Differential Effects of the hsp70-binding Protein BAG-1 on Glucocorticoid Receptor Folding by the hsp90-based Chaperone Machinery*

(Received for publication, June 21, 1999, and in revised form, September 3, 1999)

Kimon C. Kanelakis§, Yoshihiro Morishima§, Kurt D. Dittmar§, Mario D. Galigniana‡, Shinichi Takayama§, John C. Reed§, and William B. Pratt¶

From the §Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan 48109 and the ¶Burnham Institute, Cancer Research Center, La Jolla, California 92037

The heat shock protein hsp70/hsc70 is a required component of a five-protein (hsp90, hsp70, Hop, hsp40, and p23) minimal chaperone system reconstituted from reticulocyte lysate that forms glucocorticoid receptor (GR)-hsp90 heterocomplexes. BAG-1 is a cofactor that binds to the ATPase domain of hsp70/hsc70 and that modulates its chaperone activity. Inasmuch as BAG-1 has been found in association with several members of the steroid receptor family, we have examined the effect of BAG-1 on GR folding and GR-hsp90 heterocomplex assembly. BAG-1 was present in reticulocyte lysate at a BAG-1:hsp70/hsc70 molar ratio of ∼0.03, and its elimination by immunoadsorption did not affect GR folding and GR-hsp90 heterocomplex assembly. At low BAG-1:hsp70/hsc70 ratios, BAG-1 promoted the release of Hop from the hsp90-based chaperone system without inhibiting GR-hsp90 heterocomplex assembly. However, at molar ratios approaching stoichiometry with hsp70, BAG-1 produced a concentration-dependent inhibition of GR folding to the steroid-binding form with corresponding inhibition of GR-hsp90 heterocomplex assembly by the minimal five-protein chaperone system. Also, there was decreased steroid-binding activity in cells that were transiently or stably transfected with BAG-1. These observations suggest that, at physiological concentrations, BAG-1 modulates assembly by promoting Hop release from the assembly complex; but, at concentrations closer to those in transfected cells and some transformed cell lines, hsp70 is continuously bound by BAG-1, and heterocomplex assembly is blocked.

A number of signaling proteins, including several members of the nuclear receptor family, the dioxin receptor, nitric-oxide synthase, and several protein kinases, exist in cytosolic complexes with the ubiquitous and abundant heat shock protein (hsp)1 hsp90 (for review, see Refs. 1 and 2). These signaling protein-hsp90 heterocomplexes can be formed under cell-free conditions by incubating the immunoadsorbed proteins with reticulocyte lysate (3, 4). A minimal heterocomplex assembly system has been reconstituted (5–9); and five proteins, including hsp90, hsp70,2 Hop (60-kDa hsp organizer protein), hsp40 and p23, participate in the ATP/Mg 2+ -dependent and K+ -dependent assembly process (for review of heterocomplex assembly, see Refs. 10 and 11). Hip and BAG-1 (Bel-2-associated gene product-1) are co-chaperones of hsp70 that are potential participants in or regulators of this multiprotein hsp90-based chaperone system.

BAG-1 is an hsp70-binding protein that associates with the ATPase domain of the molecular chaperone with a Kd of ∼1–10 nM (12). BAG-1 was originally cloned from a mouse library as a Bel-2-binding protein and shown to have anti-apoptotic activity (13). Subsequently, BAG-1 was shown to form complexes with some signaling proteins, including receptors for hepatocyte growth factor and platelet-derived growth factor (14), the serine/threonine protein kinase Raf-1 (15), and the retinoic acid receptor (16). An isoform of the human BAG-1 protein called RAP46 (46-kDa receptor-associated protein; BAG-1M) was cloned and shown to bind to members of the nuclear receptor family, including the glucocorticoid receptor (GR) (17). The ability of BAG-1 (18, 20) and RAP46 (BAG-1M) (19) to interact with hsp70 suggests an explanation for their association with the various signaling proteins.

It is now clear that there is a conserved family of BAG-1-related proteins (12), and three forms of BAG-1 itself exist as a result of alternative initiation of translation within a common mRNA (21). The originally identified BAG-1 protein is the smallest, migrating at ∼32 kDa. Human ∼46-kDa RAP46 is the same as BAG-1M, and we will refer to it as BAG-1M in this work. A form migrating at ∼58 kDa is called BAG-1L (22). The effect of BAG-1 on steroid receptor function appears to depend upon the isoform that is expressed. For example, BAG-1M (RAP46) was found to be a negative regulator of GR activity (23), whereas BAG-1L, but not BAG-1 or BAG-1M, forms complexes with the androgen receptor and enhances androgen receptor-mediated transactivation of a reporter gene (22).

hsp70 is a protein chaperone possessing an amino-terminal domain that binds the nucleotides ADP and ATP and a carboxy-terminal domain that binds peptide, with the binding of ADP versus ATP being coupled to its peptide-binding activity (for review, see Ref. 24). hsp70 possesses weak ATPase activity and an intrinsic ADP-ATP exchange activity (25). BAG-1, BAG-1M, and BAG-1L have been reported to bind to the hsp70 ATPase domain (19, 26, 27). BAG-1 accelerates the release of ADP from hsp70 (20, 28), acting much like bacterial GrpE in the DnaK family of chaperone proteins.

* This work was supported by National Institutes of Health Grants DK31573 (to W. B. P.) and CA67329 (to J. C. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

¶ To whom to address correspondence: Dept. of Pharmacology, University of Michigan Medical School, 1301 Medical Science Research Bldg. III, Ann Arbor, MI 48109-0632. Tel.: 734-764-5414; Fax: 734-763-4450.

