BAG-1L Protein Enhances Androgen Receptor Function*

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BAG-1 is a regulator of heat shock protein (Hsp) 70/Hsc70 family proteins that interacts with steroid hormone receptors. The recently identified BAG-1 long (BAG-1L) protein, an isoform of BAG-1 that arises from translation initiation at a noncanonical CUG codon, was co-immunoprecipitated with androgen receptors (AR) from LNCaP prostate cancer cells and other cell lysates, whereas the shorter originally identified BAG-1 and BAG-1M (RAP46) proteins were not. BAG-1L, but not BAG-1 or BAG-1M (RAP46), also markedly enhanced the ability of AR to transactivate reporter gene plasmids containing an androgen response element (ARE) in PC3 prostate cancer and other cell lines. A C-terminal region deletion mutant of BAG-1L failed to co-immunoprecipitate with AR and functioned as a trans-dominant inhibitor of BAG-1L, impairing AR-induced transactivation of ARE-containing reporter plasmids. In addition, BAG-1L significantly reduced the concentrations of 5α-dihydrotestosterone (DHT) required for AR activity but did not induce ligand-independent transactivation. BAG-1L also markedly improved the ability of AR to transactivate reporter genes when cells were cultured with DHT in combination with the anti-androgen cyproterone acetate. The effects of BAG-1L on AR could not be explained by detectable alterations in the DHT-induced translocation of AR from cytosol to nucleus, nor by BAG-1L-induced increases in the amounts of AR protein. These findings implicate BAG-1L in the regulation of AR function and may have relevance to mechanisms of prostate cancer resistance to hormone-ablative and anti-androgen therapy.

Prostate cancer is the most common malignancy in the United States and the second leading cause of cancer-related death among men (1). The normal prostate gland contains a two-layer epithelium composed of a population of small round stem cells called basal cells, which line the basement membrane, and a population of larger differentiated epithelial cells called secretory cells, which secrete a variety of proteins and other substances into the lumen of the gland (2, 3). Although both basal and secretory cells contain androgen receptors (AR)1 (4, 5), only the luminal secretory epithelial cells are dependent on steroid hormone for their function, growth, and survival (4). In the absence of testosterone or related androgens, which can serve as ligands for AR, the secretory cells undergo rapid programmed cell death (6). Current treatment for metastatic adenocarcinoma of the prostate is predicated on the cell death-inducing effects of anti-androgens and hormone-ablative measures, which reduce endogenous production of androgens. However, nearly all hormone-dependent prostate cancers eventually relapse as fatal hormone-independent disease (7).

Multiple, still largely unidentified mechanisms may account for the complete independence or reduced dependence of prostate cancers on androgens (reviewed in Refs. 8–10). AR gene deletion or sequestration of the AR from the nucleus to the cytoplasm have been described in some hormone-independent tumors, implying that genetic alterations associated with tumor progression can abrogate the necessity for AR in some cases. However, many tumors may rely on other strategies that allow cancer cells to grow in low concentrations of androgens, including AR gene amplification or overexpression (11, 12) and AR mutations that permit transactivation of target genes with little or no requirement for steroid hormones (9, 13). Since most hormone-insensitive prostate cancers still retain a wild-type AR, presumably alterations in the factors that control the levels of AR and its function appear to play a major role in resistance to anti-androgen and hormone-ablative therapies. Thus, a need exists to understand more about the molecular mechanisms that govern the activity of AR.

Steroid hormones mediate their effects by binding to specific intracellular receptors that act as hormone-dependent transcription factors. Upon binding steroid ligands, the AR undergo a conformational change, translocate to the nucleus, and bind to specific DNA sequences located near or in promoter regions of target genes. After binding DNA, the receptor interacts with components of the basal transcription machinery and sometimes sequence-specific transcription factors, resulting in positive or negative effects on gene transcription (14, 15). A number of proteins have been identified that associate with the inactive or hormone-bound hormone-receptor complexes, including several heat shock family proteins and various types of transcription co-activators (reviewed in Refs. 16 and 17). However, many details remain unclear as to the molecular mechanisms by which these proteins modulate the activities of steroid hormone receptors, and even less is known about whether alterations in their expression or function might contribute to the deregulation of steroid hormone responses in cancers.

