hsp70 Interacting Protein Hip Does Not Affect Glucocorticoid Receptor Folding by the hsp90-Based Chaperone Machinery Except To Oppose the Effect of BAG-1[†]

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ABSTRACT: Reticulocyte lysate contains a chaperone system that assembles glucocorticoid receptor (GR)·hsp90 heterocomplexes. Using purified proteins, we have prepared a five-protein heterocomplex assembly system consisting of two proteins essential for heterocomplex assembly—hsp90 and hsp70—and three proteins that act as co-chaperones to enhance assembly—Hop, hsp40, p23 [Morishima, Y., Kanelakis, K. C., Silverstein, A. M., Dittmar, K. D., Estrada, L., and Pratt, W. B. (2000) *J. Biol. Chem. 275*, 6894−6900]. The hsp70 co-chaperone Hip has been recovered in receptor·hsp90 heterocomplexes at an intermediate stage of assembly in reticulocyte lysate, and Hip is also thought to be an intrinsic component of the assembly machinery. Here we show that immunodepletion of Hip from reticulocyte lysate or addition of high levels of Hip to the purified five-protein system does not affect GR·hsp90 heterocomplex assembly or the activation of steroid binding activity that occurs with assembly. Despite the fact that Hip does not affect assembly, it is recovered in GR·hsp90 heterocomplexes assembled by both systems. In the five-protein system, Hip prevents inhibition of assembly by the hsp70 co-chaperone BAG-1, and cotransfection of Hip with BAG-1 opposes BAG-1 reduction of steroid binding activity in COS cells. We conclude that Hip is not a component of the assembly machinery but that it could play a regulatory role in opposition to BAG-1.

A multiprotein chaperone system originally identified in rabbit reticulocyte lysate forms complexes between the ubiquitous and essential heat shock protein hsp90[1] and a variety of proteins involved in signal transduction (e.g., steroid and dioxin receptors, multiple signaling protein kinases) (see ref *1* for review). Genetic studies have shown this association with hsp90 to be critical for signaling via several systems (*2*−*5*), and binding to hsp90 is required for some of the steroid receptors and the dioxin receptor to have ligand binding activity (*6*−*8*). Both biochemical observations (*9*, *10*) and data derived from GR mutants (*11*, *12*) support the idea (*7*) that the hsp90-based chaperone machinery directs the ATP-dependent partial unfolding of the GR ligand binding domain to open a hydrophobic steroid binding cleft to access by steroid. This hsp90 heterocomplex assembly system is widely distributed (probably ubiquitous) among animal and plant cells (*13*), with complete conservation of both hsp90 (*14*) and hsp70[2] (*13*) function, suggesting that the system is essential for fundamental processes in eukaryotic cells.

An hsp90 heterocomplex assembly system has been reconstituted from five purified proteins—hsp90, hsp70, Hop (60-kDa hsp organizer protein), hsp40, and p23 (*14*−*17*). These proteins can function together as a machinery (*18*), with hsp90 and hsp70 being required for opening the steroid binding cleft in the GR and the other proteins (Hop, hsp40, and p23) acting as co-chaperones that increase the efficiency of stable GR·hsp90 heterocomplex assembly (*19*). Assembly of GR·hsp90 heterocomplexes consists of at least two sequential ATP-dependent steps (*20*). Initially, hsp70 binds to the receptor, and in a step promoted by both ATP and hsp40 (provided as the yeast homologue YDJ-1), the receptor is converted to a state that can bind hsp90. In a subsequent step involving hsp90, Hop, and p23, an ATP-dependent conversion of the GR to the steroid binding form takes place (*20*). Because the receptor-bound hsp90 must assume its ATP-dependent conformation to yield steroid binding activity (*21*) and to bind p23 (*22*), which is present in the final GR·hsp90 heterocomplex, it seems that hsp90 is converted from its ADP-dependent conformation to its ATP-dependent conformation in this second step.