1 The abbreviations used are: hsp, heat shock protein; GR, glucocorticoid receptor; GFP, green fluorescent protein; DMEM, Dulbecco’s modified Eagle’s medium; TES, 2-(2-hydroxy-1,1-bis(hydroxymethyl)amino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

2 In this paper, we use the term hsp70 collectively to refer to both the heat shock-induced hsp70 and the constitutively expressed heat shock cognate hsc70.
chaperone cycle (Ref. 29; for review of BAG-1 regulation of hsp70, see Ref. 30). However, unlike GrpE, which enhances the folding activity of DnaK, BAG-1 forms a ternary complex with hsp70 and a non-native substrate, uncoupling the ATP-dependent release of the hsp70-associated substrate (31).

Because hsp70 is absolutely required for formation of heterocomplexes between the GR and hsp90 and for changing the folding state of the GR hormone-binding domain to form the high affinity steroid-binding conformation (32), we have asked in this work how BAG-1 affects GR-hsp90 heterocomplex assembly and GR folding by the multiprotein hsp90-based chaperone system. BAG-1 itself was the dominant BAG-1 isoform in rabbit reticulocyte lysate, where it was present at a low concentration with a BAG-1:hsp70 molar ratio of ~0.03. Elimination of BAG-1 from lysate had little or no effect on its ability to assemble GR-hsp90 heterocomplexes and to generate steroid-binding activity. Addition of low concentrations of purified recombinant BAG-1 to a five-protein minimal assembly system consisting of purified hsp90, hsp70, Hop, YDJ-1, and p23 promoted release of Hop from assembly complexes without inhibiting GR-hsp90 heterocomplex assembly. At concentrations approaching stoichiometry with hsp70, however, BAG-1 produced a concentration-dependent inhibition of GR folding to the steroid-binding form, with corresponding inhibition of GR glucocorticoid-binding activity. Thus, at the low levels that exist in reticulocyte lysate, BAG-1 appears to modify heterocomplex assembly by promoting Hop release from the hsp90-based chaperone system; but at higher levels that may be closer to those achieved in transfected cells and some types of transformed cells (27), it has the potential to regulate GR folding in a negative manner.

**EXPERIMENTAL PROCEDURES**

**Materials**

6,7-3H)Triamcinolone acetonide (42.8 Ci/mmol) and 3H-labeled goat anti-mouse and anti-rabbit IgGs were obtained from NEN Life Science Products. Untreated rabbit reticulocyte lysate was from Green Hectares (Oregon, WI). Charcoal-stripped bovine calf serum, protein A-Sepharose, and goat anti-rabbit horseradish peroxidase conjugates were from Sigma, and donkey anti-rabbit IgG was from Pierce. TransFast reagent was from Promega (Madison, WI), and Complete-Mini protease inhibitor mixture was from Roche Molecular Biochemicals (Mannheim, Germany). The BuGR2 monoclonal IgG against the GR and the 2G6 monoclonal anti-p48 IgG against Hip were from Affinity Bioreagents (Golden, CO). The AC88 monoclonal IgG against hsp90, the N27F3-4 anti-72/73-kDa hsp monoclonal IgG (anti-hsp70), and the anti-p40 rabbit polyclonal antibody were from Stracksen Biotech Corp. (Victoria, British Columbia, Canada). The J33 monoclonal IgG against p23 and Escherichia coli cells expressing human p23 were gifts from Dr. David Toft (Mayo Clinic). E. coli cells expressing YDJ-1 were a gift from Dr. Avrom Caplan (Mount Sinai School of Medicine). The DS14F5 monoclonal IgG against Hop and E. coli cells expressing Hop were kindly provided by Dr. David F. Smith (University of Nebraska Medical School). The BAG-1 (C-16) affinity-purified rabbit polyclonal antibody was from Santa Cruz Biotechnologies (Santa Cruz, CA). Hybridoma cells producing the FIGR monoclonal IgG against the GR were generously provided by Dr. Jack Bodwell (Dartmouth Medical School). The baculovirus for mouse GR was kindly provided by Dr. Edwin Sanchez (Medical College of Ohio). Construction of the pcDNA3-hu-BAG-1 plasmid for expression of human BAG-1 has been described previously (33), as was the GM-701 human fibroblast cell line stably expressing FLAG-BAG-1 (28). The cDNA for expressing GFP-GR, described previously (34), was provided by Dr. Paul Housley (University of South Carolina School of Medicine).

**Methods**

**Cell Culture and Transfection**—Monkey kidney COS-7 cells were grown in DMEM supplemented with 10% fetal bovine serum. When cells were confluent, they were rinsed three times with serum-free DMEM and incubated for an additional hour in DMEM with 5% serum. Cells were then transfected with 2 μg/ml GFP-GR cDNA and 3 μg/ml pcDNA3 vector or pcDNA3-hu-BAG-1. The DNAs were preincubated for 10 min at room temperature with 6 μl of TransFast reagent/μg of DNA in 200 μl of DMEM, and the mixtures were added to the cell cultures. After 1 h at 37 °C, the medium was replaced with DMEM containing 10% fetal bovine serum, and the cells were cultured for 24 h. The medium was then replaced with 10% fetal bovine serum for an additional 24 h of incubation. The cells were washed three times with Earle’s balanced saline and suspended in 1 volume of buffer containing 10 mM Hepes, pH 7.35, 1 mM EDTA, 20 mM sodium molybdate, 20 mM sodium vanadate, and 1 tablet of Complete-Mini protease inhibitor mixture/3 ml of buffer. Cells were ruptured by Dounce homogenization, centrifuged for 1 h at 100,000 × g to prepare the cytosol.