Recently, an isoform of the human BAG-1 protein (known as RAP46 (see below)) (18, 19) has been reported to bind several steroid hormone receptors in vitro, including AR (19). It is unknown, however, what effect if any, BAG-1 has on the functions of these steroid-dependent transcription factors. Interestingly, BAG-1 and its alternative isoform RAP46 were recently shown to bind tightly to heat shock protein (Hsp) 70/Hsc70 family proteins and modulate their chaperone activity in vitro (20–22). In this regard, BAG-1 appears to function analogously to bacterial GrpE, stimulating the exchange of ADP for ATP on
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Hsc70 (22). It seems plausible therefore that BAG-1 could alter the bioactivity of AR and other steroid hormone receptors, given that many steroid hormone receptors are constitutively bound to heat shock proteins and that their hormone binding affinity and DNA binding activity can be increased in the presence of Hsp90 and Hsp70, respectively, under some circumstances (23–25).

The human and murine BAG-1 proteins are predicted to be amino acids 230 and 219 base pairs in length, respectively, based on cDNA cloning (18, 19, 26, 27). However, recently longer isoforms of the human and mouse BAG-1 proteins have been identified that can arise by translation initiation from noncanonical CUG codons located upstream and in frame with the originally described BAG-1 open reading frames (27, 28). This longer isoform of BAG-1 contains a basic motif resembling nuclear localization sequences and preferentially targets to nuclei. The human BAG-1 and BAG-1 long (BAG-1L) proteins migrate as ~36-kDa and 57–58-kDa proteins, respectively in SDS-PAGE experiments. In addition, a less abundant isoform of BAG-1 that migrates at ~46–53 kDa has been described and termed either BAG-1M or RAP46. The BAG-1M (RAP46) protein arises from translation initiation at an AUG codon located upstream of the usual start site in the BAG-1 mRNA (27, 29). BAG-1L (RAP46) is produced in human, but not mouse, cells. 2

Like BAG-1, the BAG-1L and BAG-1M proteins also bind to Hsp70 and Hsc70. 2 BAG-1 is ubiquitously expressed, whereas BAG-1L is found preferentially in steroid hormone-dependent tissues such as testis, ovary, breast, and prostate. 2 Although little is known about the expression of BAG-1 and BAG-1L in cancers, both proteins were detected by immunoblotting in 9 of 9 prostate cancer cell lines tested. 2 In this report, we present evidence that the BAG-1L protein may play an important role in the AR signaling pathway, in that it can form complexes with AR and enhance the androgen-dependent transactivation function of this steroid hormone receptor.

MATERIALS AND METHODS

Plasmids—The plasmids pcDNA3-hu-BAG-1L and pcDNA3-hu-BAG-1 were generated as described previously (26). 2 Translation of the longer form BAG-1L protein arises from translation initiation from a noncanonical in frame first CTG (BAG-1L) was forced by mutation of the noncanonical in frame first CTG (lacking the last 47 amino acids of the human BAG-1 protein) was generated by polymerase chain reaction using pcDNA3-BAG-1L as a template and the EcoRI-containing forward primer 5'-GGGAAATTCAGTCGGCGATGCTC-3' together with the XhoI containing reverse primer 5'-CCCTCGGATATTCGGCAGATGAGTTCGAGGATCAGCTGGTCG-3'. After digestion of the polymerase chain reaction product using EcoRI and XhoI sites, the resulting ~36-kDa form of BAG-1 and ~46–53-kDa BAG-1M (RAP46) proteins. The plasmid pcDNA3-hu-BAG-1L (ΔC) (lacking the last 47 amino acids of the human BAG-1 protein) was generated by polymerase chain reaction using pcDNA3-BAG-1L as a template and the EcoRI-containing forward primer 5'-GGGAAATTCAGTCGGCGATGCTC-3' together with the XhoI containing reverse primer 5'-CCCTCGGATATTCGGCAGATGAGTTCGAGGATCAGCTGGTCG-3'. After digestion of the polymerase chain reaction product at the EcoRI and XhoI sites, the resulting ~0.8-kilobase pair fragments were subcloned into EcoRI/XhoI-digested pcDNA3. pcDNA3-AR contains the cDNA for the wild-type AR (29). The reporter pCI plasmid contains the full-length mouse mammary tumor virus long terminal repeat sequences linked with the chloramphenicol acetyltransferase (CAT) gene (29, 30). pcCMV-p53wt expression vector, MYH101–81 containing the p53 response element and the TATA box from the BAX promoter, and pUCSV3-CAT containing a SV40 early region promoter have been described (31, 32).

