and bound proteins were subjected to SDS-10% PAGE and visualised by autoradiography.

3.7.2 N- and C-terminus of BAG-1L bind to different domains of androgen receptor

To identify the regions of the AR involved in binding to BAG-1L, *in vitro* binding of different domains of the AR to BAG-1L and its mutants was analysed. BAG-1L and its mutants were expressed in bacteria as GST fusion proteins, immobilised on the glutathione-agarose beads and incubated with radioactively labelled *in vitro* translated AR, lacking the HBD and the hinge region (N-termAR), in the absence or presence of DHT, or with radioactively labelled □1 and □5 transactivation functions of the AR AF1 domain. The radioactively labelled AR bound to the BAG-1 proteins was subjected to the SDS PAGE and the receptor mutants were visualised by autoradiography.

The results of these analyses show that AR, lacking the HBD and the hinge region (N-termAR), can bind to BAG-1L and BAG-1M, but not to the BAG-1 mutants lacking the C-terminus (**Fig.3.14A**), indicating that the N-terminal domain of the AR binds to the C-terminal sequences of BAG-1L.

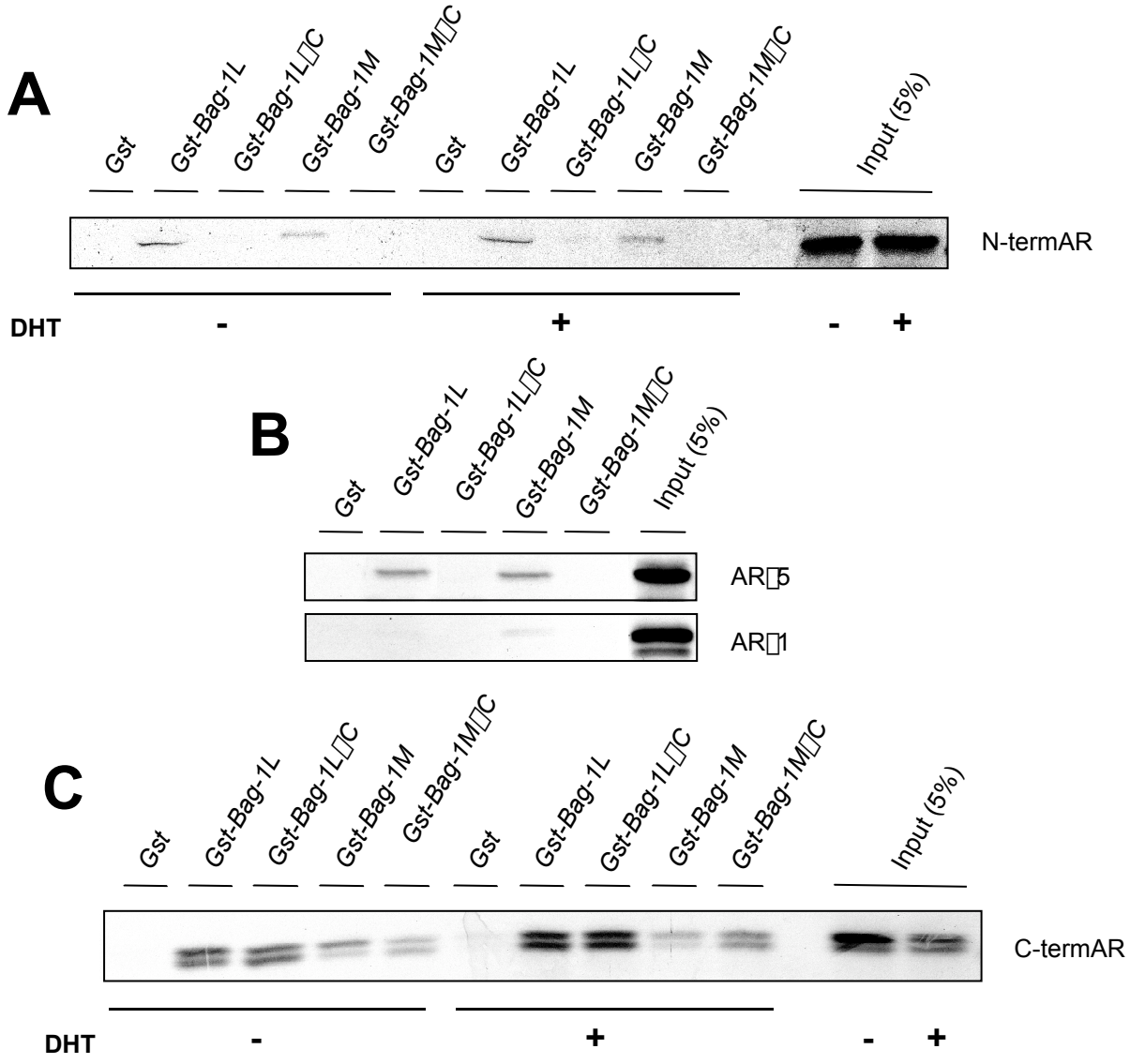

FIGURE 3.14 ***In vitro*** **interaction of BAG-1 proteins with different domains of the AR**

Bacterially expressed GST fusion proteins were bound to glutathione-agarose, incubated with in vitro-translated, radiolabeled N-termAR (A), ARΔ5 and ARΔ1(B) or C-termAR (C) and subjected to the GST pull-down assay. Input and bound proteins were analyzed by SDS-10% PAGE and autoradiography.

To specify which region of AR N-terminus is involved in this interaction with the C-terminal domain of BAG-1L, the binding of the BAG-1 proteins to the 5 and 1 domains of the AR was investigated. The results show that AR 5 binds BAG-1L and BAG-1M with the same efficiency as the intact AR N-terminus, while 1 revealed no significant interaction with either of BAG-1 proteins (**Fig.3.14B**). Deletion of the C-terminal domain of BAG-1L and BAG-1M abolished binding to the AR 5, indicating that the C-terminus of BAG-1 interacts with the 5 domain of the AR.

In contrast to the AR N-terminus, interaction of the C-terminus of the AR (C-termAR) with BAG-1L is not sensitive to the deletion of the C-terminal BAG domain, and the binding is strong for the intact BAG-1L and BAG-1LC (**Fig.3.14C**). Binding of the C-termAR to both BAG-1L proteins is increased in the presence of DHT. However, binding of this AR mutant to BAG-1M and BAG-1MC is low and is not affected by the hormone, indicating that N-terminus of BAG-1L is important for the binding of BAG-1L to the C-terminus of the AR and that this interaction is hormone dependent. The *in vitro* interaction pattern of the AR and its domains with BAG-1 proteins is summarised in **Table 3.1**:

Table 3.1: Interaction pattern of the intact AR and its domains with BAG-1 proteins

	BAG-1L	BAG-1LC	BAG-1M	BAG-1MC
ARwt	++	+	++	-
N-termAR	++	-	++	-
1	-/+	-	-/+	-
5	++	-	++	-
C-termAR	++[1]	++[1]	+	+

1: Interaction is increased in presence of DHT

To prove that N-terminal sequences of BAG-1L binds the C-terminus of the AR, a fusion protein consisting of the first 127 amino acids of BAG-1L, fused to GST (GST-BAG-1L1-127), was generated, and its *in vitro* binding to the N- and C-terminal deletion mutants of the AR was analysed. GST-BAG-1L was used as a positive and GST alone as a negative control in the pull down experiments. BAG-1L N-terminus is able to bind the C-terminus of (C-termAR) in both the absence and presence of DHT (**Fig.3.15A**). However, as in case of the BAG-1L□C (**Fig.3.14C**), in presence of hormone, the binding is increased, indicating that the N-terminal amino acids of BAG-1L are involved in hormone dependent binding to the C-terminus of the AR. The specificity of this binding is confirmed by the fact, that the N-terminal sequence of the AR did not bind to the GST-BAG-1L N-terminus fusion protein neither in the presence or absence of DHT, although it did bind to GST-BAG-1L which was used as a positive control (**Fig.3.15B**).

The C-terminus of BAG-1L interacts with the ATPase domain of Hsp70 and it is possible that Hsp70 is involved in interaction of the C-terminal domain of BAG-1L with the AR□5. To determine whether the N-terminus of BAG-1L also binds AR via interaction with Hsp70, binding of Hsp70 to the GST fused N-terminus of BAG-1L was studied. Binding of Hsp70 to the immobilised GST and GST fused BAG-1L was analysed as a negative or positive control, respectively. The result of this experiment showed that Hsp70 did not interact with the N-terminus of BAG-1L, though, as predicted, it effectively bound the intact BAG-1L protein (**Fig.3.15C**). This indicates, that the N-terminal sequences of BAG-1L bind directly to the C-terminus of the AR.

Taken together these results suggest that BAG-1L binds to the □5 domain of the AR with its C-terminal region and its N-terminal sequences bind directly in a hormone dependent manner to the carboxyl-terminus of the AR. As BAG-1M lacks a portion of the N-terminus of BAG-1L, this protein is less able to interact with the C-terminus of the AR.