GM-701 human fibroblasts (the parental line and a subline stably transfected with FLAG-tagged murine BAG-1) were grown in DMEM supplemented with 10% fetal bovine serum. When cells were ~80% confluent, they were harvested, and the cytosol was prepared as described for COS-7 cells.

**Expression of Mouse GR in S9 Cells**—S9 cells were grown in SFM9000 II serum-free medium (Life Technologies, Inc.) supplemented with Cytomax (Kemp Biotechnology, Rockville, MD) in suspension cultures maintained at 27 °C with continuous shaking (150 rpm). Cultures were infected in log phase of growth with recombinant baculovirus at a multiplicity of infection of 3.0. Cultures were supplemented with 1% glucose at infection and 24 h post-infection as described by Srinivasan et al. (34). Cells were harvested in the G1 phase of the cell cycle, resuspended in 1.5 volumes of buffer (10 mM Hepes, pH 7.5, 1 mM EDTA, 20 mM molybdate, and 1 mM phenylmethylsulfonyl fluoride), and ruptured by Dounce homogenization. The lysate was then centrifuged at 100,000 × g for 30 min, and the supernatant was collected, aliquoted, flash-frozen, and stored at -70 °C.

**Immunoadsorption of GR and Hop—Receptors were immunoadsorbed from 50-μl aliquots of S9 cell lysate by rotation for 2 h at 4 °C with 14 μl of protein A-Sepharose precoated to 7 μl of FIGR ascites suspended in 300 μl of TEG buffer (10 mM TES, pH 7.6, 50 mM NaCl, 4 mM EDTA, and 10% glycerol). Prior to incubation with reticulocyte lysate or various mixtures of purified proteins as noted, immunoadsorbed receptors were stripped of associated hsp90 by incubating the immune pellet for an additional 2 h at 4 °C with 1 ml of 0.5 mM KCI in TEG buffer. The pellets were then washed once with 1 ml of TEG buffer, followed by a second wash with 1 ml of 10 mM Hepes, pH 7.4. For immunoadsorption of Hop, 300-μl aliquots of reticulocyte lysate were immunoadsorbed to 5 μl of protein A-Sepharose prebound with DS14F5 antibody against Hop or with nonimmune mouse IgG. The samples were rotated at 4 °C for 2 h, and immune pellets were washed three times with 1 ml of TEG buffer and with 20 mM sodium molybdate.

**Glucocorticoid Receptor Heterocomplex Reconstitution**—FIGR immune pellets containing GRs stripped of hsp90 were incubated with 50 μl of rabbit reticulocyte lysate, with combinations of lysate DE52 fractions A–C, or with various mixtures of proteins (20 μg of purified hsp90, 20 μg of purified hsp70, 2 μl of lysate from bacteria expressing Hop, 6 μg of purified p23, and 2 μl of lysate from bacteria expressing YDJ-1) and/or inhibitor mixture/3 ml of buffer. The assay mixtures were incubated for 30 min at 30 °C with suspension of the pellets by shaking the tubes every 2 min. At the end of the incubation, the pellets were washed twice with 1 ml of ice-cold TEG buffer and assayed for steroid-binding capacity and, in some experiments, for receptor-associated proteins.

**Assay of Steroid-Binding Capacity**—Immune pellets to be assayed for steroid binding were incubated overnight in 50 μl of 10 mM Hepes, pH 7.5, 1 mM EDTA, 20 mM molybdate plus 50 mM [3H]triamcinolone acetonide. Samples were then washed three times with 1 ml of TEG buffer and counted by liquid scintillation spectrometry. The steroid binding is expressed as counts/min of [3H]triamcinolone acetonide bound per FIGR immune pellet prepared from 50 μl of S9 cytosol.

For assay of steroid binding in the cytosol from COS-7 cells or GM-701 cells, 100 μl of cytosol was incubated overnight with 50 mM [3H]triamcinolone acetonide (±1000-fold excess of radiometric dexamethasone). Bound steroid was separated from free steroid by adding 1 ml of a 1:1 mixture of dextran-coated charcoal and 0.2% (w/v) dextran in 10 mM Hepes and 1 mM EDTA, pH 7.35). The radioactivity in the supernatant was assayed, and the specific binding was normalized for the cytosol protein concentration.

**Western Blotting**—To assay the GR and associated proteins and Hop and associated proteins, immune pellets were resolved on 10% SDS-polyacrylamide gels and transferred to Immobilon-P membranes. The
membranes were probed with 2 μg/ml BuG2R2 for GR, 1 μg/ml AC88 for hsp90, 1 μg/ml N27F3-4 for hsp70, 0.1% DS14F5 mouse ascites for Hop, and 0.5% anti-hsp40 antibody or 0.1% JJ3 mouse ascites for p23. The immunoblots were then incubated a second time with the appropriate 125I- or horseradish peroxidase-conjugated counterantibody to visualize the immunoreactive bands.

Protein Purification—hsp90 and hsp70 were purified from rabbit reticulocyte lysate by sequential chromatography on DE52, hydroxyapatite, and ATP-agarose as described previously (32). Human p23 (36) was purified from 10 ml of bacterial lysate by chromatography on DE52 as described (8), followed by hydroxyapatite chromatography. Fractions containing p23 were identified by immunoblotting, pooled, concentrated by Amicon filtration to ~1.5 ml, dialyzed against HKB buffer, aliquoted, and stored at ~70 °C. The bacterial expression of YDJ-1 has been described previously (37, 38), as has the expression of human hsp organizer protein (Hop) (5). In this work, bacterial lysate expressing YDJ-1 or Hop was added to the heterocomplex assembly system without purification of the recombinant proteins. Murine BAG-1 was expressed as a GST fusion protein in E. coli and purified by adsorption to glutathione-agarose, followed by thrombin cleavage and ion-exchange and Superdex-75 gel filtration chromatography as described previously (20). Purified BAG-1 (3 mg/ml) was stored at ~70 °C in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% β-mercaptoethanol, and 1 mM EDTA. After unfreezing, the solution was cleared of insoluble protein by centrifugation at 100,000 × g.

