Bag1 is a regulator of heat shock protein 70 kDa (Hsp70/Hsc70) family proteins that interacts with steroid hormone receptors. Four isoforms of Bag1 have been recognized: Bag1, Bag1S, Bag1M (RAP46/HAP46), and Bag1L. Although Bag1L, Bag1M, and Bag1 can bind the androgen receptor (AR) in vitro, only Bag1L enhanced AR transcriptional activity. Bag1L was determined to be a nuclear protein by immunofluorescence microscopy, whereas Bag1, Bag1S, and Bag1M were predominantly cytoplasmic. Forced nuclear targeting of Bag1M, but not Bag1 or Bag1S, resulted in potent AR coactivation, indicating that Bag1M possesses the necessary structural features provided it is expressed within the nucleus. The ability of Bag1L to enhance AR activity was reduced with the removal of an NH2-terminal domain of Bag1L, which was found to be required for efficient nuclear localization and/or retention. In contrast, deletion of a conserved ubiquitin-like domain from Bag1L did not interfere with its nuclear targeting or AR regulatory activity. Thus, both the unique NH2-terminal domain and the COOH-terminal Hsc70-binding domain of Bag1L are simultaneously required for its function as an AR regulator, whereas the conserved ubiquitin-like domain is expendable.

Steroid receptors play a crucial role in the development and maintenance of many organs. The androgen receptor (AR) is a ligand-activated transcription factor that is a member of the nuclear receptor superfamily (1). One tissue that exhibits profound dependence on the ligand for the nuclear hormone receptor is the prostate gland. In normal prostate, androgens play a role in hormone-independent prostate cancers. Many hormone-insensitive tumors have been found to retain a wild-type AR gene. Moreover, the AR gene is sometimes amplified, or its transcriptional activity may be increased in advanced prostate cancer (6). Therefore, a need exists to understand more about the factors that control the functions of the AR so that the mechanisms responsible for resistance to endocrine therapy can be revealed and eventually alleviated.

In the absence of the ligand, AR and most steroid hormone receptors are maintained in an inactive state complexed with heat shock proteins. Upon binding of the cognate ligand, the receptor dissociates from the inactive complex and translocates to the nucleus in which it binds specific response elements in the promoter and/or enhancer regions of responsive genes (reviewed in Ref. 7). Once bound to the nuclear response element, the nuclear receptor up-regulates or down-regulates transcription by transmitting signals directly to the transcriptional machinery via direct protein-protein interactions. In addition, another class of proteins called coactivators is recruited and serves as bridging molecules between the transcription initiation complex and the nuclear receptor (reviewed in Ref. 8).

Recently, an isoform of the human Bag1 protein (known as Bag1L) has been reported to bind the AR and enhance transcriptional activity in the presence of the ligand (9). Bag1 contains a COOH-terminal “BAG” domain that binds the ATPase domain of heat shock protein 70 kDa (Hsp70/Hsc70) family proteins (10–14) and modulates the activity of Hsc70/Hsp70 family chaperones in vitro and in vivo. Through this interaction with Hsc70, Bag1 is able to interact with a variety of intracellular proteins and regulate diverse cellular processes relevant to cancer including cell division, cell survival, and cell migration (15–20). However, it is also possible that other non-Hsc70-binding domains in the NH2-terminal portion of Bag1 mediate interactions with target proteins, thus providing a mechanism for directing Hsc70 family chaperones to specific proteins in the cells. For example, Bag1 contains a ubiquitin-like (UBL) domain, which has been proposed to permit its direct binding to the 26 S proteosome (21). However, the significance of this and other NH2-terminal regions in Bag1 for
transactivation of AR or other steroid hormone receptors is unknown.

It has been shown that at least four isoforms of Bag1 protein can arise from alternative initiation of translation within a common mRNA: Bag1S, Bag1L, Bag1M (RAP46/HAP46), and Bag1L (22, 23). These isoforms all contain the Hac70-binding BAG domain near the COOH terminus as well as the upstream UBL domain, but they differ in the lengths of their amino-terminal regions. Additional motifs have been recognized within the NH2-terminal segment of the Bag1 proteins including candidate nuclear localization sequences (NLS) and variable numbers of TXSEEX repeat sequences (23, 24). Bag1L, the longest isoform, contains both an SV40-Large-T-like and nucleoplasmin-like candidate NLS preceded by a unique −50 amino acid-domain. This Bag1 isoform is predominantly nuclear (23). Bag1M (RAP46/HAP46) contains only a portion of the candidate NLS and has been shown to reside in the cytosol unless stimulated to traffic into the nucleus by associating with other proteins, such as the glucocorticoid receptor (24). Bag1 and the shorter and rarer isoform of Bag1S are predominantly found in the cytosol (23).