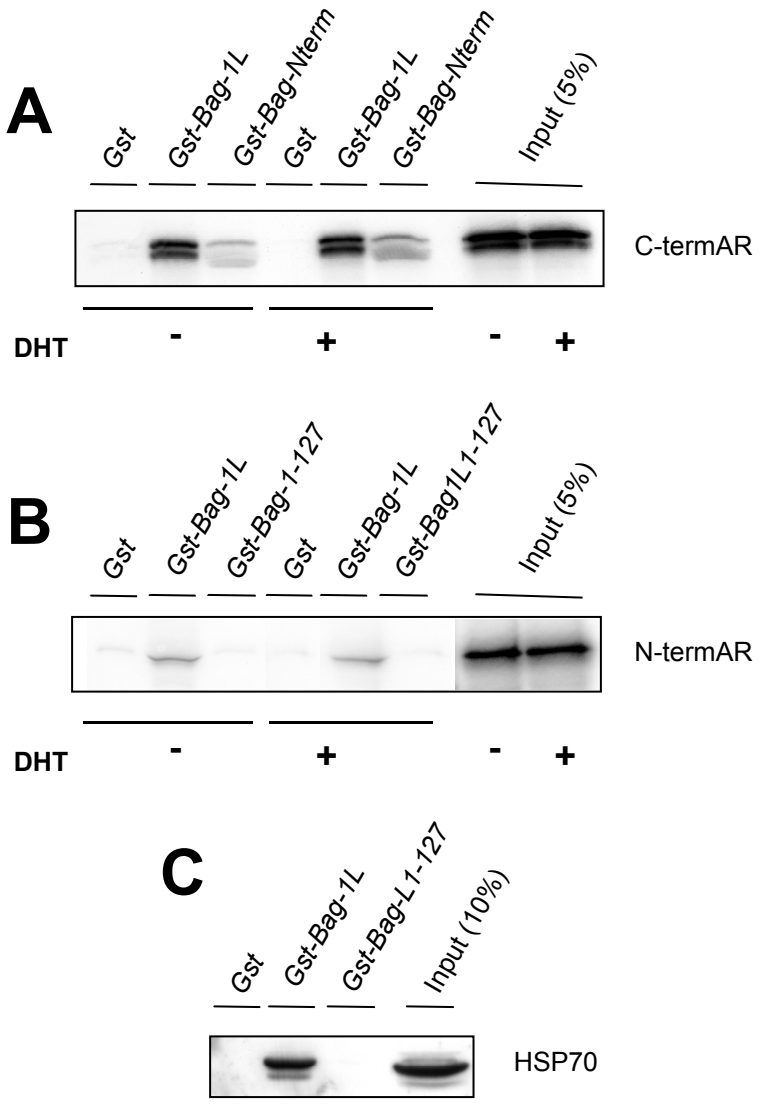

FIGURE 3.15 In vitro interaction of N-terminus of BAG-1 proteins with different domains of the AR and Hsp70

Bacterially expressed GST fusion proteins were bound to glutathione-agarose, incubated with in vitro-translated, radiolabeled C-termAR (**A**) or N-termAR (**B**), or with cell lisate containing endogenous Hsp70 protein (**C**) and subjected to the GST pull-down assay. Input and bound proteins were analyzed by SDS-10% PAGE and either autoradiography (**A, B**) or Western blot analysis with Hsp70-specific Ab (**C**).

3.8 BAG-1L, Hsp70 and androgen receptor bind together to the androgen response elements on the prostate specific antigen promoter

As BAG-1L, AR and Hsp70 can localize in the nucleus and these proteins interact with each other, it is possible that they form a multiprotein complex on the androgen response elements of target genes. To find out whether BAG-1L and Hsp70 play a role in the regulation of the transcription activation function of the AR under physiological conditions, the recruitment of the three proteins to the promoter of an androgen responsive gene, PSA (Schuur *et al*, 1996; Cleutjens *et al*, 1997; Zhang *et al*, 1997), was studied.

To choose the cell system for this experiment, two prostate cancer cell lines, LNCaP and 22Rv1, expressing endogenous AR, were analysed for the levels of the endogenous BAG-1L and Hsp70 by Western blot. An AR negative PC3 cell line was used as a control.

Both LNCaP and 22Rv1 cells expressed equal amounts of AR (**Fig.3.16**, lanes 1 and 2), while PC3 (negative control) showed no AR expression (**Fig.3.16**, lane 3). The levels of Hsp70 were also similar in LNCaP and 22Rv1 cell lines (**Fig.3.16**, lanes 1 and 2). However, the level of BAG-1L protein was much higher in 22Rv1 cells than in LNCaP (**Fig.3.16**, lanes 2 and 1). Thus, as 22Rv1 cell expressed high amounts of all three proteins, this cell line was chosen for further studies.

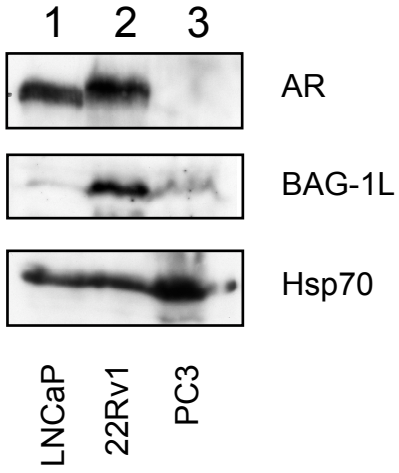

FIGURE 3.16 Different levels of expression of AR, BAG-1L and Hsp70 in 3 different prostate carcinoma cell lines

22Rv2, LNCaP and PC3 cell were grown in appropriate medium supplemented with 5% CCS for 72 hours before harvesting. Cell lysates were subjected to the SDS PAGE and levels of AR, BAG-1L and Hsp70 proteins were analyzed by Western blot.

Androgen responsive expression of the PSA gene in the 22Rv1 cell line was verified. Total RNA was extracted from these cells following treatment with ethanol as vehicle or with 10^{-7}M DHT for 5 hours and quantitative real-time PCR was performed in collaboration with Dr. A.Nestl. This experiment showed that treatment with DHT resulted in about 4.5 fold increase of the PSA expression (**Fig.3.17A**), indicating that expression of PSA in 22Rv1 cell line is induced by androgens.

To address the question whether DHT mediated activation of expression of the PSA gene corresponds with hormone dependents recruitment of AR, BAG-1L and Hsp70 to the PSA promoter, chromatin immunoprecipitation (ChIP) assay was carried out. 22Rv1 cells were cultured for 72 hours in phenol-free RPMI-1640 medium supplemented with 5% charcoal treated foetal bovine serum (CCS) to remove the residual steroids. Thereafter the cells were treated either with vehicle or 10^{-7} M DHT for 30 minutes followed by formaldehyde cross-linking of the protein-DNA complexes. The cells were then harvested