RESULTS

BAG-1 Exists in Multiprotein hsp Heterocomplexes—Hop is a 60-kDa protein that interacts with hsp70 and hsp90 (39) via separate binding sites (40), acting as an adaptor protein (41) in the hsp90-based chaperone machinery. We have shown previously that immunoadsorption of Hop from reticulocyte lysate is accompanied by co-immunoadsorption of hsp90, hsp70, and hsp40 as an hsp90-Hop-hsp70-hsp40 heterocomplex (8). This complex immunoadsorbed from reticulocyte lysate or prepared from purified proteins is able to convert the GR to the high affinity steroid-binding conformation (6, 8). Formation of a heterocomplex between hsp90 and the hormone-binding domain of the GR is required to produce the proper folding state for steroid binding (42, 43).

In Fig. 1, we investigated whether either BAG-1 or Hip is present in the multiprotein GR-hsp90 heterocomplex assembly machine that we call a foldosome (44). As shown in Fig. 1, immunoadsorption of rabbit reticulocyte lysate with a monoclonal antibody against Hop yielded co-adsorption of hsp90 and hsp70 as well as the hsp70-associated proteins hsp40, Hip, and BAG-1. hsp70 possesses separate non-overlapping binding sites for Hop, hsp40, and Hip (18). However, Hop and BAG-1 compete for binding to the ATPase domain of hsp70 (28), and they must exist in separate hsp90-Hop-hsp70-hsp40 heterocomplexes. It should be noted that, to visualize Hip and BAG-1, the autoradiograms for these proteins required a much longer exposure time than for the major proteins in Fig. 1. Thus, it seems that only a fraction of the multiprotein hsp heterocomplexes isolated from reticulocyte lysate contain either Hip or BAG-1.

Fractionation of Reticulocyte Lysate—In Fig. 2, the rabbit reticulocyte lysate proteins were fractionated by chromatography on DE52. The dominant BAG-1 isoform is ~32-kDa BAG-1, which eluted early in the salt gradient with another slower migrating immunoreactive species that we refer to as BAG-1M (Fig. 2A). A trace amount of an immunoreactive species migrated as expected for BAG-1L, but eluted at higher salt concentrations, indicating that it is more acidic than BAG-1 and BAG-1M. Reticulocyte lysate contained very little BAG-1 relative to both Hip and the established components of the hsp90-based chaperone system: hsp90, hsp70, Hop, hsp40, and p23 (Fig. 2A). The reticulocyte lysate proteins were combined into three DE52 subfractions designated A, B, and C (Fig. 2B).

In Fig. 3A, immune pellets containing GRs stripped of hsp90 were incubated with DE52 subfractions A–C in various combinations. Maximum generation of steroid-binding activity required all three fractions of reticulocyte lysate. Because of a low level of cross-contamination of components between fractions, some steroid-binding activity was generated when just two subfractions were present (Fig. 3A). As shown in Fig. 3B, substitution of fraction B with purified hsp90 and of fraction C with purified p23 yielded a reconstituted system that was entirely dependent upon the presence of lysate fraction A.

Removal of BAG-1 from Lysate Fraction A—To determine if BAG-1 in reticulocyte lysate was affecting (either negatively or positively) GR folding, BAG-1 was eliminated from lysate fraction A by immunoadsorption. As shown in Fig. 4A, adsorption of fraction A with protein A-Sepharose prebound with anti-BAG-1 antibody (I) eliminated BAG-1 from fraction A. Substitution of fraction A with either nonimmune IgG-adsorbed (A/NI) or anti-BAG-1 antibody-adsorbed (A/I) fraction A yielded comparable levels of GR steroid-binding activity and resulted in assembly of similar GR-hsp90 heterocomplexes (Fig. 4B). Thus, we suggest that, at the levels that are present in reticulocyte lysate, BAG-1 does not affect the ability of the GR to be folded to the steroid-binding state by the hsp90-based chaperone system.

Effects of a High Concentration of BAG-1 on GR Folding and Heterocomplex Assembly—To determine the effect of BAG-1 on GR folding at concentrations approaching stoichiometry with hsp70, we performed experiments in which stripped GR immune pellets were incubated with a minimal folding system (8) consisting of purified hsp90, hsp70, and p23 and lysate from bacteria expressing Hop and YDJ-1 in the presence of increas-
The concentration of BAG-1 is presented as a molar ratio of purified recombinant human p23, respectively.

A

B

**Fig. 3.** Reconstitution of the GR-folding activity from DE52 fractions of reticulocyte lysate. **A**, DE52-resolved fractions A–C of reticulocyte lysate are all required for generation of steroid-binding activity. The GR was immunoadsorbed to protein A-Sepharose from replicate aliquots of Sf9 cytosol, and the immune pellets were stripped of hsp90 with salt. Stripped immune pellets (Str) were then incubated for 30 min at 30 °C with 50 μl of normal unfraccionated reticulocyte lysate (RL) or with 10 μl of DE52 fraction pools A–C of reticulocyte lysate alone or in the combinations indicated in the presence of 100 mM KC1 and an ATP-regenerating system. The immune pellets were then washed and incubated with 50 nM [3H]triamcinolone acetonide to determine steroid-binding activity. **B**, substitution of lysate DE52 fractions B and C with 20 μg of purified rabbit hsp90 and 6 μg of purified recombinant human p23, respectively.