Bag1 proteins have been reported to interact with and regulate the activity of several members of the nuclear receptor superfamily. For example, Bag1M and Bag1L have been found to repress the activity of the glucocorticoid receptor (24, 25), and Bag1 represses the transcriptional activity of retinoic acid receptors (27). Conversely, Bag1L but not Bag1M or Bag1 can potentiate the transcriptional activity of the AR (9). In this report, we have extended structure-function analysis of Bag1 protein with respect to their regulation of the AR.

MATERIALS AND METHODS

Plasmids—The plasmids pcDNA3-Bag1L, pcDNA3-Bag1L_C, and pcDNA-Bag1 have been described previously (9), pcDNA3-Bag1M was generated from pcDNA3-Bag1L/Bag1M (9) by mutating the initiation codon that gave rise to Bag1 from an ATG to an ATC so that this construct now can give rise only to Bag1M.

The eDNAs encoding various fragments of Bag1 were generated by polymerase chain reaction from the plasmid pcDNA3-Bag1L (9) using the following forward (F) and reverse (R) primers containing EcoRI and XhoI sites: Bag1L 5′-GGAATTCGAGCGGATGGGTTCCCG-3′ (F1) and 5′-CCCTCGAGTCATCGCGAGGCGGAAAG-3′ (R1); Bag1LΔ1-50 primer 5′-GGAATTCGAGCGGATGGGTTCCCG-3′ (F2) and R1; Bag1LΔ1-16 5′-GGAATTCGAGCGGATGGGTTCCCG-3′ (F3) and R1; Bag1MΔ385 5′-GGAATTCGAGCGGATGGGTTCCCG-3′ (F4) and 5′-CCCTCGAGTCATCGCGAGGCGGAAAG-3′ (R2); and Bag1C83 5′-GGAATTCGAGCGGATGGGTTCCCG-3′ (F5) and 5′-CCCTCGAGTCATCGCGAGGCGGAAAG-3′ (R1). The polymerase chain reaction products were digested with EcoRI and XhoI and then directly cloned into the EcoRI and XhoI sites of the mammalian expression vector pcDNA3 (Bag1M and Bag1Δ1-50) or pcDNA3-Myc (Bag1LΔ1-16, Bag1MΔ385, and Bag1C83).

The GST-Bag1 fusion proteins were generated from pGEX-4T-Bag1L, pGEX-4T-Bag1L_C, pGEX-4T-Bag1LΔ1-50, pGEX-4T-Bag1M, pGEX-4T-Bag1M_C, and pGEX-4T-1-Bag1C83. These plasmids were all generated by subcloning the appropriate cDNAs from pcDNA3 clone into the EcoRI and XhoI sites of pGEX-4T-1 (Amersham Pharmacia Biotech). To generate the nuclear-targeted Bag1 proteins, the appropriate cDNA was subcloned from pcDNA3 clone into the EcoRI and XhoI sites of pcDNA3-NLS (generated by the insertion of an oligo-nucleotide containing the SV40-Large-T-like NLS into the HindIII-EcoRI sites of pcDNA3).1

The reporter plC1 plasmid contains the full-length mouse mammary tumor virus long terminal repeat sequence linked with the chloramphenicol acetyltransferase (CAT) gene (28, 29). The pSG5-AR plasmid contains the cDNA for the wild-type AR (28).

Cell Culture—The monkey kidney COS-7 cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in a humidified atmosphere with 5% CO2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 3 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.). One day prior to experiments, cells were transferred into charcoal-treated fetal bovine serum (CT-FBS) and Dulbecco's modified Eagle's medium minus Phenol Red to reduce background levels of steroids. R1881 (PerkinElmer Life Sciences) was dissolved in ethanol and added to the cultures at a minimum dilution of 0.0001% (v/v). Control cells received an equivalent amount of solvent only.

Transfections and Enzyme Assays—COS-7 cells at 60% confluency in 12-well plates (Nunc) were transfected by a lipofection method. 1.1 μg of DNA was diluted into 176.5 μl of Opti-MEM (Life Technologies, Inc.) and combined with 3 μl of LipofectAMINE (Life Technologies, Inc.) in 185.5 μl of Opti-MEM. After incubation for 20 min, 0.375 ml of Opti-MEM was added, and the mixtures were overlaid onto monolayers of cells. After 5 h with 5% CO2, for 5 h at 37 °C, 0.75 ml of Opti-MEM containing 20% CT-FBS was added to the cultures. At 32–38 h after transfection, cells were stimulated with 0.1 nM R1881. Cell extracts were prepared 48 h after transfection. For reporter gene assays, cell lysates were made as described previously (30), and assays for β-galactosidase and CAT activity were performed. All transfection experiments were carried out in duplicate, repeated at least three times, and normalized for β-galactosidase activity.