Cell Culture—The human prostate cancer cell lines LN-CaP and PC3, the transformed human embryonal kidney 293, and the monkey kidney COS7 cell lines were obtained from the American Type Culture Collection (Rockville, MD). The ALVA31 human prostate cancer cell line was generously provided by Dr. G. Miller (University of Colorado, Denver, CO). Cells were maintained in a humidified atmosphere with 5% CO2 in RPMI 1640 or Dulbecco’s modified Eagle’s medium (293 and COS7) supplemented with 10% FCS, 3 mM glutamine, and 100 units/ml penicillin, and 100 mg/ml streptomycin (Life Technologies, Inc.). Two days prior to experiments, cells were transfected into CT-FCS to reduce background levels of steroids. 5α-Dihydrotestosterone (DHT) (Sigma) and cyproterone acetate (CPA) (Sigma) were dissolved in dimethyl sulfoxide and added to the cultures at a minimum dilutions of 0.0001% (v/v). Control cells received an equivalent amount of solvent only.

Infection and Enzyme Assays—COS7, PC3, and 293T cells at 60% confluency were transiently transfected with expression plasmid pCMV-βgal by a standard calcium phosphate precipitate method (33). The medium was replaced with fresh charcoal-treated fetal calf serum/Dulbecco’s modified Eagle’s medium 1 h before transfection. The total amount of plasmid DNA used was normalized to 2.5 μg/well and 8 μg/plate for transfection in 12-well and 6-cm2 plates, respectively, by the addition of empty plasmid. For reporter gene assays, a β-galactosidase expression plasmid pCMV-βgal was co-transfected with the CAT reporter gene to normalize the transfection efficiency. Cells were exposed to the precipitate for 5 h at 37 °C. For COS7 and PC3 cells, a glycerol shock was applied. Cells were exposed to 15% glycerol in HBSS buffer (25 mM HEPES pH 7.05, 0.75 mM NaHPO4, 140 mM NaCl) for 4 min. The glycerol was removed by washing three times with PBS and replacement with fresh charcoal-treated fetal calf serum medium. For 293 cells, the medium was replaced without applying a shock.

ALVA31 cells were transfected by a lipofection method. Briefly, 1.3 μg of DNA was diluted into 50 μl of Opti-MEM medium (Life Technologies) and combined with 3.3 μl of Lipofectamine (Life Technologies) in 50 μl of Opti-MEM. After incubation for 20 min, 0.35 ml of Opti-MEM containing the mixture was overlayed onto cells.

At 32–36 h after transfection, cells were stimulated with 0.001–10 nM DHT or 0.1 nM R1881 (ALVA31). Cell extracts were prepared 48 h after transfection. For reporter gene experiments, cells were lysates were made as described in Ref. 34 and assayed for CAT and β-galactosidase activity. All transfection experiments were carried out in triplicate, repeated at least three times, and normalized for β-galactosidase activity.

Cell Extracts and Subcellular Fractionation—For gene expression experiments, cells were washed two times in PBS and lysed in radio-immuno precipitation buffer (35) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.28 trypsin inhibitory units/ml apro- nitin, 50 μg/ml leupeptin, 1 μg benzamidine, 0.7 μg/ml pepstatin). Protein for localization experiments, nuclear and nonnuclear fractions were prepared according to the method of Schreiber et al. (36). Briefly, cells were collected and washed two times with ice-cold PBS. Cell pellets were resuspended in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2.5 mM dithiothreitol, protease inhibitors) to a final concentration of 1 mg protein/ml. 200 μl of buffer A was added and the cell pellet was disrupted with a Dounce homogenizer. After centrifugation, supernatants (cytoplasmic fractions) were collected, and the nuclear pellets were washed twice in the same buffer. Pellets were finally resuspended in buffer B (20 mM HEPES, pH 7.9, 400 mM NaCl, 25% glycerol, 0.1 mM EDTA, 0.1 mM EGTA, 2.5 mM dithiothreitol, protease inhibitors) and vigorously shaken for 10 min, and the postnuclear supernatants were collected. Fractions were normalized based on the bicinchoninic acid method (Pierce) prior to SDS-PAGE/immunoblot assay.

Immunoblotting—Aliquots containing 25 μg of protein were subjected to SDS-PAGE using 10% gels, followed by electrotransfer to Immobilon-P transfer membranes (Millipore Corp., Bedford, MA). Immunodetection was accomplished using 1:1000 (v/v) of anti-BAG-1 monoclonal antibodies or 26, 37) or polyclonal rabbit AR antisem (Clone AR N20, Santa Cruz Biotechnology, Inc., Santa Barbara, CA), followed by horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech). Detection was performed using an enhanced chemiluminescence detection method (ECI, Amersham Pharmacia Biotech) or the Vector SG substrate (Vector Laboratories, Burlingame, CA).