**Fig. 4.** Elimination of BAG-1 from lysate fraction A does not affect GR folding or GR-hsp90 heterocomplex assembly. **A**, immunodepletion of BAG-1 from fraction A. Protein A-Sepharose pellets prebound with nonimmune IgG (NI) or with anti-BAG-1 antibody (I) were incubated with 50 μl of fraction A for 2 h at 4 °C and then washed three times with 1 ml of TEGM buffer, and the pellet and a portion (−20%) of the supernatant (Super) were assayed for BAG-1 by immunoblotting. **B**, reconstitution of GR-hsp90 heterocomplexes and steroid-binding activity with BAG-1-depleted fraction A. Stripped GR immune pellets (Str) were incubated for 30 min at 30 °C with fraction A (A), with fraction A incubated with nonimmune IgG (A(NI)), or with fraction A depleted with anti-BAG-1 antibody (A(I)) in the presence of purified rabbit hsp90, purified human p23, 100 mM KC1, and an ATP-regenerating system. The GR, hsp90, hsp70, and Hop in the washed immune pellets were assayed by SDS-PAGE and Western blotting, and a portion of each immune pellet was then incubated with 50 nM [3H]triamcinolone acetonide to determine steroid-binding activity. Steroid binding data are means ± S.E. of samples from three experiments.

**Fig. 5.** Effect of Low Concentrations of BAG-1—Although in vitro studies of BAG-1 effects on protein folding assays have used BAG-1 (or BAG-1M) at concentrations that are stoichiometric or slightly higher with respect to hsp70, BAG-1 produced ~50% inhibition of GR folding when present at a molar ratio of 0.4. The inhibition of folding was prevented by heat inactivation of the BAG-1 protein at 100 °C (data not shown).

Geldanamycin is a benzoquinone ansamycin antibiotic that binds to the nucleotide-binding site of hsp90 and specifically blocks hsp90 function (45–47). When steroid receptor-hsp90 heterocomplexes are assembled in reticulocyte lysate in the presence of geldanamycin, they cannot bind steroid. In this case, the complexes still contain the adaptor protein Hop in the absence of inhibitor (lane 2) or in the presence of 10 μM geldanamycin (lane 4) or in the presence of BAG-1 (lane 6) at a BAG-1:hsp70 molar ratio of 1.2. In contrast to complexes assembled in the presence of geldanamycin, which had an increased amount of Hop, Hop protein was eliminated from GR-hsp90 heterocomplexes assembled in the presence of BAG-1. By comparing lanes 5 and 6, it can be seen that the added BAG-1 was present in the GR-hsp90 heterocomplex. As shown in Fig. 7, incubation of preassembled complexes with BAG-1 did not affect either steroid-binding activity or the composition of the GR-hsp90 heterocomplex.

**Fig. 6.** Composition of the GR-hsp90 heterocomplexes assembled in the purified minimal folding system in the absence of inhibitor (lane 2) or in the presence of 10 μM geldanamycin (lane 4) or in the presence of BAG-1 (lane 6) at a BAG-1:hsp70 molar ratio of 1.2. In contrast to complexes assembled in the presence of geldanamycin, which had an increased amount of Hop, Hop protein was eliminated from GR-hsp90 heterocomplexes assembled in the presence of BAG-1. By comparing lanes 5 and 6, it can be seen that the added BAG-1 was present in the GR-hsp90 heterocomplex. As shown in Fig. 7, incubation of preassembled complexes with BAG-1 did not affect either steroid-binding activity or the composition of the GR-hsp90 heterocomplex.

**Fig. 7.** Reproducibility of the observation that geldanamycin (lane 4) or BAG-1 (lane 6) assembles the GR-hsp90 heterocomplex without Hop. As shown in Fig. 6, incubation of preassembled complexes with BAG-1 did not affect either steroid-binding activity or the composition of the GR-hsp90 heterocomplex. **A**, reconstitution of GR-hsp90 heterocomplex assembly were inhibited in a concentration-dependent fashion. By excising and counting the 125I-labeled bands for GR and hsp90 in several experiments, we have determined that the decrease in steroid-binding activity at each BAG-1 concentration was accompanied by a comparable decrease in the hsp90:GR ratio (Fig. 8B).

**Fig. 8.** BAG-1 Overexpression Decreases Steroid-binding Activity in Vivo—To determine if overexpression of BAG-1 would affect GR steroid-binding activity in cells, COS-7 cells were cotransfected with cDNAs expressing a fusion protein containing the mouse GR and a cDNA expressing BAG-1. As shown in Fig. 9A, transfection with BAG-1 reduced steroid-binding activity from the transfected GR by ~65% (lane 4) with respect to the vector control (lane 3). We also examined the effect of BAG-1 on the endogenous GR in GM-701 fibroblasts stably transfected with BAG-1 (26). As shown in Fig. 9B, steroid-binding activity in BAG-1-overexpressing cells (lane 2) was ~50% of that in the parent cell line (lane 1). On the original autoradiogram, a thin band of endogenous BAG-1 was visible both in untransfected COS-7 cells and in the parental GM-701 cell line that was not visible in the Western blot photographs of Fig. 9, A (lanes 1–3).
Reconstituted in the presence of 10 mM purified GR lysate containing Hop, 2 mM immune pellets were incubated for 30 min at 30 °C with 20 mM SDS-PAGE and Western blotting, and a portion of each immune pellet was incubated with [3H]triamcinolone acetonide to determine steroid-binding activity. Lanes 1 and 4, nonimmune and immune pellets, respectively, reconstituted in the presence of 10 μM geldanamycin; lanes 3 and 6, nonimmune and immune reconstituted pellets, respectively, incubated at 30 °C; lanes 5 and 6, reconstituted pellets incubated at 30 °C with BAG-1 at a BAG-1:hsp70 molar ratio of 1.2. The steroid-binding activities (black bars) are means ± S.E. from three experiments expressed as a percent of the 0 °C control.