In Vitro Binding Assays—The AR was in vitro translated in reticulocyte lysates (TNT lysates, Promega) containing [35S]methionine and then preincubated with 10 nM R1881 for 30 min. Glutathione S-transferase (GST) fusion proteins were immobilized on glutathione-Sepharose and blocked in NET-N buffer (20 mM Tris, pH 8.0, 20 mM NaCl, 1 mM EDTA) containing 0.1% Nonidet P-40 and 15% milk for 30 min. The GST proteins were incubated for 2 h at 4 °C with 10 nM of R1881 treated in vitro translated AR in NET-N buffer containing 0.1% Nonidet P-40, proteinase inhibitors, and 10 nM R1881. The beads were washed three times in NET-N buffer containing 0.5% Nonidet P-40 and then boiled in Laemmli SDS sample buffer. The use of equivalent amounts of intact GST fusion proteins and successful in vitro translated of the AR was confirmed by SDS-PAGE analysis using Coomasie Blue staining or autoradiography, respectively.

Immunofluorescence—Transfected COS-7 cells were fixed with a −20 °C chilled mix of methanol and acetone (1:1) for 2 min at 20 °C. After fixation the cells were blocked with phosphate-buffered saline containing 3% bovine serum albumin, 2% FBS, and 0.1% goat serum and then incubated for 4 h at 20 °C with anti-Bag1 antibody (Dako Corp., Carpinteria, CA) diluted 1:50 in blocking solution (31). After this incubation, cells were rinsed three times for 10 min with phosphate-buffered saline at 20 °C and then incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG (Dako Corp.), diluted 1:50 in blocking solution for 2 h at 37 °C. Excess secondary antibody was thoroughly washed off with phosphate-buffered saline. The slides were then treated with Mowiol containing 1,4-diazabicyclo[2.2.2]octane, and glass coverslips were applied. The stained slides were observed using a laser-scanning confocal microscope (Bio-Rad 1024MP).

Hormone Binding Assays—COS-7 cells at 60% confluency in 10-cm2 plates were transiently transfected either with empty vectors or a combination of expression vectors encoding for AR, Bag1L, or Bag1 with a lipofection procedure. 10 μg of DNA was incubated with 26 μl of LipofectAMINE (Life Technologies, Inc.) for 20 min, and the mixtures were overlaid onto monolayers of cells. After culturing with 5% CO2 at 37 °C for 5 h, 1 volume of Opti-MEM containing 20% charcoal-stripped fetal bovine serum (CT-FBS) was added to the cells. Cultures were transferred into 24-well plates 1 day later and grown in CT-FBS medium for an additional 24 h. To determine the hormone binding affinities of the transfected AR, cells were incubated for 2 h with increasing concentrations (0.1–10 nM) of [3H]R1881 (88 ±Ci/mmol, PerkinElmer Life Sciences) in the presence or absence of a 100-fold molar excess of cold R1881. Cells were then washed three times with ice cold phosphate-buffered saline, and radioactivity was determined by scintillation counting. Specific binding was calculated by subtracting the counts/min of samples transfected with the AR expression plasmid that was treated with the same concentration of the hormone. The results represent means of three independent experiments.

RESULTS

BAG Domain of Bag1 Is Sufficient to Bind AR in Vitro—The human Bag1 protein exists as four isoforms (Fig. 1A), which all contain the same COOH-terminal Hsc70-binding domain and an upstream ubiquitin-like domain but differ in the lengths of their NH2-terminal regions (23). The Bag1L and Bag1M (RAP46) isoforms of the human Bag1 protein have been previously shown to bind the AR in vitro (9, 25). To determine
whether other isoforms of the human Bag1 protein could interact with the AR, we performed in vitro protein interaction assays. The Bag1 isoforms were fused to GST and incubated with in vitro translated radiolabeled AR. As shown in Fig. 1B, the AR specifically interacted with Bag1L and Bag1 but not with the control proteins GST and GST-CD40. Mutants of Bag1L and Bag1, which lack the COOH-terminal Hsc70-binding domain (ΔC), were unable to interact with the AR in these assays, indicating that the BAG domain is required for interactions with the AR. Moreover, a GST-fusion protein containing only the last COOH-terminal 83 amino acids, GST-Bag1C83, was sufficient for binding to AR under these conditions (Fig. 1C). Therefore, we conclude that the BAG domain of Bag1 protein is necessary and sufficient for associating with the AR.