Co-immunoprecipitations—LN-CaP cells (2 × 107) were collected at 70% confluency and lysed in HKMEN buffer (10 mM HEPES, pH 7.2, 142 mM KCl, 5 mM MgCl2, 2 mM EGTA, 0.2% Nonidet P-40, protease inhibitors). Cells lysates were passed several times through a 30-gauge needle to disrupt the nuclei. Alternatively, COS7 cells were transiently transfected with AR and BAG-1 expression plasmids several times in PBS, and treated with 1 mM dimethyl-3,3' -dithiobispropionimidate (Pierce) in PBS for 30 min on ice. After extensive washing in ice-cold PBS, cells were lysed in radiimmuno precipitation buffer containing protease inhibitors. Immunoprecipitations were performed in HKMEN either using the IgG1, anti-BAG-1 monoclonal RS608 (26) or a polyclonal rabbit AR antisem (clone AR PA1–110 ABR, Inc.) conjugated to protein G-agarose (Zymed, San Fran-
The AR-encoding plasmid pSG5-AR (0.4 μg) was co-transfected with a standard calcium phosphate precipitate method into PC3 prostate cancer cells, COS7 monkey kidney cells, and 293T human embryonic kidney cells with 0.5 μg of pLCI reporter plasmid, 0.2 μg of pCMV-β-gal and increasing amounts of BAG-1 expression plasmids as indicated. The total amount of plasmid DNA used was normalized to 2.5 μg/well by the addition of empty plasmid. Thirty-two hours after transfection, cell extracts were prepared and assayed for CAT and β-galactosidase activity at 48 h. Data were normalized using β-galactosidase, and results are expressed as -fold transactivation relative to DHT-stimulated cells transfected with AR expression vector in combination with pcDNA3 control plasmids. All transfection experiments were carried out in triplicate wells and repeated at least three times. The BAG-1 expression plasmid pcDNA3-BAG-1/BAG-1M produces approximately equivalent amounts of the BAG-1 and BAG-1M proteins. pcDNA3-BAG-1/BAG-1M produces an enhanced chemiluminescence detection method.

cisco, CA). Control immunoprecipitations were performed using IgG1 or rabbit preimmune serum. Immune complexes were analyzed by SDS-PAGE/immunoblot assay using anti-BAG-1 monoclonal antibody with the absence of DHT. However, cells transfected with the BAG-1L-producing plasmid displayed greater sensitivity to androgen compared with control transfected cells or cell over-expressing BAG-1/BAG-1M. The BAG-1L-mediated increases in AR-induced transactivation of the ARE-CAT reporter gene were detected at concentrations as low as 0.01 nM DHT and were substantially higher than control cells or BAG-1/BAG-1M-expressing cells over a broad range of hormone concentrations (0.01-10 nM). The effects of BAG-1L were dependent on AR, since co-transfections lacking the AR-encoding plasmid failed to result in ARE-CAT plasmid reporter gene transactivation above background levels (not shown).

To further examine the specificity of BAG-1L-mediated enhancement of AR transcriptional activity, the effects of BAG-1L on expression of other reporter genes were evaluated. The human BAG-1M (RAP46) protein had been shown to bind to AR in vitro (19). We therefore asked whether BAG-1 family proteins can influence the transcriptional activity of this steroid hormone receptor. For these experiments, three different cell lines were transiently co-transfected with plasmids encoding various BAG-1 isoforms and AR, together with a ARE-containing CAT reporter plasmid. The cells were then cultured in the presence or absence of DHT. In the presence of hormone, BAG-1 family proteins increased the transcriptional activity of AR in a concentration-dependent manner, with the plasmid producing the BAG-1L protein displaying far more effect than the plasmid encoding for both BAG-1 and BAG-1M (Fig. 1). The extent of BAG-1L-mediated up-regulation of AR-induced transactivation varied among cell lines, with COS7 and PC3 demonstrating as much as ~5-fold increases when transfected with BAG-1L but 293T cells exhibiting only a modest effect. Immunoblot analysis confirmed the production of the BAG-1, BAG-1M, BAG-1L, and AR proteins in the transfected cells and demonstrated production of similar amounts of BAG-1 and BAG-1M compared with BAG-1L (see below for examples). Thus, differences in the relative amounts of BAG-1, BAG-1M, and BAG-1L proteins produced could not account for the greater potency of BAG-1L.