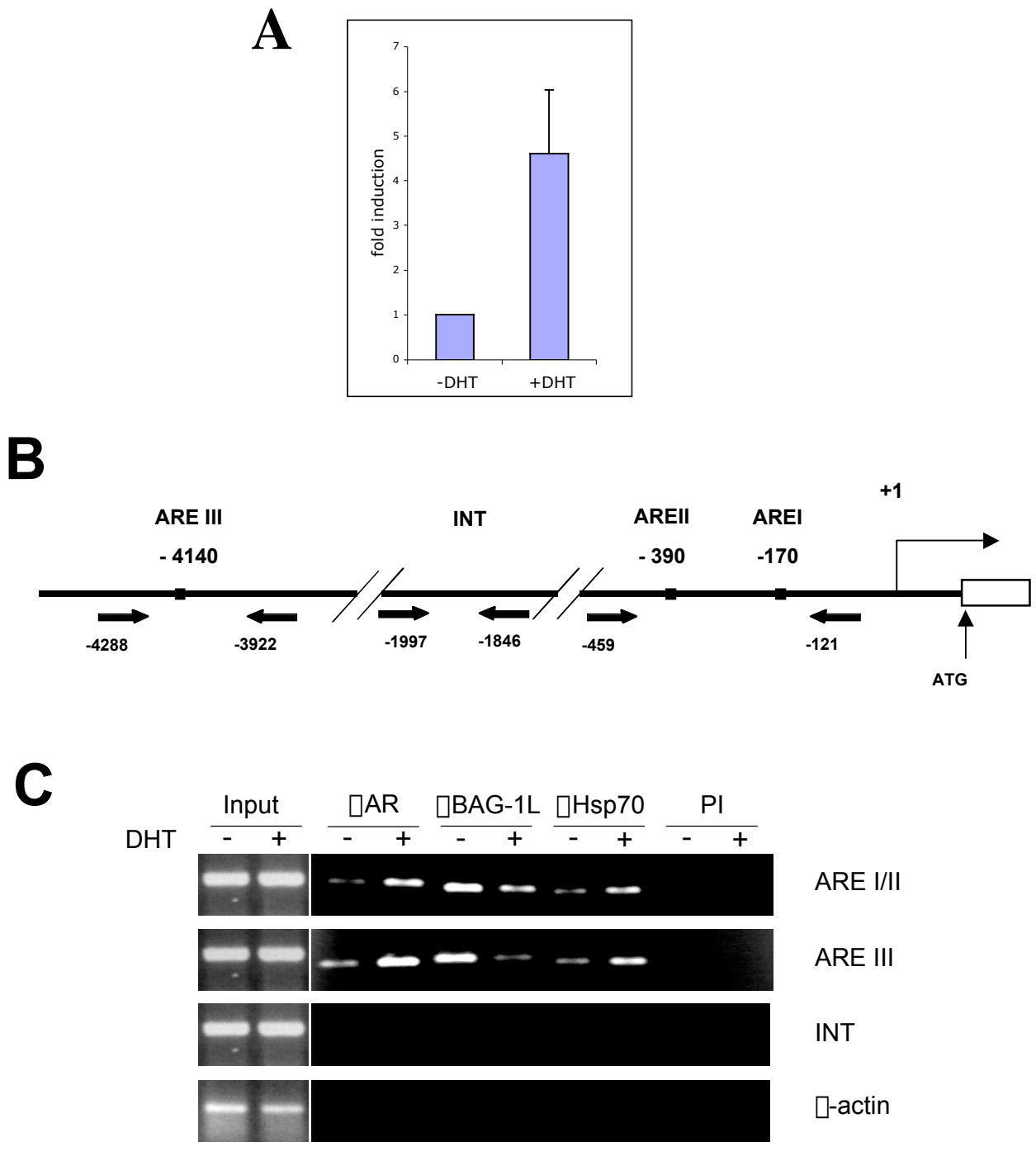

FIGURE 3.17 DHT mediated activation of the expression of the PSA gene corresponds with hormone dependent recruitment of AR and Hsp70 to PSA promoter

A. Total RNA was extracted from 22Rv1 cells treated with ethanol alone as vehicle or stimulated with 10^{-7} DHT for 5 hours. A real-time RT-PCR for human -actin and human PSA was performed. After PCR reaction, quantification of PSA mRNA against the normalisation control -actin was calculated based on

the CT value. The PCR was repeated three times and the average value for the induction and the standard deviation was calculated. **B.** A schematic representation of the PSA promoter showing the regions specifically amplified in the PCR after the ChIP assay. **C.** Soluble chromatin was prepared from 22Rv1 cells treated with DHT for 30 min and immunoprecipitated with antibody against AR (αAR), BAG-1L (αBAG-1L) or Hsp70 (αHsp70), along with preimmune serum (PI) as control. The final DNA extractions were amplified using pairs of primers that cover different PSA promoter fragments.

and sonicated to obtain soluble chromatin with an average DNA fragment length ~500-1000 bp. Aliquots of the sonicated extracts were immunoprecipitated with antibody against either AR, BAG-1L or Hsp70. The presence of the specific promoter regions in the chromatin immunoprecipitates was analysed by PCR using specific pairs of primers spanning the androgen responsive regions in the PSA promoter. PSA has three AR binding sites: two AREs in the proximal promoter region (ARE I and ARE II) and one about 4600 base upstream the transcription start (ARE III) (**Fig.3.17B**) in the enhancer region. The PCR primers were designed to cover all the three regions. One of the pairs covers the region, containing both proximal androgen responsive elements (ARE I/II), and the other – ARE in the enhancer. As a negative control, primers for the androgen insensitive promoter region of the same gene and for an unrelated promoter (β-actin) were used.

The results demonstrated that AR was present at all three AREs on the PSA promoter even in the absence of hormone (**Fig.3.17C**). Treatment of 22Rv1 cells with DHT increased the recruitment of the AR to the AREs containing regions of the PSA promoter. Recruitment of Hsp70 to the PSA promoter AREs revealed a similar pattern. In contrast, occupancy of the promoter AR binding sites by BAG-1L was strong in the absence of hormone and was significantly reduced upon the DHT treatment. No DNA was recovered by PCR when the ARE-negative PSA promoter region or the β-actin promoter were analysed or when the specific antibodies were replaced with pre-immune serum, attesting to the specificity of the assay conditions. Thus, the results demonstrate that DHT mediated activation of expression of the PSA gene corresponded with hormone dependent recruitment of AR and Hsp70 to the AREs of the PSA promoter, indicating their possible role in androgen-mediated gene expression. BAG-1L is also recruited to the AREs on the PSA promoter, suggesting that this protein also plays a role in regulation of

the AR-mediated transcription. The fact, that DHT treatment has a repressing effect on precipitation of the PSA promoter fragments by the antibody against BAG-1L, does not exclude the presence of BAG-1L at the AREs in the promoter of the PSA gene in the presence of hormone. The reduction in the DNA recovery could be caused by the decrease in affinity of the antibody to BAG-1L due to the conformational changes in the BAG-1L molecule upon the DHT treatment.

4. DISCUSSION

4.1 BAG-1L as a positive regulator of transcription activation by the androgen receptor

The AR is a ligand inducible transcription factor, activating the expression of target genes. However, it does not activate transcription alone, a number of proteins with transactivation function have been identified as associating with the receptor (reviewed McKenna and O'Malley, 2002). These factors form multiprotein complexes with the receptor to enhance its transactivation function. In addition co-chaperones whose main function is to transform the AR into a conformation competent for hormone binding have also been shown to act in the nucleus to enhance the transcriptional activation function of the receptor. Though several molecular chaperones and cochaperones have been reported to modulate the transactivation by the AR (Freeman *et al*., 2000; Fliss *et al*., 1997; Rao *et al*., 2001). The group of J.Reed was the first to report that the cochaperone BAG-1L enhances the transcription activation function of the AR (Froesch *et al*., 1998). However, the mechanism of this regulation is so far not clear.

BAG-1L belongs to a family of molecular cochaperones whose main function is to serve as a nucleotide exchange factor for the molecular chaperone Hsp70, allowing it to bind to various substrates to bring them to the correct conformation. However, in addition BAG-1 proteins also modulate the action of nuclear receptors. The most intensively studied is the negative regulation of the transactivation function of the GR by BAG-1M (Schneikert *et al*., 1999 and 2000). Negative regulation of the transactivation function of other nuclear receptors such as RAR/RXR heterodimer and Vit D3 receptor has also been reported (Liu *et al*., 1998; Witcher *et al*., 2001). It is therefore intriguing that BAG-1L does not negatively regulate the transactivation function of the AR but rather enhances the activity of this receptor.

In the work presented here a number of experiments has been performed to determine the mechanism by which BAG-1L enhances the transactivation by the AR.

Functional experiments with deletion mutants of the AR demonstrated that the N-terminus of the receptor is necessary for the BAG-1L-mediated enhancement of the transcription activation function of the AR. The N-terminus of the AR contains two independent transactivation functions, □1 and □5, at amino acids 101-370 and 360-528, respectively (Jenster *et al*., 1995). The work presented here showed that a region between

amino acids 280 and 488 of the AR is important for the BAG-1L-mediated regulation of the AR function. This region can be further narrowed down to the τ5 domain of the AR. The τ5 domain of the AR was reported to be the site of interaction with nuclear receptor coactivators SRC1, TIF2 and CBP (Bevan *et al*., 1999; Berrevoets *et al*., 1998; Fronsdal *et al*., 1998) and to play a role in binding to the components of the basal transcription machinery TFIIF and TBP (McEwan and Gustafsson, 1997). The fact that BAG-1L acts via ARτ5 might be suggestive for the role of BAG-1L in recruitment of AR co-activators to the androgen responsive promoter. Bound to the androgen receptor, BAG-1L might provide either additional interaction surface for the co-factors or, alternatively, stabilize the AR in the conformation favorable for the receptor-co-factor association.

4.2 The N- and C-terminal sequences of BAG-1L are necessary for its action

In the work presented here, BAG-1M, lacking the first 70 N-terminal amino acids of BAG-1L, was shown to enhance the transactivation by the AR to a much lower extent than the wild type BAG-1L. This difference was thought to be due to differences in cellular localization of the two proteins since BAG-1L is localized in the nucleus and BAG-1M is mainly cytoplasmic, as it lacks a nuclear localization signal. The forced nuclear targeting of BAG-1M by appendage of an exogenous NLS, however, did not improve the ability of BAG-1M to enhance the transactivation function of the AR to that of BAG-1L. This result does not agree with the previously published data, that targeting BAG-1M to the nucleus is sufficient for making BAG-1M as efficient a modulator of the AR transactivation function as BAG-1L (Knee *et al*., 2001). This contradiction might be possibly explained by the sensitivity of the cell system used. In the work of Knee *et al*. the Cos-7 cell line was used, where the effect of BAG-1L on the activity of the AR was about 2.5-3 fold, while the present work was carried out in PC3 cells where BAG-1L effect reached more than 10 fold. The rather large degree of enhancing activity of BAG-1L in AR response in this cell line will make the detection of slight differences more evident. The results presented in this work therefore point to an additional role of the N-terminus of BAG-1L in the enhancing AR response.