Nuclear Targeting of the Cytoplasmic Bag1 Is Insufficient for Potentiating AR Activity—Although all isoforms of Bag1 interact with the AR in vitro, only the Bag1L protein significantly enhances the transcriptional activity of the AR in vivo (9). Because the ligand-bound AR is localized to the nucleus (7), only isoforms of the Bag1 protein that are nuclear would be expected to enhance the transcriptional activity of the AR. Therefore, we checked the compartmentalization of the Bag1 proteins by immunofluorescence. COS-7 cells were transfected with plasmids encoding the various Bag1 isoforms followed by immunostaining with an anti-Bag1 monoclonal antibody and analysis by confocal laser-scanning microscopy. Bag1L is exclusively a nuclear protein, whereas all other Bag1 isoforms are predominantly cytosolic (Fig. 2, left panels). These findings are consistent with the presence of both nucleoplasmic-like and SV40-LargeT-like nuclear-targeting sequence in the Bag1L protein (22, 23) but not Bag1M, Bag1, or Bag1S. Although the Bag1M (RAP46/HAP46) isoform contains a region of basic residues suggestive of a nuclear targeting sequence (25), it is evidently transported inefficiently into the nucleus. Interestingly, the Bag1L protein may be associated with nuclear sub-structures given the speckled pattern of the immunofluorescence observed (Fig. 2).

The difference in cellular distribution of the Bag1 proteins represents a possible explanation for the inability of the cytosolic Bag1 isoforms to enhance the transcriptional activity of the AR. To address this issue, we constructed plasmids that express Bag1L, Bag1M, Bag1, or Bag1S fused to SV40-LargeT-like nuclear-targeting sequences. Nuclear localization of these
NLS-Bag1 proteins was verified by transfecting COS-7 cells with plasmids expressing the nuclear-targeting Bag1 isoforms and then immunostaining with an antibody that recognizes Bag1 (Fig. 2, right panels). Confocal laser-scanning microscopy analysis revealed that all the Bag1 isoforms are located exclusively in the nucleus of transfected cells.

The effects of these nuclear-targeted Bag1 isoforms on AR transactivation activity were then tested by transfection into COS-7 cells together with an AR-expressing plasmid and an ARE-containing reporter gene. The cells were stimulated with R1881 to activate AR. As shown in Fig. 3A, the fusion of heterologous nuclear-targeting sequences to the 5' end of Bag1L did not alter its ability to enhance the transactivation activity of the AR. Targeting of Bag1M to the nucleus but not Bag1 or Bag1S was sufficient to enhance the transactivation activity of the AR. Taken together, these results suggest that the first 70 amino acids of Bag1L are expendable for AR coactivation, whereas the NH2-terminal region of Bag1M corresponding to amino acids 71-115 of Bag1L is required in conjunction with nuclear localization for enhancing AR function.

Immunoblot analysis showed that the levels of all Bag1 isoforms produced in cells (except Bag1S) were comparable to the nuclear-targeted isoforms, excluding quantitative differences in the levels of these proteins as a trivial explanation for the results (Fig. 3B). The difference in the level of expression of Bag1S and NLS-Bag1S makes it difficult to conclude that NLS-Bag1S is without effect on AR-mediated transactivation. The absence of Bag1S from the nucleus would argue against its role.

The NH2-terminal Region of Bag1L Is Required for Efficient Nuclear Localization—The observation that Bag1L is the only isoform that is nuclear suggests that the unique NH2-terminal domain of Bag1L might perform a role in nuclear targeting or retention. We therefore generated two NH2-terminal truncation mutants of Bag1L lacking either the first 16 amino acids of Bag1L (Bag1LΔ1–16) or retaining the nuclear-targeting sequences of Bag1L but lacking the NH2-terminal 50 amino acids that differentiate it from the Bag1M protein (Bag1MΔ1–50).

Both the Bag1LΔ1–16 and Bag1MΔ1–50 proteins retain the candidate nuclear-targeting sequences of Bag1L and therefore should target to nuclei similar to Bag1L. To explore this option, COS-7 cells were transfected with plasmids encoding Bag1LΔ1–16, Bag1MΔ1–50, or Bag1L, and the localization of the resulting proteins was determined by immunofluorescence confocal microscopy. Bag1L and Bag1LΔ1–16 exhibited essentially the same compartmentalization pattern within the cell (Fig. 4B, upper panels), demonstrating a nuclear speckled pattern of immunostaining. In contrast, the Bag1MΔ1–50 protein was more promiscuous in its subcellular localization. Whereas Bag1LΔ1–50 was found in the same nuclear substructures as Bag1L, the protein was also found in a diffuse cytosolic staining pattern in transfected cells. This finding suggests that amino acids 17–50 of Bag1M may contain sequences necessary for optimal retention of Bag1L in the nucleus.