**FIG. 7.** BAG-1 does not affect preassembled GR-hsp90 heterocomplexes. Stripped GR immune pellets were incubated for 10 min at 30 °C with the minimal assembly system. The immune pellets were then washed once with TEGM buffer and once with 10 mM Hepes, pH 7.4; and the washed pellets were suspended in HKD buffer containing the ATP-regenerating system and 20 mM molybdate. The samples were incubated for an additional 10 min at 0 or 30 °C, and steroid-binding activity and the GR and its associated proteins were assayed. Lanes 1 and 2, nonimmune and immune reconstituted pellets, respectively, incubated at 0 °C; lanes 3 and 4, nonimmune and immune reconstituted pellets, respectively, incubated at 30 °C; lanes 5 and 6, reconstituted pellets incubated at 30 °C with BAG-1 at a BAG-1:hsp70 molar ratio of 1.2. The steroid-binding activities (black bars) are means ± S.E. from three experiments expressed as a percent of the 0 °C control.

**DISCUSSION**

Previous studies have established that a system containing hsp90, hsp70, Hop, YDJ-1, and p23 is sufficient to assemble stable steroid receptor-hsp90 heterocomplexes (5–9). Although the hsp70-binding proteins BAG-1 and Hip are not required for assembly, they may nevertheless play a role in modulating the assembly process. Here, we have examined the role of BAG-1 in GR folding in reticulocyte lysate. Upon immunoadsorption of Hop from reticulocyte lysate, only a small fraction of the hsp90-Hop-hsp70-hsp40 foldosome complexes contained BAG-1 (Fig. 1). This suggests that any activity of BAG-1 in GR-hsp90 heterocomplex assembly by reticulocyte lysate would likely involve a dynamic association with multiple receptor-hsp90-Hop-hsp70 complexes during the assembly process.

We prepared a partially reconstituted GR-hsp90 heterocomplex assembly system in which hsp70 and its co-chaperones hsp40, BAG-1, and Hip were present at the same stoichiometry relative to each other that existed in whole reticulocyte lysate (Figs. 2 and 3). Selective elimination of BAG-1 by its immunoadsorption from this system had little or no effect on the ability of the system to convert the GR to the steroid-binding form and no apparent effect on the composition of the GR-hsp90 heterocomplex (Fig. 4).

We have previously noted that GR-hsp90 heterocomplexes assembled by reticulocyte lysate contain little or sometimes no Hop, whereas those assembled by the minimal reconstituted system of purified hsp90, hsp70, Hop, YDJ-1, and p23 contain Hop (6). This led to the notion that reticulocyte lysate may contain an activity that facilitates the exit of Hop from the receptor heterocomplex during its assembly (6). It is interesting that addition of BAG-1 to the purified minimal assembly system at a BAG-1:hsp70 molar ratio of 0.07, which is in the range of the ratio existing in reticulocyte lysate, resulted in a GR-hsp90 heterocomplex that contained very little Hop relative to the BAG-1-free system (Fig. 8A, cf. lanes 2 and 4). It is possible that BAG-1 at concentrations that are substoichiometric with respect to hsp70 can facilitate the release of Hop from receptor-bound hsp70 in the GR-hsp90 heterocomplex without affecting GR folding. Promoting the release of Hop would facilitate the formation of new hsp90-Hop-hsp70 complexes and thus facilitate the hsp90-based chaperone cycle.

Johnson et al. (49) have shown that Hop binds preferentially to the ADP-bound form of hsp70. Inasmuch as BAG-1 reportedly binds to the ATPase domain of hsp70 (19, 26) and promotes the release of ADP (20, 28), it may alter the conformation of hsp70 to reduce its affinity for Hop, thus promoting Hop dissociation during GR-hsp90 heterocomplex assembly. Hip is
then assayed. After incubation with 125I-labeled counterantibody, the bands for the GR, hsp90, and hsp70 on immunoblots (A) were excised and counted to determine the relative hsp90:GR (white bars) and hsp70:GR (hatched bars) ratios shown in B. Steroid binding and the ratios are expressed as a percentage of the no-BAG-1 control. Steroid binding and the hsp90:GR ratio represent the means ± S.E. from three experiments, and the hsp70:GR ratio is an average of two experiments with the range indicated by the vertical lines. Lanes 1 and 2, nonimmune and immune pellets, respectively, reconstituted without BAG-1; lanes 3–8, pellets reconstituted in the presence of BAG-1: hsp70 molar ratios of 0.07 (lanes 3 and 4), 0.25 (lanes 5 and 6), and 0.5 (lanes 7 and 8). Note that 20 μg of hsp70 was present in all samples and that only the BAG-1 concentration was varied.

thought to stabilize the ADP-bound conformation of hsp70 (28) and may have the opposite effect compared with BAG-1. In the purified minimal assembly system where no Hop is present, it may be easier to observe an effect of BAG-1 at low molar ratios with respect to hsp70 than in a more complex assembly system, such as DE52 fraction A of reticulocyte lysate (Figs. 3B and 4), where Hop is also present.