The C-terminal region of BAG-1, which is required for Hsp/Hsc70 binding, was also found to be essential in potentiation of AR activity. BAG-1L, lacking Hsp70-binding domain, failed to modulate the transactivation function of the AR. This finding suggests a role of Hsp/Hsc70 in the mechanism of action of BAG-1L in modulation of the

transactivation function of the AR. The involvement of Hsp/Hsc70 in the regulatory action of BAG-1L was confirmed in experiments in which overexpression of Hsp70 resulted in the enhancement of the BAG-1L-mediated effect on the transactivation function of the AR. At the same time dominant negative mutant of Hsp70 repressed the regulatory function of BAG-1L on transactivation by the AR. These studies therefore indicated that Hsp/Hsc70 is connected with the regulation of AR function by BAG-1L. In this connection, it is interesting to note that the ability of BAG-1M to regulate the transactivation function of the GR was also shown to require the C-terminal Hsp/Hsc70 binding site of BAG-1M (Schneikert *et al.*, 1999). Thus, it is possible that involvement of Hsp70 is a general requirement for the regulation of the nuclear receptors by BAG-1 proteins.

4.3 Hsp70 in regulatory action of BAG-1L

In vitro binding studies showed that BAG-1L could interact with the □5 domain of the AR through its C-terminal region. As this domain of BAG-1L also binds Hsp/Hsc70, this presents certain complications in the elucidation of the mechanism of action of BAG-1L.. The regulating action of BAG-1L can occur in different ways:

a) □5 and Hsp/Hsc70 compete for binding to the C-terminus of BAG-1L

b) Both Hsp70 and □5 could bind to different regions of the C-terminus of BAG-1L

or

c) Hsp70 may first bind to the C-terminus of BAG-1L bringing about a conformational change that allows BAG-1L to bind to □5 through its C-terminal binding site. Hsp70 acts as molecular chaperone and is therefore not a component of this final functional unit.

The results presented in this work do not easily allow the various mechanisms to be differentiated. However, co-transfection experiments performed with Hsp70 expression vectors revealed the following possibilities.

Overexpression of the intact Hsp70 or Hsp70 ATPase domain that interacts with the C-terminus of BAG-1L resulted in the increase in effect of BAG-1L on the transactivation function of □5 as well as the AR. Thus whatever mechanism is used by Hsp70, this molecular chaperone is important for the enhancing action of BAG-1L on transactivation by the AR. Further importance of the role of Hsp70 was shown by the fact that overexpression of the substrate binding domain of Hsp70 exerted a strong dominant

negative effect on the BAG-1L-mediated enhancement of the AR response but not of the transactivation function of 5. As the substrate-binding domain of Hsp70 is known to bind the HBD of steroid receptors (Smith and Toft, 1993), it is possible that this domain sequesters the AR making it unavailable for the action of BAG-1L. This dominant negative effect was not observed with 5, which lacks the HBD of the AR, and does not interact with the substrate-binding domain of Hsp70.

For Hsp70 to influence the action of BAG-1L, it must be localized in the nucleus. Using laser confocal microscopy techniques it was demonstrated in this work that Hsp70 can co-localize with BAG-1L and activated AR in the nucleus. Molecular chaperones are thought to bring steroid receptors into the conformations that are competent to bind steroid ligands in the cytoplasmic compartment of the cell (Pratt and Toft, 1997). The results of this study indicating that the components of the chaperone–co-chaperone machinery modulate the transcriptional activation function of the AR in the nucleus, define e novel action of these proteins. These results are however consistent with recent reports that describe a new function of molecular chaperones in transcriptional activation at a level downstream of hormone binding (Landel *et al*., 1994; DeFranco *et al*., 1998). For example, nuclear co-localization of Hsp70 with a steroid receptor was already observed in case of the down-regulation of the activity of glucocorticoid receptor by BAG-1M, where Hsp70 and BAG-1M were carried into the nucleus by the GR upon hormone treatment (Schneikert *et al*., 1999 and 2000). However in the work presented here no evidence was found for cotransport of Hsp70 into the nucleus by the AR. Hsp70 on its own seem to be present mainly in the cytoplasmic compartment but some molecules are nuclear. Whatever is responsible for the nuclear translocation of Hsp70 is not clear.

By chromatin immunoprecipitation assay the recruitment of AR, BAG-1L and Hsp70 to the PSA promoter could be demonstrated. The result of this experiment revealed a significant increase of the occupancy of both proximal androgen responsive elements AREI and AREII, as well as the AREIII at the enhancer region of the PSA promoter by AR and Hsp70 upon hormone treatment. Surprisingly, BAG-1L appeared to be recruited to the promoter in the absence of hormone, while hormone treatment, in contrast to the increase in AR and Hsp70, resulted in reduction of the amount of DNA fragments precipitated with antibody against BAG-1L. The reason for this result is not known. One of the possible explanations is that in the absence of androgen, BAG-1L, which is

constitutively nuclear, is bound to the promoter region on its own. BAG-1M has been reported to bind DNA directly and, furthermore, the region responsible for this binding has been shown to be important for the regulation of the GR transactivation function (Zeiner *et al*., 1999; Schmidt *et al*., 2003). As BAG-1 proteins are translated from the same mRNA and BAG-1L is the longest isoform, it contains these sequences of BAG-1M, and thus might be able to bind DNA. Upon hormone treatment BAG-1L facilitates the recruitment of the AR and Hsp70 to the promoter and either detaches from the AR binding site region, or, alternatively, remains on the promoter, but its conformation changes in such a way that the DNA-bound protein is not recognized by the BAG-1L antibody used in the experiment. However, there is no doubt that recruitment of the proteins to the promoter is specific, as they did not occupy the promoter of an androgen nonresponsive gene.

4.4 Interaction of N-terminus of BAG-1L with the HBD of the AR

It was also shown in this work that N-terminus of BAG-1L interacts with the C-terminal region of the AR. BAG-1M that lack the first 70 N-terminal amino acids has a compromised affinity for the HBD of the AR indicating that the extreme N-terminal sequences of BAG-1L are involved in the interaction of this protein with the AR. Thus BAG-1L makes multiple interactions with the AR. In chromatin immunoprecipitation experiments the antibody against the extreme N-terminus of BAG-1L were used to precipitate the protein-DNA complexes. Interaction of the N-terminus of BAG-1L with the HBD of the AR is increased in the presence of androgen, indicating conformational changes in either AR or BAG-1L molecules upon ligand binding. These conformational could lead to the lower affinity of the BAG-1L antibody to the protein, thus possibly explaining the reduction in the recovery of the DNA fragments, precipitated with the BAG-1L antibody, upon DHT treatment.

4.5 Androgen receptor N/C-terminal interaction

The work presented here show that the □5 domain of the AR is involved in the amino- to carboxyl-terminal intermolecular interaction of the receptor. This interaction is important for maximal activation of the full-length receptor (Berrevoets *et al*., 1998; Ikonen *et al*., 1998; Karvonen *et al*., 1997; Langley *et al*., 1998). Disruption of the AR N- to C-terminal functional communication leads to the repression of the receptor activity (Shenk *et al*.,

2001; Liao *et al*., 2002) and is linked to androgen insensitivity syndrome (Thompson *et al*., 2001). The ability of BAG-1L to bind both N- and C-termini of the AR allows the speculation that BAG-1L stabilizes the N/C interaction, thus further enhancing activity of the full-length AR. This possibly occur through affecting the recruitment of coactivators, as intermolecular N/C terminal interaction of the AR is reported to be important for the formation of the functionally active complex with such factors as SRC1, TIF2 and CBP (Saitoh *et al*., 2002).

Conclusion

Collectively, the results presented here provide additional proof for the hypothesis, that along with the well established roles in protein synthesis and trafficking (Ellis, 1997; Gottesman *et al*., 1997), molecular chaperones and co-chaperones also play a role in transcriptional regulation. This role in the regulation of transcription has previously been proposed for the bacterial chaperones DnaK and DnaJ which regulate the function of the prokaryotic transcription factor σ^{32} (Gamer *et al*., 1992). A human nuclear localized chaperone, regulating DNA binding and transcriptional activity of bZIP proteins, has also been described (Virbasius *et al*., 1999). BAG-1M and Hsp70 have been shown to downregulate the transactivation function of the GR (Schneikert *et al*., 1999 and 2000). Recently a molecular chaperone Cdc37 was reported to regulate the transactivation function of nuclear receptors (Rao *et al*., 2001; Freeman *et al*., 2000; Freeman and Yamamoto, 2002).

The work presented here gives an insight into the molecular mechanism of the upregulation of the transcription activation function of the AR by cochaperone BAG-1L. The results obtained so far, allow a model for the effect of BAG-1L in regulating the nuclear activity of the AR to be proposed (**Fig.4.1**). Present on the promoter of the AR target gene BAG-1L might facilitate the recruitment of the activated AR to the AREs. It may further promote N- and C-termini interaction of the AR, an effect that is stabilized by Hsp70, as shown schematically below, and possibly provide additional interaction surface for the recruitment of the AR co-activators. Whether all androgen response elements are regulated by AR-BAG-1L-Hsp70 complex in the same way is not clear. However the studies carried out with the PSA promoter might turn out to be a prototype of regulation of androgen responsive genes because of the rather ubiquitous pattern of expression of Hsp70 and BAG-1L.