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[1] Abbreviations: hsp, heat shock protein; GR, glucocorticoid receptor; PAGE, polyacrylamide gel electrophoresis; TES, 2-{[2-hydroxy-1,1-bis (hydroxymethyl)ethyl]amino}ethanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein.

[2] In this paper, we will use the term hsp70 collectively to refer to both the heat-shock induced hsp70 and the constitutively expressed heat shock cognate hsp70.

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Although GR·hsp90 heterocomplex assembly can be carried out by the purified five-protein system, two hsp70 co-chaperones that have been detected in steroid receptor heterocomplexes are potential participants in, or regulators of, this hsp90-based chaperone system. A murine protein BAG-1 (Bcl-2-associated gene product-1) (*23*, *24*) and the human homolog, which is called RAP46 [46-kDa receptor-associated protein (*25*)] or Hap46 [hsp70-associating protein (*26*)], have been recovered with several nuclear receptors (*25*, *27*), including the GR. BAG-1 and Hap46 bind to the amino-terminal ATP-binding domain of hsp70 (*26*, *28*, *29*) and under some circumstances may (a) accelerate the dissociation of ADP, thus promoting conversion of hsp70 to its ATP-bound conformation (*29*, *30*, *31*), or (b) uncouple ATP hydrolysis from peptide substrate binding/release reactions (*32*). BAG-1 is present in reticulocyte lysate at a low molar ratio (~0.03−0.06) with respect to hsp70 (*33*). At similar low molar ratios, purified BAG-1 does not affect GR unfolding to the steroid binding state by the purified five-protein system; however, at high levels approaching stoichiometry with hsp70, GR unfolding and GR·hsp90 heterocomplex assembly are inhibited (*33*). Recently, Hap46 has been found to affect GR-mediated transactivation independent of any potential cytoplasmic action on GR·hsp90 heterocomplex assembly (*34*, *35*).

A 48-kDa protein recovered in progesterone receptor·hsp90 heterocomplexes at early times during assembly in reticulocyte lysate (*36*) was subsequently shown (*37*) to be the hsp70 co-chaperone Hip (hsc70-interacting protein) discovered by Höhfeld et al. (*38*). Hip binds to the ATPase domain of hsp70, stabilizing the ADP state, which has a high affinity for nonnative substrate protein (*38*). Hip and BAG-1 compete with each other in binding to the hsp70 ATPase domain (*29*, *39*). Small amounts of both Hip and BAG-1 are present in hsp90·Hop·hsp70 complexes immunoadsorbed from reticulocyte lysate, suggesting at least two subtypes of complexes depending upon the hsp70 co-chaperone that is present (*33*). Unlike mature progesterone receptor·hsp90 heterocomplexes, which do not contain Hip, so-called intermediate receptor heterocomplexes formed in reticulocyte lysate in the presence of the hsp90 inhibitor geldanamycin do contain Hip (*40*). Thus, Hip has been considered to be a functional component at an intermediate stage of the heterocomplex assembly process in reticulocyte lysate. Prapapanich et al. (*41*) prepared a mutant form of Hip that caused a concentration-dependent inhibition of receptor·hsp90 heterocomplex assembly in reticulocycte lysate, and they concluded that Hip was required for progression from an early receptor complex with hsp70 into later complexes containing hsp90.

It is generally accepted that Hip is an important functional component of the receptor·hsp90 heterocomplex assembly machinery (reviewed in refs *42* and *43*), but Hip function in assembly has not yet been directly tested. Because functional receptor heterocomplexes with hsp90 can be assembled by the purified five-protein system, it is clear that Hip is not essential for assembly or for generating the steroid binding state (*16*−*19*). Here, we show that immunodepletion of Hip from reticulocyte lysate does not affect either GR unfolding or GR·hsp90 heterocomplex assembly. Addition of high levels of purified Hip to the five-protein assembly system does not affect the rate or extent of GR·hsp90 heterocomplex assembly or generation of steroid binding activity, despite the fact that Hip is present in both intermediate receptor heterocomplexes prepared in the presence of geldanamycin and in GR·hsp90 heterocomplexes with functional steroid binding sites. Hip does, however, prevent BAG-1 inhibition of GR unfolding by the five-protein chaperone system, and transfection of Hip opposes BAG-1 reduction of steroid binding activity in vivo. Our data are consistent with the conclusion that Hip is not an intrinsic component of the hsp90-based chaperone machinery. However, Hip could play a regulatory role in opposition to BAG-1.