Other studies of BAG-1 effects on hsp70 chaperone activity in vitro have been performed at BAG-1:hsp70 molar ratios of 1 or more (28, 31). These ratios are much higher than in reticulocyte lysate (0.03–0.06), but probably approach ratios existing in transfected cells overexpressing BAG-1 and in some types of tumors (27). At high BAG-1:hsp70 molar ratios, we found that BAG-1 inhibited the generation of steroid-binding activity (Figs. 5, 6, and 8) and the assembly of GR:hsp90 heterocomplexes (Figs. 6 and 8). A similar situation is seen with geldanamycin, which binds to the nucleotide-binding site of hsp90 and blocks its conversion to the ATP-dependent conformation (46). hsp90 in the GR:hsp90 heterocomplex must be in the ATP-bound conformation to be capable of hormone binding (50). Because this conversion is prevented, GR:hsp90 heterocomplexes generated in the presence of geldanamycin do not bind steroid. Also, Toft and co-workers (49, 50) have shown that ADP-bound hsp90 has a high affinity for Hop and that binding of ATP markedly reduces Hop-binding affinity. Thus, the GR:hsp90 heterocomplex assembled in the presence of geldanamycin contains abundant amounts of Hop and does not bind steroid (Fig. 6, lane 4). In contrast to the situation with geldanamycin, these heterocomplexes assembled in the presence of BAG-1 are free of Hop (Fig. 6 and 8), suggesting that both hsp70 and hsp90 in the assembled heterocomplexes are no longer in the ADP-bound conformation that possesses high affinity for Hop.

In summary, at the low levels at which BAG-1 exists in reticulocyte lysate, BAG-1 appears to modulate the GR:hsp90 assembly process by promoting Hop release from the assembly complex. When it is present at high levels in the cell-free assembly system or when it is overexpressed (Fig. 9), BAG-1 affects GR folding in a negative manner to yield decreased steroid-binding activity.

Acknowledgments—We thank David Smith and David Toft for providing antibodies and cDNAs for Hop and p23, respectively; Avrom Caplan for providing the YDJ-1 cDNA; Paul Housley for the GFP-GR cDNA; Jack Bodwell for providing FGR-producing hybridoma cells; and Ed Sanchez for providing the mouse GR baculovirus.

REFERENCES

1. Pratt, W. B., and Toft, D. O. (1997) Endocr. Rev. 18, 306–360
2. Pratt, W. B. (1997) Annu. Rev. Pharmacol. Toxicol. 37, 297–326
3. Smith, D. F., Schwalter, D. B., Kost, S. L., and Toft, D. O. (1990) Mol. Endocrinol. 4, 1704–1711
4. Scherrer, L. C., Dalman, F. C., Massa, E., Meshinchi, S., and Pratt, W. B. (1990) J. Biol. Chem. 265, 21397–21400
5. Dittmar, K. D., Hutchison, K. A., Owens-Grillo, J. K., and Pratt, W. B. (1996) J. Biol. Chem. 271, 12833–12839
6. Dittmar, K. D., and Pratt, W. B. (1997) J. Biol. Chem. 272, 13047–13054
7. Dittmar, K. D., Demady, D. R., Stancato, L. F., Krishna, P., and Pratt, W. B. (1997) J. Biol. Chem. 272, 21213–21220
8. Dittmar, K. D., Banach, M., Galgiani, M. D., and Pratt, W. B. (1998) J. Biol. Chem. 273, 7558–7566
9. Komano, H., Stensgard, B., Charleworth, M. C., McMahon, N., and Toft, D. (1998) J. Biol. Chem. 273, 32973–32979
10. Toft, D. O. (1998) Trends Endocrinol. Metab. 9, 238–243
11. Pratt, W. B., and Dittmar, K. D. (1998) Trends Endocrinol. Metab. 9, 244–252
12. Takayama, S., Xie, X., and Reed, J. C. (1999) J. Biol. Chem. 274, 781–786
13. Takayama, S., Sato, T., Krajewski, S., Kochel, K., Irie, S., Millan, J. A., and Reed, J. C. (1995) Cell 80, 279–284
14. Bardelli, A., Longati, P., Albero, D., Geruppi, S., Schneider, C., Ponzetto, C., and Comoglio, P. M. (1996) EMBO J. 15, 6205–6212
15. Wang, H.-G., Takayama, S., Rapp, U. R., and Reed, J. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7063–7068

FIG. 8. Effect of various concentrations of BAG-1 on GR:hsp90 heterocomplex assembly. Stripped GR immune pellets were incubated for 20 min at 30 °C with the minimal assembly system in the presence of 20 mM molybdate and various concentrations of BAG-1. Steroid-binding activity (black bars) and GR-associated proteins were then assayed. After incubation with 125I-labeled counterantibody, the bands for the GR, hsp90, and hsp70 on immunoblots (A) were excised and counted to determine the relative hsp90:GR (white bars) and hsp70:GR (hatched bars) ratios shown in B. Steroid binding and the ratios are expressed as a percentage of the no-BAG-1 control. Steroid binding and the hsp90:GR ratio represent the means ± S.E. from three experiments, and the hsp70:GR ratio is an average of two experiments with the range indicated by the vertical lines. Lanes 1 and 2, nonimmune and immune pellets, respectively, reconstituted without BAG-1; lanes 3–8, pellets reconstituted in the presence of BAG-1: hsp70 molar ratios of 0.07 (lanes 3 and 4), 0.25 (lanes 5 and 6), and 0.5 (lanes 7 and 8). Note that 20 μg of hsp70 was present in all samples and that only the BAG-1 concentration was varied.