The transcription-potentiating effect of BAG-1L was dependent on the addition of androgen to cultures. As shown in Fig. 2, AR-mediated transactivation of the ARE-CAT reporter plasmid remained at background levels when cells were co-transfected with plasmids encoding BAG-1 family proteins but cultured in the absence of DHT. However, cells transfected with the BAG-1L-producing plasmid displayed greater sensitivity to androgen compared with control transfected cells or cell over-expressing BAG-1/BAG-1M. The BAG-1L-mediated increases in AR-induced transactivation of the ARE-CAT reporter gene were detected at concentrations as low as 0.01 nM DHT and were substantially higher than control cells or BAG-1/BAG-1M-expressing cells over a broad range of hormone concentrations (0.01-10 nM). The effects of BAG-1L were dependent on AR, since co-transfections lacking the AR-encoding plasmid failed to result in ARE-CAT plasmid reporter gene transactivation above background levels (not shown).

To further examine the specificity of BAG-1L-mediated enhancement of AR transcriptional activity, the effects of BAG-1L on expression of other reporter genes were evaluated. The tumor suppressor p53 was chosen because, by analogy to steroid receptors, p53 is often associated with Hsp90 and Hsp70 in the cytoplasm and must translocate from cytosol to nucleus to exert its transcriptional regulatory action (38). Co-transfection of BAG-1Lexpressing encoding plasmid into PC3 cells with a p53-producing vector and a p53-RE-CAT reporter gene demonstrated that BAG-1L does not influence p53-mediated transactivation (Fig. 3). Similarly, BAG-1L had no effect on the constitutive expression of either a SV40 early region promoter-driven CAT or the CMV immediate early region lacZ reporter gene plasmid used for normalizing transfection efficiencies (Fig. 3 and data not shown). These viral promoter/effectors contain Sp1 binding sites, thus suggesting that BAG-1L does not nonspecifically modulate this family of transcription factors.

**BAG-1L Decreases the Response of AR to Anti-androgen CPA**—The observation that BAG-1L increased the sensitivity of AR to its ligand DHT (Fig. 2) prompted us to explore the effects of BAG-1L on the suppression of AR transactivity by the anti-androgen cyproterone acetate. For these experiments, AR and ARE-CAT were transfected into COS7 cells with either pcDNA3 control DNA or an equivalent amount of pcDNA3-BAG-1L. The cells were treated ~1.5 days later with 1 nM DHT alone or in combination with various concentrations of CPA.

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**RESULTS**

**BAG-1L Enhances AR-mediated Transactivation of an Androgen Response Element (ARE)-containing Reporter Gene**—The human BAG-1M (RAP46) protein had been shown to bind to AR in vitro (19). We therefore asked whether BAG-1 family proteins can influence the transcriptional activity of this steroid hormone receptor. For these experiments, three different cell lines were transiently co-transfected with plasmids encoding various BAG-1 isoforms and AR, together with a ARE-containing CAT reporter plasmid. The cells were then cultured in the presence or absence of DHT. In the presence of hormone, BAG-1 family proteins increased the transcriptional activity of AR in a concentration-dependent manner, with the plasmid producing the BAG-1L protein displaying far more effect than the plasmid encoding for both BAG-1 and BAG-1M (Fig. 1). The extent of BAG-1L-mediated up-regulation of AR-induced transactivation varied among cell lines, with COS7 and PC3 demonstrating as much as ~5-fold increases when transfected with BAG-1L but 293T cells exhibiting only a modest effect. Immunoblot analysis confirmed the production of the BAG-1, BAG-1M, BAG-1L, and AR proteins in the transfected cells and demonstrated production of similar amounts of BAG-1 and BAG-1M compared with BAG-1L (see below for examples). Thus, differences in the relative amounts of BAG-1, BAG-1M, and BAG-1L proteins produced could not account for the greater potency of BAG-1L.

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Relative CAT activity was then measured 12–14 h later. As shown in Fig. 4, CPA reduced in a concentration-dependent manner the DHT-induced transactivation of the ARE-CAT reporter gene plasmid in both control and BAG-1L-transfected COS7 cells. However, because AR-mediated reporter gene transactivation started at higher levels in BAG-1L transfec-
tants, approximately 2 log higher concentrations of CPA an-
drogens were generally required to reduce reporter gene activ-
ity to levels comparable with control-transfected cells (Fig. 4).