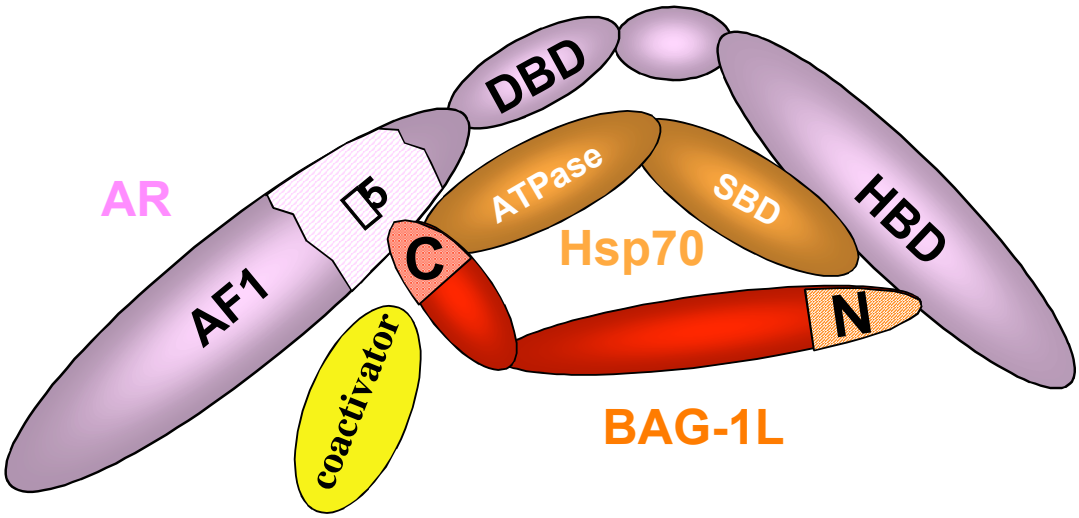

FIGURE 4.1 A schematic representation of a ternary structure of the AR-BAG-1L-Hsp70

Indicated are the domains of the AR which consist of the N-terminal transactivation function (AF1), the DNA binding domain (DBD) and the hormone binding domain (HBD). In the AF1 domain 5 transactivation function is depicted. The interaction of the NH_2- and COOH-terminal domains of BAG-1L with the HBD of the AR and ATPase domain of Hsp70 together with the AR 5, respectively, is shown.

REFERENCES

Adler, A.J., Scheller, A. and Robins, D.M. (1993) The stringency and magnitude of androgen-specific gene activation are combinatorial functions of receptor and nonreceptor binding site sequences. *Mol Cell Biol*, **13**, 6326-6335.

Alen, P., Claessens, F., Schoenmakers, E., Swinnen, J.V., Verhoeven, G., Rombauts, W. and Peeters, B. (1999) Interaction of the putative androgen receptor-specific coactivator ARA70/ELE1alpha with multiple steroid receptors and identification of an internally deleted ELE1beta isoform. *Mol Endocrinol*, **13**, 117-128.

Anfinsen, C.B. (1973) Principles that govern the folding of protein chains. *Science*, **181**, 223-230.

Anzick, S.L., Kononen, J., Walker, R.L., Azorsa, D.O., Tanner, M.M., Guan, X.Y., Sauter, G., Kallioniemi, O.P., Trent, J.M. and Meltzer, P.S. (1997) AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science*, **277**, 965-968.

Baulieu, E.E. and Jung, I. (1970) A prostatic cytosol receptor. *Biochem Biophys Res Com*, **38**: 599-606.

Baxter, J.D. and Tomkins, G.M. (1971) Specific cytoplasmic glucocorticoid hormone receptors in hepatoma tissue culture cells. *Proc. Natl. Acad. Sci. USA*, **68**: 932-937.

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.

Bellmann, K., Jaattela, M., Wissing, D., Burkart, V. and Kolb, H. (1996) Heat shock protein hsp70 overexpression confers resistance against nitric oxide. *FEBS Lett*, **391**, 185-188.

Berrevoets, C.A., Doesburg, P., Steketee, K., Trapman, J. and Brinkmann, A.O. (1998) Functional interactions of the AF-2 activation domain core region of the human androgen receptor with the amino-terminal domain and with the transcriptional coactivator TIF2 (transcriptional intermediary factor2). *Mol Endocrinol*, **12**, 1172-1183.

Bevan, C.L., Hoare, S., Claessens, F., Heery, D.M. and Parker, M.G. (1999) The AF1 and AF2 domains of the androgen receptor interact with distinct regions of SRC1. *Mol Cell Biol*, **19**, 8383-8392.

Bimston, D., Song, J., Winchester, D., Takayama, S., Reed, J.C. and Morimoto, R.I. (1998) BAG-1, a negative regulator of Hsp70 chaperone activity, uncouples nucleotide hydrolysis from substrate release. *Embo J*, **17**, 6871-6878.

Brady, M.E., Ozanne, D.M., Gaughan, L., Waite, I., Cook, S., Neal, D.E. and Robson, C.N. (1999) Tip60 is a nuclear hormone receptor coactivator. *J Biol Chem*, **274**, 17599-17604.

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.

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

Buller, R.E., Toft, D.O., Schrader, W.T. and O'Malley, B.W. (1975) Progesterone-binding components of chick oviduct. VIII. Receptor activation and hormone-dependent binding to purified nuclei. *J.Bio. Chem.*, **250**: 801-808.

Burris, T.P. and McCabe, E. R.B. (2001) Nuclear receptors and genetic disease. *Academic Press*

Butenandt, A. (1931) Über die chemische untersuchung der sexualhormone. *Ztschr. F. angwe. Chem.* **44**: 905.

Canosa, L.F., Lin, X. and Peter, R.E. (2002) Regulation of expression of somatostatin genes by sex steroid hormones in goldfish forebrain. *Neuroendocrinology*, **76**, 8-17.

Caplan, A.J. (1999) Hsp90's secrets unfold: new insights from structural and functional studies. *Trends Cell Biol*, **9**, 262-268.

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.

Catterall, J.F., Kontula, K.K., Watson, C.S., Seppanen, P.J., Funkenstein, B., Melanitou, E., Hickok, N.J., Bardin, C.W. and Janne, O.A. (1986) Regulation of gene expression by androgens in murine kidney. *Recent Prog Horm Res*, **42**, 71-109.

Chang, C., Saltzman, A., Yeh, S., Young, W., Keller, E., Lee, H.J., Wang, C. and Mizokami, A. (1995) Androgen receptor: an overview. *Crit Rev Eukaryot Gene Expr*, **5**, 97-125.

Chang, C.S., Kokontis, J. and Liao, S.T. (1988) Structural analysis of complementary DNA and amino acid sequences of human and rat androgen receptors. *Proc Natl Acad Sci U S A*, **85**, 7211-7215.

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.

Chen, Q., Kaji, H., Sugimoto, T. and Chihara, K. (2001) Testosterone inhibits osteoclast formation stimulated by parathyroid hormone through androgen receptor. *FEBS Lett*, **491**, 91-93.

Chen, S. and Smith, D.F. (1998) Hop as an adaptor in the heat shock protein 70 (Hsp70) and hsp90 chaperone machinery. *J Biol Chem*, **273**, 35194-35200.

Cheung, J. and Smith, D.F. (2000) Molecular chaperone interactions with steroid receptors: an update. *Mol Endocrinol*, **14**, 939-946.

Claessens, F., Alen, P., Devos, A., Peeters, B., Verhoeven, G. and Rombauts, W. (1996) The androgen-specific probasin response element 2 interacts differentially with androgen and glucocorticoid receptors. *J Biol Chem*, **271**, 19013-19016.

Claessens, F., Celis, L., De Vos, P., Heyns, W., Verhoeven, G., Peeters, B. and Rombauts, W. (1993) Functional androgen response elements in the genes coding for prostatic binding protein. *Ann N Y Acad Sci*, **684**, 199-201.

Claessens, F., Dirckx, L., Delaey, B., Decourt, J.L., Hemschoote, K., Peeters, B. and Rombauts, W. (1989) The androgen-dependent rat prostatic binding protein: comparison of the sequences in the 5' part and upstream region of the C1 and C2 genes and analysis of their transcripts. *J Mol Endocrinol*, **3**, 93-103.

Cleutjens, K.B., van der Korput, H.A., Ehren-van Eekelen, C.C., Sikes, R.A., Fasciana, C., Chung, L.W. and Trapman, J. (1997) A 6-kb promoter fragment mimics in transgenic mice the prostate-specific and androgen-regulated expression of the endogenous prostate-specific antigen gene in humans. *Mol Endocrinol*, **11**, 1256-1265.

Colquhoun-Flannery, W. and Carruth, J.A. (1996) Low adrenal androgen output in patients with abnormal laryngeal epithelium. *Clin Otolaryngol*, **21**, 439-441.

David, K., Dingemanse, E., Freud, J. and Laqueur, E. (1935) Über krystallinisches mannliches hormon aus hoden (testosteron), wirksamer als aus harn order aus cholesterin bereitetes androsterone. *Ztschr F physiol Chem*, **233**: 281.