EXPERIMENTAL PROCEDURES

Materials. [6,7-^{3}H]Triamcinolone acetonide (42.8 Ci/mmol), ^{125}I-conjugated goat anti-mouse and anti-rabbit IgGs were obtained from DuPont NEN. Untreated rabbit reticulocyte lysate was from Green Hectares (Oregon, WI). Protein A-Sepharose and goat anti-mouse horseradish peroxidase conjugates were from Sigma, and donkey anti-rabbit IgG was from Pierce (Rockford, IL). Complete-Mini protease inhibitor cocktail was from Boehringer (Mannheim, Germany). The BuGR2 monoclonal IgG antibody against the GR and the 2G6 monoclonal anti-p48 IgG against Hip were from Affinity Bioreagents (Golden, CO). The AC88 monoclonal IgG against hsp90 and the N27F3−4 anti-72/73-kDa hsp monoclonal IgG (anti-hsp70) were from StressGen (Victoria, BC, Canada). The BAG-1 (C-16) affinity-purified rabbit polyclonal antibody was from Santa Cruz Biotechnologies (Santa Cruz, CA). The JJ3 monoclonal IgG against p23 was described previously (*44*). *E. coli* expressing YDJ-1 was a gift from Dr. Avrom Caplan (Mount Sinai School of Medicine). The DS14F5 monoclonal IgG against Hop, *E. coli* expressing Hop, and human Hip cDNA were kindly provided by Dr. David F. Smith (Mayo Clinic, Scottsdale, AZ). Hybridoma cells producing the FiGR monoclonal IgG against the GR were generously provided by Dr. Jack Bodwell (Dartmouth Medical School). The cDNA for expressing GFP-GR, described previously (*45*), was provided by Dr. Paul Housley (University of South Carolina School of Medicine). The baculovirus for mouse GR was described previously (*20*). Construction of the pcDNA3-hu-BAG-1 plasmid for expression of human BAG-1 has been described previously (*46*). Geldanamycin was obtained from the Drug Synthesis and Chemistry Branch of the Developmental Therapeutics Program, National Cancer Institute.

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 2.4 μg/mL GFP-GR cDNA, 0.3 μg/mL pcDNA3 vector or pcDNA3-hu-BAG-1, and 16 μg/mL pCI vector or pCI-Hip. The DNAs were preincubated for 10 min at room temperature with 9 μL of TransFast reagent/μg of DNA in 200 μL of DMEM, and the mixtures were added to the cell cultures. After 2 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 by 10% charcoal-stripped bovine calf serum for an additional 24 h of incubation. The cells were washed three times with Earle's balanced saline and suspended in 1 vol 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 and centrifuged for 30 min at 100000*g* to prepare the cytosol.

To achieve the concentrations of cDNAs used in triple transfection, the concentration of pcDNA3-hu-BAG-1 was first titrated on cotransfection with GFP-GR cDNA, and 0.3 mg/mL was the lowest concentration to yield 50% inhibition of steroid binding activity. When pCI vector was added in a triple transfection, it was determined that total cDNA levels higher than 20 mg/mL resulted in some decrease in GFP-GR expression. Thus, the amount of pCI-HIP was set at 16 mg/mL to yield the highest Hip:BAG-1 ratio possible without an effect of high DNA levels on GFP-GR expression.