In Vivo Binding of BAG-1L to the AR—Although BAG-1M (RAP46) has been reported to bind AR in vitro, the interaction of these proteins has not been demonstrated previously in cells. Co-immunoprecipitation assays were therefore performed using lysates prepared from untransfected LN-CaP cells, which constitutively express high levels of the BAG-1, BAG-1M, BAG-
1L, and AR proteins (39). A polyclonal anti-AR antiserum or a preimmune control serum was employed for immunoprecipita-
tions, and the resulting immune complexes were subjected to
SDS-PAGE/immunoblot analysis using the anti-BAG-1 monoclo-
al antibody KS6C8. As a control, BAG-1 proteins were also
immunoprecipitated using the same anti-BAG-1 monoclonal
antibody. Alternatively, an IgG1 control antibody was em-
ployed to confirm specificity.

As shown in Fig. 5, the BAG-1L protein was readily detected in association with anti-AR immune complexes (lane 5). In contrast, the BAG-1 and BAG-1M proteins did not co-immunoprecipitate with AR but were found in anti-BAG-1 immune complexes, confirming their presence in LN-CaP cells under these conditions. The specificity of these results was confirmed by the absence of BAG-1 family and AR proteins in immune complexes prepared using IgG1 control monoclonal antibody or the preimmune control serum. Although BAG-1L could be detected in AR-containing immune complexes, the reciprocal experiment involving the use of anti-BAG-1 antibody in attempts to co-immunoprecipitate AR proved unsuccessful. Additional experiments suggested that this was due to antibody-induced disruption of BAG-1L interactions with AR (data not shown). Attempts to determine whether BAG-1L can associate with AR in the absence of steroid hormone have been hampered by the rapid turnover of unliganded AR, resulting in lower levels of AR and making quantitative comparisons difficult. However, thus far, we have detected association of BAG-1L with AR only when androgens have been present.

The C-terminal Hsc70-binding Domain of BAG-1L Is Required for Interactions with AR—Previously, we showed that the last 47 amino acids of the BAG-1 protein are required for binding to the ATPase domain of Hsc70 (20). We therefore compared a mutant of BAG-1L lacking this carboxyl-terminal domain, BAG-1L (ΔC), with the wild-type BAG-1L protein. Association with AR was examined after treatment with the reversible chemical cross-linker dimethyl-3,3′-dithiobispropionimadate in total cell lysates derived from transiently trans-
fected COS7 cells. As shown in Fig. 6A, anti-AR immunoprecipitations contained BAG-1L protein, as determined by immunoblot analysis using anti-BAG-1 antibody. In contrast, the BAG-1L (ΔC) protein was not detected in anti-AR immune complexes. The BAG-1L and BAG-1M isoforms of BAG-1 also did

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\begin{array}{|c|c|c|}
\hline
\text{RE} & \text{TA} & \text{BAG-1L} \\
\hline
\text{SV40} & - & - \\
\text{SV40} & - & + \\
p53RE & - & - \\
p53RE & p53 & - \\
p53RE & p53 & + \\
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The BAG-1L (ΔC) mutant protein fails to interact with AR and functions as a trans-dominant inhibitor of the wild-type BAG-1L protein. A, COS7 cells were transiently transfected with equivalent amounts of AR and one of the following BAG-1 expression plasmids: pcDNA3-BAG-1L, pcDNA3-BAG-1L (ΔC), or pcDNA3-BAG-1/BAG-1M (produces approximately equivalent amounts of BAG-1 and BAG-1M proteins). Two days later, cells were treated with the reversible chemical cross-linker dimethyl-3,3′-dithiobispropionimidate (1 mM in PBS) for 30 min, washed several times in ice-cold PBS and lysed in radioimmune precipitation buffer. Immunoprecipitations were performed as described in Fig. 5 using the anti-AR polyclonal antisera or a preimmune control serum, followed by immunoblotting using the anti-BAG-1 monoclonal KS-6C8 and an ECL-based detection method. The positions of the BAG-1L, BAG-1M, and BAG-1 protein are indicated. In B and C, COST or ALVA31 cells were transiently co-transfected as described with 0.4 or 0.2 μg of pSG5-AR, 0.5 or 0.3 μg of the murine mammary tumor virus CAT reporter plasmid pLCI, and 0.2 or 0.1 μg of pcDNA3-BAG-1L (ΔC) (dark circles) or an equivalent amount of pcDNA3 control plasmid (total DNA normalized by the addition of pcDNA3 control plasmid). D, COST cells were transiently transfected as in B, except 0.3 μg of pcDNA3-BAG-1L was included in all transfections, and pcDNA3-BAG-1L (ΔC) was compared with pcDNA3-BAG-1/BAG-1M, which produces approximately equivalent amounts of the wild-type BAG-1 and BAG-1M proteins (solid triangles). The total amount of plasmid DNA used was normalized to 2.5 μg/well by the addition of pcDNA3 control plasmid. Approximately 1.5 days after transfection, cells were stimulated with 0, 0.1, or 0.3 mM DHT for reporter gene assays. The cells were then stimulated with 0, 0.1, or 10 nM DHT. Nuclear (A) and nonnuclear (B) fractions were prepared 2 days after transfection. Nuclear and cytoplasmic extracts (35 μg of total protein) were subjected to SDS-PAGE/immunoblot assay and probed with antibodies to BAG-1 and AR.