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.

Dittmar, K.D., Banach, M., Galigniana, M.D. and Pratt, W.B. (1998) The role of DnaJ-like proteins in glucocorticoid receptor.hsp90 heterocomplex assembly by the reconstituted hsp90.p60.hsp70 foldosome complex. *J Biol Chem*, **273**, 7358-7366.

Dittmar, K.D. and Pratt, W.B. (1997) Folding of the glucocorticoid receptor by the reconstituted Hsp90-based chaperone machinery. The initial hsp90.p60.hsp70-dependent step is sufficient for creating the steroid binding conformation. *J Biol Chem*, **272**, 13047-13054.

Ellis, R.J. (1997) Molecular chaperones: avoiding the crowd. *Curr Biol*, **7**, R531-533.

Enmark, E. and Gustafsson, J.A. (1996) Orphan nuclear receptors--the first eight years. *Mol Endocrinol*, **10**, 1293-1307.

Evans, R.M. (1988) The steroid and thyroid hormone receptor superfamily. *Science*, **240**, 889-895.

Fang, S., Anderson, K.M. and Liao, S. (1969) Receptor proteins for androgens. On the role of specific proteins in selective retention of 17β-hydroxy-5α-androstan-3-one by rat ventral prostate in vivo and in vitro. *J Biol Chem*, **244**: 6584-6595.

Ferrando, A.A., Sheffield-Moore, M., Yeckel, C.W., Gilkison, C., Jiang, J., Achacosa, A., Lieberman, S.A., Tipton, K., Wolfe, R.R. and Urban, R.J. (2002) Testosterone administration to older men improves muscle function: molecular and physiological mechanisms. *Am J Physiol Endocrinol Metab*, **282**, E601-607.

Fliss, A.E., Fang, Y., Boschelli, F. and Caplan, A.J. (1997) Differential in vivo regulation of steroid hormone receptor activation by Cdc37p. *Mol Biol Cell*, **8**, 2501-2509.

Freeman, B.C., Felts, S.J., Toft, D.O. and Yamamoto, K.R. (2000) The p23 molecular chaperones act at a late step in intracellular receptor action to differentially affect ligand efficacies. *Genes Dev*, **14**, 422-434.

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

Fronsdal, K., Engedal, N., Slagsvold, T. and Saatcioglu, F. (1998) CREB binding protein is a coactivator for the androgen receptor and mediates cross-talk with AP-1. *J Biol Chem*, **273**, 31853-31859.

Fu, M., Wang, C., Reutens, A.T., Wang, J., Angeletti, R.H., Siconolfi-Baez, L., Ogryzko, V., Avantaggiati, M.L. and Pestell, R.G. (2000) p300 and p300/cAMP-response

element-binding protein-associated factor acetylate the androgen receptor at sites governing hormone-dependent transactivation. *J Biol Chem*, **275**, 20853-20860.

Gamer, J., Bujard, H. and Bukau, B. (1992) Physical interaction between heat shock proteins DnaK, DnaJ, and GrpE and the bacterial heat shock transcription factor sigma 32. *Cell*, **69**, 833-842.

Gasc, J.M., Renoir, J.M., Faber, L.E., Delahaye, F. and Baulieu, E.E. (1990) Nuclear localization of two steroid receptor-associated proteins, hsp90 and p59. *Exp Cell Res*, **186**, 362-367.

Gast, A., Schneikert, J. and Cato, A.C. (1998) N-terminal sequences of the human androgen receptor in DNA binding and transrepressing functions. *J Steroid Biochem Mol Biol*, **65**, 117-123.

Gebauer, M., Zeiner, M. and Gehring, U. (1998) Interference between proteins Hap46 and Hop/p60, which bind to different domains of the molecular chaperone hsp70/hsc70. *Mol Cell Biol*, **18**, 6238-6244.

Genazzani, A.R., Monteleone, P. and Gambacciani, M. (2002) Hormonal influence on the central nervous system. *Maturitas*, **43 Suppl 1**, S11-17.

George, F.W. and Wilson, J.D. (1994) Sex determination and differentiation. In: Knobil, E., Neill; J.D. (Eds.), The physiology of Reproduction. *Raven Press Ltd, New York* Chapter I

Gething, M.J. and Sambrook, J. (1992) Protein folding in the cell. *Nature*, **355**, 33-45.

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

Gorski, J., Toft, D., Shyamala, G., Smith, D. and Notides, A. (1968) Hormone receptors: studies on the interaction of estrogen with uterus. *Recent Progr Horm Res*, **24**: 45.

Gottesman, S., Wickner, S. and Maurizi, M.R. (1997) Protein quality control: triage by chaperones and proteases. *Genes Dev*, **11**, 815-823.

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

Hattori, H., Kaneda, T., Lokeshwar, B., Laszlo, A. and Ohtsuka, K. (1993) A stress-inducible 40 kDa protein (hsp40): purification by modified two-dimensional gel electrophoresis and co-localization with hsc70(p73) in heat-shocked HeLa cells. *J Cell Sci*, **104 (Pt 3)**, 629-638.

He, B., Kemppainen, J.A., Voegel, J.J., Gronemeyer, H. and Wilson, E.M. (1999) Activation function 2 in the human androgen receptor ligand binding domain

mediates interdomain communication with the NH(2)-terminal domain. *J Biol Chem*, **274**, 37219-37225.

He, B., Kemppainen, J.A. and Wilson, E.M. (2000) FXXLF and WXXLF sequences mediate the NH2-terminal interaction with the ligand binding domain of the androgen receptor. *J Biol Chem*, **275**, 22986-22994.

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.

Hendrick, J.P. and Hartl, F.U. (1993) Molecular chaperone functions of heat-shock proteins. *Annu Rev Biochem*, **62**, 349-384.

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.

Hohfeld, J., Minami, Y. and Hartl, F.U. (1995) Hip, a novel cochaperone involved in the eukaryotic Hsc70/Hsp40 reaction cycle. *Cell*, **83**, 589-598.

Ikonen, T., Palvimo, J.J. and Janne, O.A. (1998) Heterodimerization is mainly responsible for the dominant negative activity of amino-terminally truncated rat androgen receptor forms. *FEBS Lett*, **430**, 393-396.

Jensen, E.V., De Sombre, E. and Jungblut, P.W. (1967) Interaction of estrogens with receptor sites *in vivo* and *in vitro*. *Proc. 2nd Int. Congr. Hormonal Steroids* (eds L. Martini, F.Franschini and M.Motto),**132**: 492-500.

Jensen, E.V., Suzuki, T., Kawashima, T., Stumpf, W.E., Jungblut, P.W. and De Sombre, E.R. (1968) A two-step mechanism for interaction of estradiol with rat uterus. *Proc Natl Acad Sci USA*, **59**: 632.

Jenster, G., van der Korput, H.A., Trapman, J. and Brinkmann, A.O. (1995) Identification of two transcription activation units in the N-terminal domain of the human androgen receptor. *J Biol Chem*, **270**, 7341-7346.

Jing, I. and Baulieu, E.E. (1972) Testosterone cytosol "receptor" in the rat levator ani muscle. *Nature New Biol*, **237**: 24-26.

Johnson, B.D., Schumacher, R.J., Ross, E.D. and Toft, D.O. (1998) Hop modulates Hsp70/Hsp90 interactions in protein folding. *J Biol Chem*, **273**, 3679-3686.

Kanelakis, K.C., Morishima, Y., Dittmar, K.D., Galigniana, M.D., Takayama, S., Reed, J.C. and Pratt, W.B. (1999) Differential effects of the hsp70-binding protein BAG-1 on glucocorticoid receptor folding by the hsp90-based chaperone machinery. *J Biol Chem*, **274**, 34134-34140.

Kang, H.Y., Yeh, S., Fujimoto, N. and Chang, C. (1999) Cloning and characterization of human prostate coactivator ARA54, a novel protein that associates with the androgen receptor. *J Biol Chem*, **274**, 8570-8576.

Karvonen, U., Kallio, P.J., Janne, O.A. and Palvimo, J.J. (1997) Interaction of androgen receptors with androgen response element in intact cells. Roles of amino- and carboxyl-terminal regions and the ligand. *J Biol Chem*, **272**, 15973-15979.

Kim, M.Y., Hsiao, S.J. and Kraus, W.L. (2001) A role for coactivators and histone acetylation in estrogen receptor alpha-mediated transcription initiation. *Embo J*, **20**, 6084-6094.

Knee, D.A., Froesch, B.A., Nuber, U., Takayama, S. and Reed, J.C. (2001) Structure-function analysis of Bag1 proteins. Effects on androgen receptor transcriptional activity. *J Biol Chem*, **276**, 12718-12724.

Koh, S.S., Li, H., Lee, Y.H., Widelitz, R.B., Chuong, C.M. and Stallcup, M.R. (2002) Synergistic coactivator function by coactivator-associated arginine methyltransferase (CARM) 1 and beta-catenin with two different classes of DNA-binding transcriptional activators. *J Biol Chem*, **277**, 26031-26035.