Expression of Mouse GR in Sf9 Cells. Sf9 cells were grown in SFM900 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 0.1% glucose at infection and 24 h postinfection as described by Srinivasan et al. (*47*). Cells were harvested, washed in Hanks BSS, resuspended in 1.5 vol of buffer (10 mM Hepes, pH 7.5, 1 mM EDTA, 20 mM molybdate, and 1 mM PMSF) with 1 tablet of Complete-Mini protease inhibitor mixture per 3 mL of buffer, and ruptured by Dounce homogenization. The lysate was then centrifuged at 100000*g* for 30 min, and the supernatant was collected, aliquoted, flash-frozen and stored at −70 °C.

Glucocorticoid Receptor Heterocomplex Reconstitution. Glucocorticoid receptors were immunoadsorbed from 50 μL aliquots of Sf9 cytosol by rotation for 2 h at 4 °C with 14 μL of protein A-Sepharose precoupled to 7 μL of FiGR ascites suspended in 200 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 immunopellet an additional 2 h at 4 °C with 300 μL of 0.5 M NaCl in TEG. The pellets were then washed once with 1 mL of TEG followed by a second wash with 1 mL of Hepes buffer (10 mM Hepes, pH 7.4). FiGR immunopellets containing GR stripped of chaperones were incubated with 50 μL of rabbit reticulocyte lysate or with a mixture of five proteins (20 μg of purified hsp90, 15 μg of purified hsp70, 0.6 μg of purified human Hop, 6 μg of purified human p23, and 0.4 μg of purified YDJ-1) adjusted to 50 μL with HKD buffer (10 mM Hepes, 100 mM KCl, and 5 mM dithiothreitol, pH 7.35) containing 20 mM sodium molybdate. All incubations contained 5 μL of an ATP-regenerating system (50 mM ATP, 250 mM creatine phosphate, 20 mM MgOAc, and 100 units/mL creatine phosphokinase). The assay mixtures were incubated for 20 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 TEGM buffer (TEG with 20 mM sodium molybdate) and assayed for steroid binding capacity and, in most experiments, for receptor-associated proteins.

Assay of Steroid Binding Capacity. Immune pellets to be assayed for steroid binding were incubated overnight at 4 °C in 50 μL HEM (10 mM Hepes, pH 7.5, 1 mM EDTA, 20 mM molybdate) buffer plus 50 nM [^{3}H]triamcinolone acetonide. Samples were then washed three times with 1 mL of TEGM and counted by liquid scintillation spectrometry. The steroid binding is expressed as counts/min of [^{3}H]-triamcinolone acetonide bound/FiGR immunopellet prepared from 50 μL of Sf9 cytosol.

Using Sf9 overexpressed mouse GR saves a lot of time and expense compared to reconstituting heterocomplexes with GR immunoadsorbed from mouse L cell cytosol, but the extent of reconstitution is less. With L cell GR, we reactivated 75−100% of the steroid binding activity with reticulocyte lysate (*48*), but with mouse GR from Sf9 cells, we reactivate 13−15% of the receptors (*19*). The amount of GR immunoadsorbed from Sf9 cytosol has been assayed, and from the specific activity of the [^{3}H]triamcinolone acetonide, we calculate that 40 000 cpm bound/GR immunopellet from 50 μL of Sf9 cytosol represents ∼0.13 mol of steroid bound/mol of GR (*20*).

For assay of steroid binding in the cytosol from COS-7 cells, 100 μL of cytosol was incubated overnight with 50 nM [^{3}H]triamcinolone acetonide (±1000-fold excess of radioinert dexamethasone). Bound steroid was separated from free steroid by adding 1.5 vol of dextran-coated charcoal suspension [1% (w/v) 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 GR and associated proteins, immune pellets were resolved on 12% SDS−polyacrylamide gels, and transferred to Immobilon-P membranes. The membranes were probed with 0.25 μg/mL BuGR for GR, 1 μg/mL AC88 for hsp90, 1 μg/mL N27F3−4 for hsp70, 0.1% DS14F5 mouse ascites for Hop, 1 μg/mL 2G6 for Hip, or 1 μg/mL BAG-1 (C-16) for BAG-1. The immunoblots were then incubated a second time with the appropriate ^{125}I-conjugated or horseradish peroxidase-conjugated counter-antibody to visualize the immunoreactive bands.