To contrast these subcellular localization results with coac- tivation function, COS-7 cells were cotransfected with the AR, an ARE-containing reporter gene, and plasmids encoding Bag1L, Bag1LΔ1–16, or Bag1LΔ1–50. The transfected cells were then stimulated with the synthetic androgen R1881. Bag1LΔ1–50 but not Bag1LΔ1–16 exhibited a decreased ability to enhance the transcriptional activity of the AR (Fig. 4D). Taken together, these observations suggest that the correct nuclear targeting/retention of Bag1L is required for optimal functional interactions of Bag1L and the AR.

To confirm this finding, we fused a nuclear-targeting sequence to Bag1L, Bag1LΔ1–16, and Bag1LΔ1–50 and transiently transfected the NLS-fusion constructs into COS-7 cells. Confocal immunofluorescence analysis revealed that NLS-Bag1L, NLS-Bag1LΔ1–16, and NLS-Bag1LΔ1–50 exhibit essentially the same nuclear pattern within the cell (Fig. 4B, lower panels). Targeting of Bag1L and Bag1LΔ1–16 to the nucleus did not significantly alter their effect on the AR, however, nuclear-targeting of Bag1LΔ1–50 markedly improved its ability to enhance AR transcriptional activity (Fig. 4D). Immu-
Bag1L, which are lacking the NH2-presented showing deletion mutants of Bag1L, which are lacking the NH2-terminal 16 (Bag1LΔ1−16) or 50 amino acids (Bag1LΔ1−50). B, COS-7 cells were translated with the following Bag1 expression plasmids: pcDNA3-Bag1L, pcDNA3-Bag1LΔ1−16, pcDNA3-Bag1LΔ1−50, pcDNA3-NLS-Bag1L, pcDNA3-NLS-Bag1LΔ1−16, or pcDNA3-NLS-Bag1LΔ1−50. At 30 h post-transfection, cells were fixed, stained, and examined by laser confocal microscopy. C, COS-7 cells were transfected as in A, and after 30 h the cells were lysed in radioimmune precipitation buffer. Cell extracts (25 µg of total protein) were subjected to SDS-PAGE/immunoblot assay and probed with an antibody to Bag1. D, COS-7 cells were transfected with 0.06 µg of pSG5-AR, 0.5 µg of pLCI, 0.04 µg of pCMV-β-galactosidase, and various amounts of the Bag1 expression plasmids used in B. Total DNA was maintained at 1.1 µg by the addition of pcDNA3 control plasmid. At 30 h after transfection, cells were stimulated with 1 nM R1881. Cell extracts were prepared and assayed for CAT and β-galactosidase activity at 40 h after transfection (mean ± S.E., n = 2).

Immunoblot analysis revealed that all Bag1 isoforms were expressed at similar levels (Fig. 4C).

The Ubiquitin-like Domain of Bag1L Is Not Required for Enhancing Transcriptional Activity of AR—The Bag1 protein contains a UBL domain that is conserved within the Bag1 homologues of Caenorhabditis elegans and Schizosaccharomyces pombe (32). To explore whether this region has functional significance in the enhancement of AR-mediated transcription, we constructed a deletion mutant of Bag1L lacking this region (Bag1LΔUBL) (Fig. 5A). COS-7 cells were cotransfected with plasmids expressing Bag1LΔUBL, AR, and an ARE-containing reporter gene plasmid and then stimulated with R1881. As illustrated in Fig. 5B, deletion of the UBL domain from Bag1L did not alter its ability to enhance AR-mediated transcription in reporter gene assays, suggesting that this region of Bag1L is not required for functional interactions of Bag1L and the AR. Immunoblot analysis showed that the levels of Bag1LΔUBL produced in cells was comparable to Bag1L (Fig. 5C). COS-7 cells were transfected with plasmids encoding Bag1LΔUBL or Bag1L, and the localization of the resulting proteins was determined by immunofluorescence confocal microscopy. Bag1L and Bag1LΔUBL exhibited essentially the same compartmentalization pattern within the cell (Fig. 5D), demonstrating a nuclear speckled pattern of immunostaining.