Kosano, H., Stensgard, B., Charlesworth, M.C., McMahon, N. and Toft, D. (1998) The assembly of progesterone receptor-hsp90 complexes using purified proteins. *J Biol Chem*, **273**, 32973-32979.

Kotaja, N., Karvonen, U., Janne, O.A. and Palvimo, J.J. (2002a) The nuclear receptor interaction domain of GRIP1 is modulated by covalent attachment of SUMO-1. *J Biol Chem*, **277**, 30283-30288.

Kotaja, N., Vihinen, M., Palvimo, J.J. and Janne, O.A. (2002b) Androgen receptor-interacting protein 3 and other PIAS proteins cooperate with glucocorticoid receptor-interacting protein 1 in steroid receptor-dependent signaling. *J Biol Chem*, **277**, 17781-17788.

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.

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.

Langley, E., Kemppainen, J.A. and Wilson, E.M. (1998) Intermolecular NH2-/carboxyl-terminal interactions in androgen receptor dimerization revealed by mutations that cause androgen insensitivity. *J Biol Chem*, **273**, 92-101.

Langley, E., Zhou, Z.X. and Wilson, E.M. (1995) Evidence for an anti-parallel orientation of the ligand-activated human androgen receptor dimer. *J Biol Chem*, **270**, 29983-29990.

Laudet, V., Hänni, C., Coll, J., Catzeflis, F. and Stehelin, D. (1992) Evolution of the nuclear receptor gene supefamily. *EMBO J.,* **11**: 1003-1013.

Le Douarin, B., Nielsen, A.L., Garnier, J.M., Ichinose, H., Jeanmougin, F., Losson, R. and Chambon, P. (1996) A possible involvement of TIF1 alpha and TIF1 beta in the epigenetic control of transcription by nuclear receptors. *Embo J*, **15**, 6701-6715.

Lee, D.K. (2002) Androgen receptor enhances myogenin expression and accelerates differentiation. *Biochem Biophys Res Commun*, **294**, 408-413.

Lee, D.K., Duan, H.O. and Chang, C. (2000) From androgen receptor to the general transcription factor TFIIH. Identification of cdk activating kinase (CAK) as an androgen receptor NH(2)-terminal associated coactivator. *J Biol Chem*, **275**, 9308-9313.

Li, H., Gomes, P.J. and Chen, J.D. (1997) RAC3, a steroid/nuclear receptor-associated coactivator that is related to SRC-1 and TIF2. *Proc Natl Acad Sci U S A*, **94**, 8479-8484.

Liao, G., Chen, L.Y., Zhang, A., Godavarthy, A., Xia, F., Ghosh, J.C., Li, H. and Chen, J.D. (2003) Regulation of Androgen Receptor Activity by the Nuclear Receptor Corepressor SMRT. *J Biol Chem*, **278**, 5052-5061.

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.

Lubahn, D.B., Joseph, D.R., Sullivan, P.M., Willard, H.F., French, F.S. and Wilson, E.M. (1988) Cloning of human androgen receptor complementary DNA and localization to the X chromosome. *Science*, **240**, 327-330.

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.

MacLean, H.E., Warne, G.L. and Zajac, J.D. (1997) Localization of functional domains in the androgen receptor. *J Steroid Biochem Mol Biol*, **62**, 233-242.

Mainwaring, W.I.P. (1969) A soluble androgen receptor in the cytoplasm of rat prostate. *J. Endocrinol*, **45**: 531-541.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P. and et al. (1995) The nuclear receptor superfamily: the second decade. *Cell*, **83**, 835-839.

Marver, D., Goodman, D. and Edelman, I.S. (1972) Relationships between renal cytoplasmic and nuclear aldosterone-receptors. *Kidney Int.*, **1**: 210-223.

McEwan, I.J. and Gustafsson, J. (1997) Interaction of the human androgen receptor transactivation function with the general transcription factor TFIIF. *Proc Natl Acad Sci U S A*, **94**, 8485-8490.

McGuire, J.L. and DeDella, C. (1971) In vitro evidence for a progestogen receptor in the rat and rabbit uterus. *Endocrinology*, **88**, 1099-1103.

McKay, L.I. and Cidlowski, J.A. (2000) CBP (CREB binding protein) integrates NF-kappaB (nuclear factor-kappaB) and glucocorticoid receptor physical interactions and antagonism. *Mol Endocrinol*, **14**, 1222-1234.

McKenna, N.J. and O'Malley, B.W. (2002) Minireview: nuclear receptor coactivators--an update. *Endocrinology*, **143**, 2461-2465.

McMahon, C., Suthiphongchai, T., DiRenzo, J. and Ewen, M.E. (1999) P/CAF associates with cyclin D1 and potentiates its activation of the estrogen receptor. *Proc Natl Acad Sci U S A*, **96**, 5382-5387.

Milgrom, E., Atger, M. and Baulieu, E.E. (1970) Progesterone in uterus and plasma. IV-progesterone receptor(s) in guinea pig uterus cytozol. *Steroids,* **16**: 741-754.

Mink, S., Haenig, B. and Klempnauer, K.H. (1997) Interaction and functional collaboration of p300 and C/EBPbeta. *Mol Cell Biol*, **17**, 6609-6617.

Mink, S., Kerber, U. and Klempnauer, K.H. (1996) Interaction of C/EBPbeta and v-Myb is required for synergistic activation of the mim-1 gene. *Mol Cell Biol*, **16**, 1316-1325.

Mohan, P.F., Ihnen, J.S., Levin, B.E. and Cleary, M.P. (1990) Effects of dehydroepiandrosterone treatment in rats with diet-induced obesity. *J Nutr*, **120**, 1103-1114.

Mooradian, A.D., Morley, J.E. and Korenman, S.G. (1987) Biological actions of androgens. *Endocr Rev*, **8**, 1-28.

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.

Muller, J.M., Isele, U., Metzger, E., Rempel, A., Moser, M., Pscherer, A., Breyer, T., Holubarsch, C., Buettner, R. and Schule, R. (2000) FHL2, a novel tissue-specific coactivator of the androgen receptor. *Embo J*, **19**, 359-369.

Munck, A. and Brinck-Johnsen, T. (1967) Specific metabolic and and physiochemical interactions of glucocorticoids and related steroids in vivo and in vitro with rat adipose tissue and thymus cells. Proceedings of the 2nd International congress on Hormonal Steroids. *Exerpta Medica Int Congr Ser*, **132**: 472-481.

Munck, A. and Brinck-Johnsen, T. (1968) Specific and nonspecific physiochemical interactions of glucocortiocoids and related steroids with rat thymus cells in vitro. *J. Biol. Chem.*, **243**: 5556-5565.

Murtha, P., Tindall, D.J. and Young, C.Y. (1993) Androgen induction of a human prostate-specific kallikrein, hKLK2: characterization of an androgen response element in the 5' promoter region of the gene. *Biochemistry*, **32**, 6459-6464.

Ning, Y.M. and Robins, D.M. (1999) AML3/CBFalpha1 is required for androgen-specific activation of the enhancer of the mouse sex-limited protein (Slp) gene. *J Biol Chem*, **274**, 30624-30630.

Nollen, E.A., Brunsting, J.F., Song, J., Kampinga, H.H. and Morimoto, R.I. (2000) Bag1 functions in vivo as a negative regulator of Hsp70 chaperone activity. *Mol Cell Biol*, **20**, 1083-1088.

Pasqualini, J.R., Sumida, C. and Gelly, C. (1972) Mineralocorticoid receptors in the foetal compartment. *J. Steroid Biochem.*, **3**: 543-556.

Poukka, H., Aarnisalo, P., Karvonen, U., Palvimo, J.J. and Janne, O.A. (1999) Ubc9 interacts with the androgen receptor and activates receptor-dependent transcription. *J Biol Chem*, **274**, 19441-19446.

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

Quigley, C.A., De Bellis, A., Marschke, K.B., el-Awady, M.K., Wilson, E.M. and French, F.S. (1995) Androgen receptor defects: historical, clinical, and molecular perspectives. *Endocr Rev*, **16**, 271-321.

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.

Randall, V.A., Hibberts, N.A., Thornton, M.J., Hamada, K., Merrick, A.E., Kato, S., Jenner, T.J., De Oliveira, I. and Messenger, A.G. (2000) The hair follicle: a paradoxical androgen target organ. *Horm Res*, **54**, 243-250.

Rao, J., Lee, P., Benzeno, S., Cardozo, C., Albertus, J., Robins, D.M. and Caplan, A.J. (2001) Functional interaction of human Cdc37 with the androgen receptor but not with the glucocorticoid receptor. *J Biol Chem*, **276**, 5814-5820.

Rao, B.R., Wiest, W.G. and Allen, W.M. (1973) Progesterone "receptor" in rabbit uterus. I. Characterization and estradiol-17ß augmentation. *Endocrinology*, **92**: 1229-1240.

Reel, J.R., Van Dewark, S.D., Shih, Y. and Callantine, M.R. (1971) Macromolecular binding and metabolism of progesterone in the decidual and pseudopregnant rat and rabbit uterus. *Steroids*, **18**: 441-461.