Effect of BAG-1 on the cytoplasmic/nuclear ratio of the AR. COS7 cells were transiently co-transfected with 3 μg of pSG5-AR and 5 μg of either pcDNA3 (Neo), pcDNA3-BAG-1/BAG-1M, or pcDNA3-BAG-1L expression plasmids by a standard calcium phosphate method. Thirty-two hours after transfection, cells were stimulated with 0, 0.1, or 10 nM DHT. Nuclear (A) and nonnuclear (B) fractions were prepared 2 days after transfection. Nuclear and cytoplasmic extracts (35 μg of total protein) were subjected to SDS-PAGE/immunoblot assay and probed with antibodies to BAG-1 and AR.
BAG-1, BAG-1M, and BAG-1L proteins present within the nuclear and nonnuclear compartments.

**DISCUSSION**

The data presented here provide the first evidence that a recently identified longer isoform of the human BAG-1 protein (BAG-1L) can modulate the function of a steroid hormone receptor. In particular, we found that the BAG-1L protein can be co-immunoprecipitated with AR and significantly enhances AR-induced transactivation of a reporter gene, whereas the shorter BAG-1 and BAG-1M isoforms of the protein did not. Thus, despite evidence that the human BAG-1M (RAP46) protein can bind to AR and several other steroid hormone receptors in vitro (19), only the long form appears to physiologically interact with AR in cells and to regulate its function.

One factor that could contribute to the preferential binding of BAG-1L to AR in cells is that this longer isoform of BAG-1 contains a nuceloplasmic-like nuclear localization sequence within the NH2-unique domain, which is missing from the shorter BAG-1 and BAG-1M (RAP46) protein.2 In previous studies, we observed that BAG-1L targets preferentially but not exclusively to nucleus when transfected in COS7 and other cell lines, whereas BAG-1 and BAG-1M had a greater tendency to reside in the cytosol.2 This is also true for the LN-CaP cells used in this study for co-immunoprecipitations, which contain all three isoforms of BAG-1, i.e. BAG-1, BAG-1M, and BAG-1L (data not shown). Since in the presence of androgen the AR resides almost exclusively in the nucleus (40), it is conceivable that under these conditions AR interacts with nuclear and not cytoplasmic proteins. Thus, the higher nuclear levels of BAG-1L compared with BAG-1 and BAG-1M may be largely responsible for its physical and functional interactions with AR protein complexes in cells.

Alternatively, another explanation for the observation that BAG-1L but not BAG-1 or BAG-1M (RAP46) was detected in association with AR in cells could be that the unique N-terminal region of the BAG-1L protein is required for binding to AR under physiological conditions. In this regard, it should be noted that the interaction of the BAG-1M (RAP46) protein with AR and other steroid hormone receptors has only been demonstrated in vitro and only then after treatment of steroid hormone receptor complexes with high salt at elevated temperature or with urea-containing solutions, conditions that could cause protein unfolding. In contrast, conformations of BAG-1 that are competent to bind AR complexes in vivo may only be achieved when the N-terminal unique region of BAG-1L is present. The N-terminal unique domain within BAG-1L could also directly bind to AR. However, clearly the N-terminal domain of BAG-1L is insufficient for AR binding, since deletion of the C-terminal last 47 amino acids abolished interactions BAG-1L with AR in cells. The failure of the BAG-1L (ΔC) protein to form complexes with AR was not due to altered subcellular localization of this protein compared with wild-type BAG-1L (data not presented).