Bag1 Proteins Do Not Alter the Affinity of the AR for Its Ligand—Heat shock proteins and other molecular chaperones are required for placing steroid hormone receptors into a state that is competent to bind steroid ligands (33, 34). The ability of Bag1 proteins to bind and modulate the function of Hsp70/Hsc70 family molecular chaperones (10), therefore, could conceivably alter the ability of AR to bind androgenic hormones. Therefore, we determined the hormone binding affinity of the AR in whole cells in the absence or presence of Bag1 or Bag1L. As depicted in Fig. 6A, the presence of elevated levels of either Bag1 or Bag1L did not significantly influence the amount of hormone bound to the receptors at any of the hormone concentrations tested. In addition, as revealed by Scatchard analysis (Fig. 6, B–D), neither Bag1 nor Bag1L significantly altered the apparent equilibrium binding constant (Kd) for R1881. Therefore, Bag1L exerts its influence on AR-mediated transcription at a stage other than ligand binding.

DISCUSSION

The data presented here confirm that Bag1L is the only isoform of Bag1 capable of enhancing the transcriptional activity of the AR (9). Thus, despite evidence that Bag1M and Bag1 can interact with the AR in vitro, only Bag1L interacts with AR in cells (9) and alters its transactivation function. Because Bag1L is the only isoform that is constitutively present in the nucleus, one possible explanation is that the higher nuclear levels of Bag1L may be responsible for its physical and functional interactions with AR complexes. Previous studies have shown that Bag1L does not alter the ligand-dependent nuclear translocation of the AR (9).

The appendage of an exogenous nuclear-targeting sequence to Bag1M, Bag1, and Bag1S is sufficient to force these proteins into the nucleus. This forced nuclear targeting bestowed upon Bag1M but not Bag1 or Bag1S the ability to coactivate the AR. Therefore, it appears that additional structural differences in Bag1L and Bag1M compared with Bag1 and Bag1S play an important role in the differential effects of these proteins on the AR. Recent papers have suggested that the TSXEEX repeat found in 8, 8, and 2 copies, respectively, in Bag1L, Bag1M, and Bag1 is required for repression of the transcriptional activity of the glucocorticoid receptor (24). Thus, these TSXEEX motifs may be important for functional collaboration of Bag1 with AR as both Bag1L and Bag1M is effective at potentiating AR function when expressed within the nucleus, whereas Bag1 is not.

By deleting the NH2-terminal region of Bag1L, we demonstrated a function for this unique proline-rich domain for the first time. Subcellular localization experiments indicated that although Bag1L and Bag1LΔ1−16 were localized to nuclear substructures, Bag1LΔ1−50 protein was present diffusely throughout the cells. Therefore, the region between amino acids 17 and 50 of Bag1L was found to be required for its physical and functional collaboration of Bag1 with AR as both Bag1L and Bag1M is sufficient to force these proteins into the nucleus. This forced nuclear targeting bestowed upon Bag1M but not Bag1 or Bag1S the ability to coactivate the AR. Therefore, it appears that additional structural differences in Bag1L and Bag1M compared with Bag1 and Bag1S play an important role in the differential effects of these proteins on the AR. Recent papers have suggested that the TSXEEX repeat found in 8, 8, and 2 copies, respectively, in Bag1L, Bag1M, and Bag1 is required for repression of the transcriptional activity of the glucocorticoid receptor (24). Thus, these TSXEEX motifs may be important for functional collaboration of Bag1 with AR as both Bag1L and Bag1M is effective at potentiating AR function when expressed within the nucleus, whereas Bag1 is not.
in interactions with the nuclear proteins that serve to anchor Bag1L firmly in the nucleus.

In contrast, deletion of the UBL domain from Bag1L did not impair subcellular targeting or functional collaboration with the AR. A wide variety of proteins have been shown to contain UBLs (reviewed in Ref. 35). These domains can mediate direct interactions with subunits of the proteosome that recognize polyubiquitin chains on proteins that have been targeted for destruction. Recently, Bag1 was reported to bind the 26 S proteosome in vitro. The UBL domain is found within all four Bag1 isoforms and is conserved in the Bag1 homologues of other species including the yeast S. pombe and the nematode C. elegans (32), implying an evolutionarily conserved role for this domain in some aspect of Bag1 function. However, deletion of the UBL domain did not abrogate the stimulatory effect of Bag1L on AR function. Thus, whatever the function of the conserved UBL domain of Bag1 may be, it is expendable for coactivation of steroid hormone receptors.