Reid, J., Murray, I., Watt, K., Betney, R. and McEwan, I.J. (2002) The androgen receptor interacts with multiple regions of the large subunit of general transcription factor TFIIF. *J Biol Chem*, **277**, 41247-41253.

Rennie, P.S., Bruchovsky, N., Leco, K.J., Sheppard, P.C., McQueen, S.A., Cheng, H., Snoek, R., Hamel, A., Bock, M.E., MacDonald, B.S. and et al. (1993) Characterization of two cis-acting DNA elements involved in the androgen regulation of the probasin gene. *Mol Endocrinol*, **7**, 23-36.

Reutens, A.T., Fu, M., Wang, C., Albanese, C., McPhaul, M.J., Sun, Z., Balk, S.P., Janne, O.A., Palvimo, J.J. and Pestell, R.G. (2001) Cyclin D1 binds the androgen receptor and regulates hormone-dependent signaling in a p300/CBP-associated factor (P/CAF)-dependent manner. *Mol Endocrinol*, **15**, 797-811.

Riegman, P.H., Vlietstra, R.J., van der Korput, J.A., Brinkmann, A.O. and Trapman, J. (1991) The promoter of the prostate-specific antigen gene contains a functional androgen responsive element. *Mol Endocrinol*, **5**, 1921-1930.

Roche, P.J., Hoare, S.A. and Parker, M.G. (1992) A consensus DNA-binding site for the androgen receptor. *Mol Endocrinol*, **6**, 2229-2235.

Rundlett, S.E. and Miesfeld, R.L. (1995) Quantitative differences in androgen and glucocorticoid receptor DNA binding properties contribute to receptor-selective transcriptional regulation. *Mol Cell Endocrinol*, **109**, 1-10.

Russell, D.W. and Wilson, J.D. (1994) Steroid 5 alpha-reductase: two genes/two enzymes. *Annu Rev Biochem*, **63**, 25-61.

Saitoh, M., Takayanagi, R., Goto, K., Fukamizu, A., Tomura, A., Yanase, T. and Nawata, H. (2002) The presence of both the amino- and carboxyl-terminal domains in the AR is essential for the completion of a transcriptionally active form with coactivators and intranuclear compartmentalization common to the steroid hormone receptors: a three-dimensional imaging study. *Mol Endocrinol*, **16**, 694-706.

Sato, T., Kawano, H. and Kato, S. (2002) Study of androgen action in bone by analysis of androgen-receptor deficient mice. *J Bone Miner Metab*, **20**, 326-330.

Schaumburg, B.P. and Bojesen, E. (1968) Specificitay and thermodynamic properties of the corticosteroid binding to a receptor of rat thymocytes in vitro. *Biochem. Biophys. Acta*, **170**: 172-188.

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.

Schuur, E.R., Henderson, G.A., Kmetec, L.A., Miller, J.D., Lamparski, H.G. and Henderson, D.R. (1996) Prostate-specific antigen expression is regulated by an upstream enhancer. *J Biol Chem*, **271**, 7043-7051.

Shang, Y., Myers, M. and Brown, M. (2002) Formation of the androgen receptor transcription complex. *Mol Cell*, **9**, 601-610.

Sherman, M.R., Corvol, P.L. and O'Malley, B.W. (1970) Progesterone-binding components of chick oviduct. I. Preliminary characterization of cytoplasmic components. *J. Biol. Chem.*, **245**: 6085-6096.

Shenk, J.L., Fisher, C.J., Chen, S.Y., Zhou, X.F., Tillman, K. and Shemshedini, L. (2001) p53 represses androgen-induced transactivation of prostate-specific antigen by disrupting hAR amino- to carboxyl-terminal interaction. *J Biol Chem*, **276**, 38472-38479.

Smith, C.L., Onate, S.A., Tsai, M.J. and O'Malley, B.W. (1996) CREB binding protein acts synergistically with steroid receptor coactivator-1 to enhance steroid receptor-dependent transcription. *Proc Natl Acad Sci U S A*, **93**, 8884-8888.

Smith, D.F. and Toft, D.O. (1993) Steroid receptors and their associated proteins. *Mol Endocrinol*, **7**, 4-11.

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.

Swaneck, G.E., Chu, L.L.H. and Edelman, I.S. (1970) Stereospecific binding of aldosterone to renal chromatin. *J. Biol. Chem.*, **245**: 5382-5389.

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., Krajewski, S., Krajewska, M., Kitada, S., Zapata, J.M., Kochel, K., Knee, D., Scudiero, D., Tudor, G., Miller, G.J., Miyashita, T., Yamada, M. and Reed, J.C. (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., 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.

Takeshita, A., Cardona, G.R., Koibuchi, N., Suen, C.S. and Chin, W.W. (1997) TRAM-1, A novel 160-kDa thyroid hormone receptor activator molecule, exhibits distinct properties from steroid receptor coactivator-1. *J Biol Chem*, **272**, 27629-27634.

Thigpen, A.E., Davis, D.L., Milatovich, A., Mendonca, B.B., Imperato-McGinley, J., Griffin, J.E., Francke, U., Wilson, J.D. and Russell, D.W. (1992) Molecular genetics of steroid 5 alpha-reductase 2 deficiency. *J Clin Invest*, **90**, 799-809.

Thompson, J., Saatcioglu, F., Janne, O.A. and Palvimo, J.J. (2001) Disrupted amino- and carboxyl-terminal interactions of the androgen receptor are linked to androgen insensitivity. *Mol Endocrinol*, **15**, 923-935.

Toft, D. and Gorski, J. (1966) A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. *Proc Natl Acad Sci U S A*, **55**, 1574-1581.

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.

Trapman, J., Klaassen, P., Kuiper, G.G., van der Korput, J.A., Faber, P.W., van Rooij, H.C., Geurts van Kessel, A., Voorhorst, M.M., Mulder, E. and Brinkmann, A.O. (1988) Cloning, structure and expression of a cDNA encoding the human androgen receptor. *Biochem Biophys Res Commun*, **153**, 241-248.

Truica, C.I., Byers, S. and Gelmann, E.P. (2000) Beta-catenin affects androgen receptor transcriptional activity and ligand specificity. *Cancer Res*, **60**, 4709-4713.

Tsai, M.J. and O'Malley, B.W. (1994) Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem*, **63**, 451-486.

Velazquez, J.M. and Lindquist, S. (1984) hsp70: nuclear concentration during environmental stress and cytoplasmic storage during recovery. *Cell*, **36**, 655-662.

Verrijdt, G., Schauwaers, K., Haelens, A., Rombauts, W. and Claessens, F. (2002) Functional interplay between two response elements with distinct binding characteristics dictates androgen specificity of the mouse sex-limited protein enhancer. *J Biol Chem*, **277**, 35191-35201.

Virbasius, C.M., Wagner, S. and Green, M.R. (1999) A human nuclear-localized chaperone that regulates dimerization, DNA binding, and transcriptional activity of bZIP proteins. *Mol Cell*, **4**, 219-228.

Wang, H.G., Takayama, S., Rapp, U.R. and Reed, J.C. (1996) Bcl-2 interacting protein, BAG-1, binds to and activates the kinase Raf-1. *Proc Natl Acad Sci U S A*, **93**, 7063-7068.

Wang, Q., Sharma, D., Ren, Y. and Fondell, J.D. (2002) A coregulatory role for the TRAP-mediator complex in androgen receptor-mediated gene expression. *J Biol Chem*, **277**, 42852-42858.

Welch, W.J. (1992) Mammalian stress response: cell physiology, structure/function of stress proteins, and implications for medicine and disease. *Physiol Rev*, **72**, 1063-1081.

Wilson, J.D., Griffin, J.E. and Russell, D.W. (1993) Steroid 5 alpha-reductase 2 deficiency. *Endocr Rev*, **14**, 577-593.

Wilson, J.D., Shaw, G., Leihy, M.L. and Renfree, M.B. (2002) The marsupial model for male phenotypic development. *Trends Endocrinol Metab*, **13**, 78-83.

Wirth, M.P. and Hakenberg, O.W. (2000) [Testosterone and the prostate]. *Urologe A*, **39**, 418-420.

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.

Yamamoto, A., Hashimoto, Y., Kohri, K., Ogata, E., Kato, S., Ikeda, K. and Nakanishi, M. (2000) Cyclin E as a coactivator of the androgen receptor. *J Cell Biol*, **150**, 873-880.

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, F., Li, X., Sharma, M., Sasaki, C.Y., Longo, D.L., Lim, B. and Sun, Z. (2002) Linking beta-catenin to androgen-signaling pathway. *J Biol Chem*, **277**, 11336-11344.

Yuan, X., Lu, M.L., Li, T. and Balk, S.P. (2001) SRY interacts with and negatively regulates androgen receptor transcriptional activity. *J Biol Chem*, **276**, 46647-46654.

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., 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.

Zhang, J., Zhang, S., Murtha, P.E., Zhu, W., Hou, S.S. and Young, C.Y. (1997) Identification of two novel cis-elements in the promoter of the prostate-specific antigen gene that are required to enhance androgen receptor-mediated transactivation. *Nucleic Acids Res*, **25**, 3143-3150.