Protein Purification. hsp90 and hsp70 were purified from rabbit reticulocyte lysate by sequential chromatography on DE52, hydroxylapatite, and ATP-agarose as described previously (*49*). Human p23 was purified from 10 mL of bacterial lysate by chromatography on DE52, as described (*50*), followed by hydroxylapatite chromatography. For purification of YDJ-1, bacterial sonicates were cleared by centrifugation, and YDJ-1 was purified by sequential chromatography on DE52 and hydroxylapatite as described previously (*16*). The bacterial expression of YDJ-1 has been described (*51*) as has the expression of human Hop (*15*). Purification of human Hop was carried out in a similar manner by sequential chromatography on DE52 and hydroxylapatite. In all cases, the protein-containing fractions were identified by immunoblotting, and fractions from the final purification step were pooled, concentrated by Amicon filtration, dialyzed against HKD buffer, flash frozen, and stored at −70 °C.

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 previously described (*30*). The 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

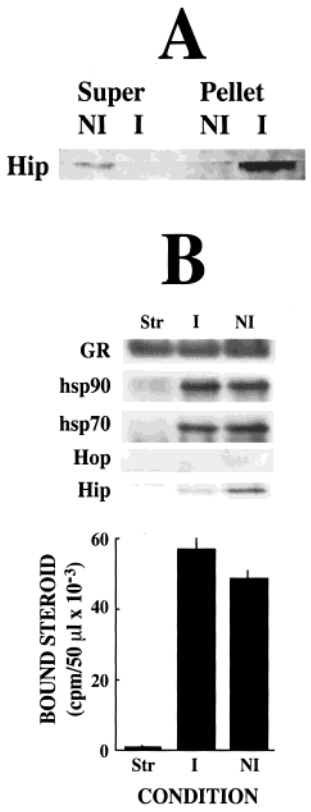

FIGURE 1: Immunodepletion of Hip from reticulocyte lysate does not affect GR unfolding or GR·hsp90 heterocomplex assembly. (A) Immunodepletion of Hip from reticulocyte lysate. Protein A-Sepharose pellets prebound with nonimmune IgG (NI) or with anti-Hip (I) were incubated with 50 μL of reticulocyte lysate for 2 h at 4 °C, then subjected to a second round of immunodepletion. The protein A-Sepharose immune pellet was washed three times with 1 mL of TEGM buffer, and the combined immune pellets and a portion (~20%) of the immunoadsorbed supernatant (super) were assayed for Hip by immunoblotting. (B) Reconstitution of GR·hsp90 heterocomplexes and steroid binding activity with reticulocyte lysate depleted of Hip by three rounds of immunoadsorption. Stripped GR immobilized on anti-GR/Sepharose (Str) was incubated 20 min at 30 °C with reticulocyte lysate absorbed with nonimmune IgG (NI) or reticulocyte lysate depleted with anti-Hip (I). GR, hsp90, hsp70, Hop, and Hip 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 [^{3}H]triamcinolone acetonide to determine steroid binding activity. The steroid binding activity is the average from two experiments with the range indicated by the vertical bars.

solution was cleared of insoluble protein by centrifugation at 100000*g*. *E. coli* expressing human Hip (*37*) were lysed by sonication in 20 mM Tris, pH 7.5, 10 mM thioglycerol, 1 mM EDTA and the protease inhibitors pepstatin (2 mg/mL), leupeptin (2 mg/mL), and 4-(2-aminoethyl) benzenesulfonyl fluoride (1 mM). Bacterial lysates were centrifuged and the soluble protein was loaded onto a DEAE-cellulose column and eluted with a 0 to 0.4 M KCl gradient. Fractions containing Hip were pooled and precipitated with 80% ammonium sulfate. The precipitate was then dissolved in the same buffer and loaded onto a 16/60 Superdex 200 sizing column. The peak containing Hip was pooled and further fractionated on a MonoQ FPLC column (10/10, Pharmacia Biotech Inc.) that was eluted with a linear gradient of 0−0.5 M KCl. The fractions containing Hip were pooled, dialyzed into 10 mM Tris-HCl, 1 mM DTT, and 1 mM EDTA, pH 7.5, and stored at −70 °C.