FIG. 9. Effect of BAG-1 overexpression on steroid-binding activity. A, transient transfection. COS-7 cells were transfected with GFPR GR cDNA and either pcDNA3 vector or pcDNA3-hu-BAG-1. After 48 h of transfection, cells were harvested, and the cytosol was prepared. Steroid-binding capacity (black bars) was assayed by charcoal assay. An aliquot of each cytosol was immunoadsorbed with the BuGR2 antibody, and GFPR GR was visualized by Western blotting. Aliquots of the cytosol were resolved by SDS-PAGE and immunoblotted for the GR, hsp70, and BAG-1. In both panels, steroid binding represents the means ± S.E. from four experiments.
16. 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, 16985–16992
17. Zeiner, M., and Gehring, U. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 11465–11469
18. Demand, J., Luders, J., and Hohfeld, J. (1998) *Mol. Cell. Biol.* 18, 2023–2028
19. Zeiner, M., Gebauer, M., and Gehring, U. (1997) *EMBO J.* 16, 5483–5490
20. Stuart, J. R., Myezka, D. G., Joss, L., Mitchell, R. S., McDonald, S. M., Xie, Z., Takayama, S., Reed, J. C., and Ely, K. R. (1998) *J. Biol. Chem.* 273, 22506–22514
21. Yang, X., Chernenko, G., Hao, Y., Ding, Z., Pater, M. M., Pater, A., and Tang, S.-C. (1998) *Oncogene* 17, 981–989
22. Froesch, B. A., Takayama, S., and Reed, J. C. (1998) *J. Biol. Chem.* 273, 11660–11666
23. Kullmann, M., Schneikert, J., Moll, J., Heck, S., Zeiner, M., Gehring, U., and Cato, A. C. B. (1998) *J. Biol. Chem.* 273, 14620–14625
24. Hartl, F. U. (1996) *Nature* 381, 571–580
25. Hiromura, M., Yano, M., Mori, H., Inoue, M., and Kido, H. (1998) *J. Biol. Chem.* 273, 5435–5438
26. Takayama, S., Bimston, D. N., Matsuzawa, S., Freeman, B. C., Aime-Sempe, C., Xie, Z., Morimoto, R. I., and Reed, J. C. (1997) *EMBO J.* 16, 4887–4896
27. Takayama, S., Krajewski, S., Krajewska, M., Kitada, S., Zapata, J., Kochel, K., Knee, D., Scudiero, D., Tudor, G., Miller, G. J., Miyashita, T., Yamada, M., and Reed, J. C. (1996) *Genomics* 35, 494–498
28. Galigniana, M. D., Scruggs, J. L., Harrington, J., Welsh, M. J., Carter-Su, C., Houssley, P. R., and Pratt, W. B. (1998) *Mol. Endocrinol.* 12, 1903–1913
29. Srivivasan, G., Post, J. F. M., and Thompson, E. B. (1997) *J. Steroid Biochem. Mol. Biol.* 60, 1–9
30. Johnson, J. L., and Toft, D. O. (1994) *J. Biol. Chem.* 269, 24989–24993
31. Caplan, A. J., Tsai, J., Casey, P. J., and Douglas, M. G. (1992) *J. Biol. Chem.* 267, 18890–18895
32. Schumacher, R. J., Hansen, W. J., Freeman, B. C., Alnemri, E., Litwack, G., and Toft, D. O. (1996) *Biochemistry* 35, 14889–14898
33. Smith, D. F., Sullivan, W. P., Marion, T. N., Zaitsu, K., Madden, B., McCormick, D. J., and Toft, D. O. (1993) *Mol. Cell. Biol.* 13, 869–876
34. Chen, S., Prapapanich, V., Rimmeron, B., and Smith, D. F. (1996) *Mol. Endocrinol.* 10, 682–693
35. Bimston, D., Song, J., Winchester, D., Takayama, S., Reed, J. C., and Morimoto, R. I. (1998) *EMBO J.* 17, 6871–6878
36. Hutchison, K. A., Dittmar, K. D., and Pratt, W. B. (1994) *J. Biol. Chem.* 269, 27894–27899
37. Grenert, J. P., Johnson, B. D., and Toft, D. O. (1999) *J. Biol. Chem.* 274, 36794–36799
38. Whitesell, L., Minnaugh, E. G., DeCosta, B., Myers, C. E., and Neckers, L. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 8324–8328
39. Sullivan W., Stensgard, B., Caucutt, G., Bartha, B., McMahon, N., Alnemri, E. S., Litwack, G., and Toft, D. (1997) *J. Biol. Chem.* 272, 8007–8012
40. Grenert, J. P., Sullivan, W. P., Fadden, P., Haystead, T. A. J., Clark, J., Minnaugh, E., Knuttsch, H., Ochel, H.-J., Schulte, T. W., Sausville, E., Neckers, L. M., and Toft, D. O. (1997) *J. Biol. Chem.* 272, 23845–23850
41. Smith, D. F., Whitesell, L., Nair, S. C., Chen, S., Prapapanich, V., and Toft, D. O. (1998) *J. Biol. Chem.* 273, 36794–36798
42. Bresnick, E. H., Dalman, F. C., Sanchez, E. R., and Pratt, W. B. (1999) *J. Biol. Chem.* 264, 4992–4997
43. Hutchison, K. A., Czar, M. J., Scherrer, L. C., and Pratt, W. B. (1992) *J. Biol. Chem.* 267, 14047–14053
44. Hutchison, K. A., Dittmar, K. D., and Pratt, W. B. (1994) *J. Biol. Chem.* 269, 27894–27899
45. Grenert, J. P., Johnson, B. D., and Toft, D. O. (1999) *J. Biol. Chem.* 274, 17528–17533