The mechanism by which BAG-1L enhances the function of the AR remains to be determined. Clearly, the ability of BAG-1 proteins to bind to and modulate the function of Hsp70/Hsc70 family molecular chaperones by increasing ADP/ATP exchange and facilitating peptide release may provide some clues. As shown here, a carboxyl deletion mutant of BAG-1L lacking the last 47 amino acids, which are required for Hsc70 binding (20), was unable to form complexes with AR. This observation therefore suggests that Hsc70 bridges BAG-1L to AR, as has been proposed for its interactions with many other proteins (21). It is known that at least three members of the Hsp family, namely Hsp90, Hsp70, and Hsp56, are associated with the inactive forms of several steroid hormone receptors in the cytoplasm and may be important for maintaining the stability of these proteins in the absence of ligand and inducing conformations that are competent to bind steroid hormone ligands. Hsp70 has also been detected in the nucleus in association with receptor-DNA complexes where it putatively increases DNA binding affinity (23, 25). Thus, BAG-1L may alter AR interactions with molecular chaperones in ways that modulate the conformation of this steroid hormone receptor and enhance its responses to steroid ligands, e.g. by stabilizing hormone binding, increasing the affinity of AR interactions with DNA target sequences, or facilitating association with coactivator proteins (39). BAG-1L, however, did not appear to detectably increase the proportion of AR that translocates into the nucleus after the addition of DHT or to cause elevations in AR protein levels as might occur if BAG-1L stimulated nuclear translocation or prolonged the half-life of this protein. Also, expression of BAG-1L did not appear to increase the amount of Hsc70 or Hsp70 that would be co-immunoprecipitated with AR (not shown). These mechanisms, therefore, seem not to be involved in the potentiation of AR function by BAG-1L.

Alternatively, BAG-1L could conceivably bind directly to AR and exert its potentiating effect on AR independently of Hsp70. An Hsp70-independent mechanism of action is suggested by at least two observations. First, androgen has been reported to induce dissociation of not only Hsp90, but also Hsc70 from AR in concert with translocation of hormone-bound receptor into the nucleus (39). Since BAG-1L forms complexes with AR in the presence of hormone, this observation implies that BAG-1L may be able to interact with and modulate AR function within the nucleus after dissociation of Hsc70. One notable caveat, however, is that Hsp70 reportedly remains associated with estrogen and progesterone receptors while bound to their target DNA elements in the nucleus (16, 23, 25). Thus, unlike Hsp90, the Hsc70 family molecular chaperones may not always dissociate from nuclear hormone receptors upon binding ligand. Indeed, we have been able to co-immunoprecipitate at least small amounts of Hsc70/Hsp70 with AR in cells cultured with androgens.3 Second, a C-terminal deletion mutant of BAG-1L that fails to bind Hsc70 functioned as a trans-dominant inhibitor of BAG-1L and reduced AR-mediated transactivation, thus suggesting the possibility of a Hsc70-independent mechanism. Although the reason why the BAG-1L (ΔC) mutant protein interferes with AR function requires further exploration, at least two possibilities can be considered. First, the BAG-1L (ΔC) protein may form dysfunctional complexes with endogenous wild-type BAG-1L, abrogating its effects on AR. However, biophysical characterization of the BAG-1 protein strongly suggests it is a monomer, unlike the functionally similar GrpE protein of prokaryotes, which is known to be a dimer (41). Thus, trans-dominant inhibition of endogenous BAG-1L may not explain why the BAG-1L (ΔC) protein inhibits AR function. Second, if BAG-1L normally bridges AR to other proteins such as transcriptional co-activators (reviewed in Ref. 28) and if the N-terminal unique domain of BAG-1L is necessary for this function, then the BAG-1L (ΔC) protein could theoretically sequester a co-factor essential for AR-mediated transcriptional activation. In this case, the BAG-1L (ΔC) protein, which does not bind to AR, would presumably prevent this hypothetical co-factor from binding to endogenous wild-type BAG-1L:AR complexes.

The role of BAG-1L in the fetal development of male reproductive organs and in the pathogenesis of prostate cancer remains to be established. In contrast to most androgen-unresponsive tissues, testes and the normal prostate gland, as well

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3 B. A. Froesch, S. Takayama, and J. C. Reed, unpublished data.
as 9 of 9 prostate cancer lines thus far tested, have been shown to express BAG-1L, in addition to the shorter ubiquitously expressed BAG-1 protein (20). The observation that BAG-1L significantly reduced the net suppressive effects of an anti-androgen on AR-mediated transactivation raises the possibility that overexpression of BAG-1L could provide a selective growth advantage for some prostate cancers during hormone ablation therapy. Although much remains to be learned about the specific mechanisms involved, the observations that (i) BAG-1L markedly enhances androgen-dependent transactivation by AR, (ii) BAG-1L reduces the efficacy of anti-androgens with respect to their suppression of AR-reporter gene transactivation, and (iii) BAG-1L (∆C) antagonizes AR-mediated transactivation all suggest that further studies of BAG-1 and BAG-1L expression and function in normal and malignant prostate and other androgen-responsive tissues are warranted.

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