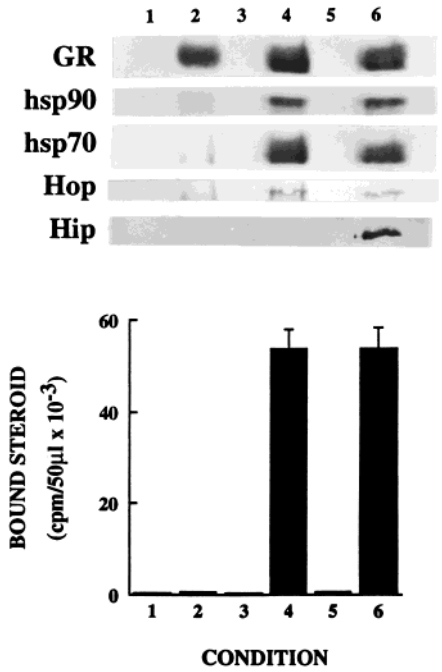

FIGURE 2: Purified Hip does not affect GR unfolding or GR·hsp90 heterocomplex assembly by the five-protein system but it is present in the reconstituted heterocomplex. GR immune pellets stripped of hsp90 were incubated for 20 min at 30 °C with purified hsp90, hsp70, Hop, YDJ-1, and p23 in the presence of 20 mM sodium molybdate and an ATP-regenerating system. GR, hsp90, hsp70, Hop, and Hip in the washed immune pellets were assayed by SDS−PAGE and Western blotting, and a portion of each immune pellet was incubated with [^{3}H]triamcinolone acetonide to determine steroid binding activity. Lanes 1 and 2, nonimmune (lane 1) and immune (lane 2) hsp90-stripped pellets; lanes 3 and 4, nonimmune (lane 3) and immune (lane 4) pellets reconstituted with hsp90 and other chaperones without Hip; lanes 5 and 6, nonimmune (lane 5) and immune (lane 6) pellets reconstituted in the presence of purified Hip at a molar ratio of 1.5:1 with respect to hsp70. The steroid binding activities (solid bars) represent the mean value from three experiments ± SEM.

RESULTS

Depletion of Hip from Reticulocyte Lysate. To determine if Hip was affecting (either negatively or positively) the function of the hsp90-based chaperone machinery in reticulocyte lysate, we immunodepleted the lysate of Hip. As shown in Figure 1A, two adsorptions with anti-Hip antibody markedly depleted Hip from the lysate. When three-times immunodepleted (I) lysate or mock depleted (NI) lysate was used to reconstitute stripped receptors (Figure 1B), both lysate preparations yielded comparable levels of hsp90 binding (Western blot) and steroid binding activity (bar graph). Hip is present in GR·hsp90 heterocomplexes prepared with mock depleted lysate, and there is about one-fifth the Hip in heterocomplexes prepared with Hip-depleted lysate. Because the immunodepletion of Hip from lysate is greater than the ~80% reduction of Hip in heterocomplexes prepared with the depleted lysate, Hip appears to be selectively enriched in the GR·hsp90 heterocomplex. The selective enrichment of Hip in receptor heterocomplexes has been noted previously by Prapapanich et al. (*41*), and it may reflect the fact that Hip binding to hsp70 is dependent upon activation of its ATPase activity by hsp40 (*38*), which is one of the earliest steps in receptor heterocomplex assembly (*20*).