Similar to the NH2-terminal unique domain, the COOH-terminal region of Bag1 that is required for Hsc70 binding was found to be essential for potentiation of AR activity. The data reported here and elsewhere indicate that the COOH-terminal Hsc70-binding domain of Bag1 and Bag1L is required for interactions with AR in vitro and for coinmunoprecipitation of Bag1L with AR from cell lysates (9). Moreover, we presented novel evidence here that the BAG domain of Bag1 is sufficient for association with the AR in vitro. It has therefore been postulated that the interaction of Bag1L with the AR may involve Hsp70. Hsp70/Hsc70 along with Hsp90 and Hsp56 are involved in maintaining nuclear receptors in an inactive conformation in the cytoplasm. Thus, it is possible that Hsc70/Hsp70 family molecular chaperones provide a bridge between the AR and Bag1L. Alternatively, Hsp70 and AR may compete for binding to the BAG domain as has been determined recently for Hsp70 and Raf-1 (36).

Molecular chaperones perform several important functions in the regulation of steroid hormone receptors. For example, a variety of chaperones including Hsc70/Hsp70 is required to achieve receptor conformations that are competent to bind steroid ligands. Thus, enhanced ligand binding represents one possible explanation for the mechanism by which Bag1L poten-
mRNA can generate up to four protein products through alteration of the levels of the Bag1L protein produced. The human bag1 mRNA simulates AR function. If true, however, we would have expected the other isoforms of Bag1 also to potentiate AR activity, given that translation and folding of the AR occurs in the cytosol where the unliganded AR resides in a complex with Hsp90 and other chaperones. Thus, the data reported here demonstrating a lack of effect of Bag1 on the ligand binding affinity of the AR are consistent with the observed differences in the ability of nuclear and cytosolic isoforms of Bag1 to enhance AR activity. In addition, because heat shock proteins sequester AR in the cytosol in an inactive state until bound by specific steroid ligands, it was also possible that Bag1 proteins might regulate cytosol to nuclear translocation of AR, however, we previously demonstrated that this is not the case (9).

Given that Bag1L does not alter the affinity of AR for steroid ligands and does not modulate nuclear translocation of AR, we speculate that it potentiates AR function either by affecting coactivator binding to AR or by altering AR affinity for DNA-binding sites. Interestingly, it has been reported that Bag1M is able to bind to DNA and stimulate basal transcription machinery directly in vitro (25). Thus, Bag1L interactions with AR could conceivably increase the affinity of AR-containing transcription complexes for DNA, thereby enhancing transcriptional output of AR-responsive genes. However, given that we failed to see any affect of Bag1L on the binding of the AR to an ARE in gel retardation assays (data not shown), this observation alone cannot account for the unique effects of Bag1L on AR.

Taken together, the data reported here imply that at least two domains within Bag1L are important for its functional collaboration with the AR, namely the TXSEEX repeats and the COOH-terminal BAG domain, which binds Hsp70/Hsc70 family molecular chaperones. Multiple explanations can be envisioned for how these two domains might participate in the regulation of AR. For example, both of these domains might directly or indirectly bind AR. In this regard, it may be relevant that several coactivators of nuclear receptors, such as GRIP-1 and SRC-1, make multiple interactions with nuclear receptors through different domains (26, 37). However, it is equally probable that the BAG domain alone mediates interactions of Bag1L with AR, whereas the TXSEEX repeats associate with other proteins such as coactivators. Difficulties in producing soluble TXSEEX repeats alone have precluded us from distinguishing these two mechanisms to date. Another aspect of Bag1L effects on AR is that they may be indirect, involving Bag1L/Hsc70-induced conformational changes in AR that enhance its interactions with coactivators or reduce interactions with corepressors.

Given that only the Bag1L isoform of Bag1 collaborates functionally with the AR, one mechanism by which tissues could alter their responsiveness to androgens is by adjusting the levels of the Bag1L protein produced. The human bag1 mRNA can generate up to four protein products through alternative mRNA translation from various canonical AUG or non-canonical CUG codons (22, 23). The mouse bag1 mRNA similarly can produce up to three protein products but lack the AUG that is responsible for generating Bag1M (RAP46) (23). In human tissues and tumor cell lines, it has also been shown that Bag1 and Bag1L are by far the most abundant isoforms of Bag1 produced with little or no Bag1M or Bag1S present (23). Interestingly, whereas Bag1 is ubiquitously expressed throughout the organs of mice and humans, Bag1L production is tissue-specific and found predominantly in hormone-sensitive tissues, such as the testis, ovary, breast, and prostate. Also, in human tumor cell lines, levels of Bag1L tend to be highest in hormone-dependent cancers, such as prostate (AR), breast (estrogen receptor (ER), progesterone receptor (PR), and lymphpoid (glucocorticoid receptor) malignancies in which steroid hormones are known to play major roles in the regulation of cell growth, differentiation, and death. Given that Bag1L enhances the transactivation function of AR and that it has been shown to render AR less inhibitable by anti-androgenic drugs (9), it will be interesting to determine whether the levels of Bag1L change during the progression of prostate cancers as they undergo conversion from hormone-responsive to hormone-refractory disease.