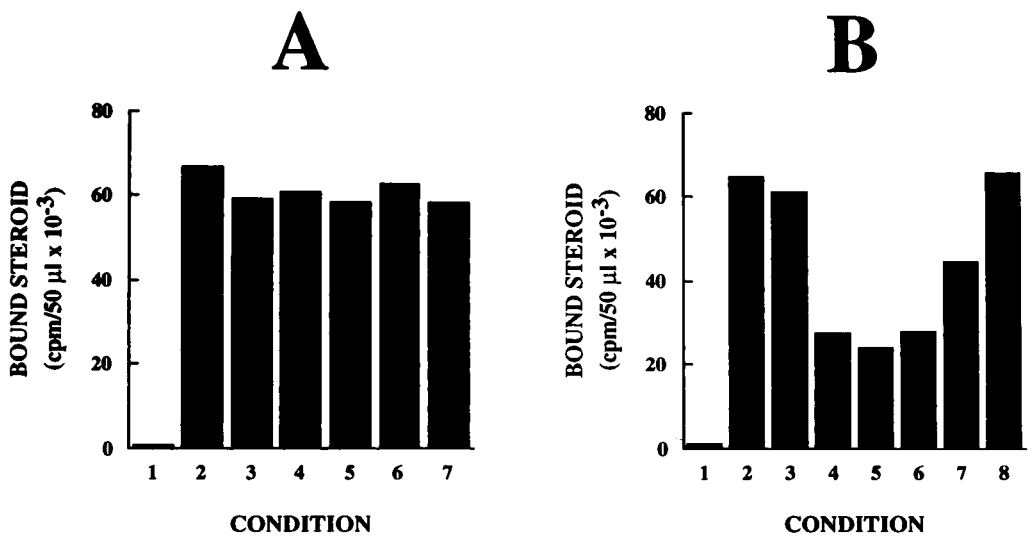

FIGURE 3: Purified Hip antagonizes BAG-1. (A) Reconstitution of GR steroid binding activity with the purified assembly system in the presence of various concentrations of Hip. Hsp90-stripped GR immune pellets were incubated for 20 min at 30 °C with the purified five-protein system in the presence of various concentrations of Hip, and steroid binding in the immune pellets was assayed. Conditions are as follows: lane 1, hsp90-stripped GR incubated with HKD buffer; lane 2, stripped GR incubated with reticulocyte lysate; lane 3, stripped GR incubated with the purified assembly system; lanes 4−7, stripped GR incubated with the purified assembly system in the presence of purified Hip at Hip:hsp70 molar ratios of 0.4 (lane 4), 0.75 (lane 5), 1.5 (lane 6), or 2.5 (lane 7). (B) Reconstitution of GR steroid binding activity with the purified system in the presence of various concentrations of Hip in the presence of BAG-1. Conditions are as follows: lane 1, stripped GR incubated with HKD buffer; lane 2, stripped GR incubated with reticulocyte lysate; lane 3, stripped GR incubated with the purified assembly system; lanes 4−8, stripped GR incubated with the purified assembly in the presence of BAG-1 at a BAG-1:hsp70 molar ratio of 0.5 without Hip (lane 4), or with Hip at Hip:hsp70 molar ratios of 0.4 (lane 5), 0.75 (lane 6), 1.5 (lane 7), 2.5 (lane 8). Note that BAG-1 is present in lanes 4−8 and only the Hip concentration is varied.

Effect of Hip Addition to the Purified Five-Protein Assembly System. As was suggested by Prapapanich et al. (*41*), the very small amount of Hip remaining in Hip-depleted reticulocyte lysate might be sufficient to satisfy any requirement for Hip in the assembly process. Thus, we examined the effect of purified Hip on GR·hsp90 heterocomplex assembly by the purified five-protein system. Stripped glucocorticoid receptors reconstituted with the purified system contained hsp90, hsp70, and some Hop (Figure 2, lane 4), and the relative amount of these components was not changed by assembly in the presence of Hip at a 1.5:1 molar ratio with respect to hsp70 (Figure 2, lane 6). The same amount of steroid binding activity is generated in the presence or absence of Hip (Figure 2, bar graph), and Hip has no effect on the rate at which steroid binding sites are activated by the five-protein system (data not shown). Despite the fact that Hip does not affect the rate or extent of GR unfolding, it enters the receptor heterocomplex (Figure 2, Western blot, lane 6).