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REFERENCES

1. Evans, R. M. (1988) Science 240, 888–895
2. Tjian, R. (1990) Science 248, 345–354
3. Lepor, H., Ross, A., and Walsh, P. C. (1982) J. Urol. 128, 335–340
4. de Vere, W. R., White, R., Meyers, F., Chu, S. G., Chamberlain, S., Siders, D., Leventhal, D., and Gumerlock, P. H. (1997) Cancer Res. 57, 314–319
5. Zeiner, M., and Gehring, U. (1995) Annu. Rev. Biochem. 63, 451–486
6. Friedman, L. P. (1999) Cell 97, 5–8
7. Zeiner, M., Lee, F., Stewart, S., and Gumerlock, P. H. (1997) EMBO J. 16, 5483–5489
8. Luders, J., Demand, J., and Hoehler, S. (2000) Mol. Cell. Biol. 20, 1083–1088
9. Takayama, S., Krajewski, S., Kochel, K., Irie, S., Millan, J. A., and Reed, J. C. (1995a) Cell 80, 279–284
10. Takayama, S., Krajewski, S., Kochel, K., Irie, S., Millan, J. A., and Reed, J. C. (1995b) Mol. Endocrinol. 9, 608–618
11. Takayama, S., Krajewska, M., Kitada, S., Zapata, J. M., Kochel, K., and Reed, J. C. (1995) Mol. Endocrinol. 9, 11660–11666
12. Takayama, S., Krajewska, M., Kitada, S., Zapata, J. M., Kochel, K., and Reed, J. C. (1997) EMBO J. 16, 4887–4896
13. Takayama, S., and Reed, J. C. (1997) EMBO J. 16, 5483–5489
14. Luders, J., Demand, J., and Hoehler, S. (2000) Mol. Cell. Biol. 20, 1083–1088
15. Takayama, S., Krajewski, S., Kochel, K., Irie, S., Millan, J. A., and Reed, J. C. (1995) Cell 80, 279–284
16. Wang, H.-G., Takayama, S., Rapp, U. R., and Reed, J. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7063–7068
17. Bardelli, A., Longati, P., Albero, D., Geruppi, S., Schneider, C., Ponzetto, C., and Comoglio, P. M. (1996) EMBO J. 15, 6205–6212
18. Clevenger, C. V., Thiekmann, K., Ngo, W., Chang, W.-P., Takayama, S., and Reed, J. C. (1997) Mol. Endocrinol. 11, 608–618
19. Takayama, S., Krajewski, S., Krajewska, M., Kitada, S., Zapata, J. M., Kochel, K., and Reed, J. C. (1998) EMBO J. 17, 2736–2747
20. Takayama, S., Krajewski, S., Kochel, K., Irie, S., Millan, J. A., and Reed, J. C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 335–340
21. Takayama, S., Xie, Z., and Reed, J. C. (1997) EMBO J. 16, 11465–11469
22. Zeiner, M., and Gehring, U. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11465–11469
23. Ma, H., Hong, H., Huang, S. M., Irvine, R. A., Webb, P., Kushnir, P. J., and Coetzee, G. A., and Stallcup, M. R. (1999) Mol. Cell. Biol. 19, 6164–6173
24. Liu, R., Takayama, S., Zheng, Y., Froesch, B., Chen, G.-Q., Zhang, X., Reed, J. C., and Zhang, X.-K. (1998) J. Biol. Chem. 273, 10685–10692
25. Lee, H. J., Kokontis, J., Wang, K. C., and Chang, C. (1993) Biochem. Biophys. Res. Commun. 194, 97–103
26. Mowszowicz, I., Lee, H. J., Chen, H. T., Mestayer, C., Portois, M. C., Cabrol, S., Huebner, K., and Reed, J. C. (1996) EMBO J. 15, 7811–7817
27. 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) Cancer Res. 58, 3116–3131
28. Schneikert, J., Huber, S., Martin, E., and Cato, A. B. (1999) Cell Biol. 164, 929–940
29. 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) Cancer Res. 58, 3116–3131
30. Nielsen, D. A., Chang, T. C., and Shapiro, D. J. (1989) Cell 57, 469–475
31. Zhai, M.-J., and O’Malle, B. W. (1998) J. Biol. Chem. 273, 12101–12108
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