As shown in Figure 3A, addition of Hip over a concentration range of 0.4−2.5 molar ratio of Hip to hsp70 has no effect on the generation of steroid binding activity by the five-protein system. However, the same amounts of Hip yield a concentration-dependent reduction in the inhibition of receptor activation produced by the hsp70 co-chaperone BAG-1 (Figure 3B). By comparing the intensity of bands developed with ^{125}I-labeled counter-antibody in immunoblots containing aliquots of rabbit reticulocyte lysate and standard amounts of purified hsp70 and Hip, we estimate the Hip:hsp70 molar ratio in reticulocyte lysate to be 0.5:1 (data not shown). Using a different method, Prapapanich et al. (*41*) have estimated the concentration of Hip in lysate to be about the same as that of hsp70. We have determined that BAG-1 is present in reticulocyte lysate at a molar ratio of 0.03−0.06 with respect to hsp70 (*33*). Thus, Hip is present in reticulocyte lysate at 10−20-fold the concentration of BAG-1. As shown in Figure 3B, at Hip:hsp70 molar ratio of 2.5:1 (lane 8), Hip blocks the inhibition of GR unfolding caused by the addition of BAG-1 at a BAG-1:hsp70 molar ratio of 0.5:1. The fact that Hip blocks the effect of BAG-1 at a Hip:BAG-1 molar ratio of 5:1 when the two hsp70 co-chaperones are added to the purified five-protein assembly system suggests that, at the Hip:BAG-1 ratio of >10:1 that exists in reticulocyte lysate, the concentration of Hip is sufficient to antagonize the action of BAG-1. When BAG-1 levels are increased, as they are in some transformed cells (*52*), BAG-1 effects could overwhelm the Hip antagonism and become dominant.

Hip Overexpression Opposes BAG-1 Action In Vivo. We have previously shown that BAG-1 overexpression in COS-7 cells decreases the steroid binding activity of cytosolic GR (*33*). To determine if overexpression of Hip would counteract the effect of BAG-1, COS-7 cells were cotransfected with cDNA expressing a fusion protein containing the mouse GR and cDNAs expressing Hip or BAG-1 or both. As shown in Figure 4, expression of Hip alone (lane 3) did not affect GR steroid binding activity, whereas expression of BAG-1 reduced steroid binding activity from the transfected GR by ∼60% (lane 4) with respect to the vector control (lane 2). Coexpression of Hip with BAG-1 (lane 5) attenuated the BAG-1 reduction in steroid binding activity. Thus, overexpression of Hip in vivo does not affect receptor steroid binding activity, except to oppose the reduction in activity caused by coexpression of BAG-1.

Hip Does Not Stabilize hsp70 Binding to GR. The ADP-bound state of hsp70 has been shown to have a high affinity for nonnative substrate protein (*38*), and Hip might reasonably be expected to stabilize the binding of hsp70 to the GR.

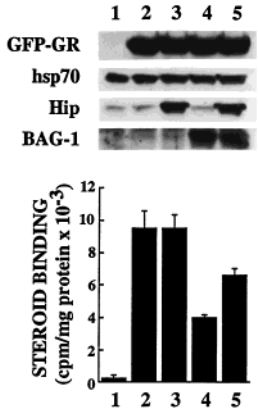

FIGURE 4: Hip overexpression attenuates BAG-1-dependent reduction of steroid binding activity. COS-7 cells were transfected with GFP-GR cDNA and pcDNA3-hu-BAG-1 or pCI-Hip or the appropriate vector as described in the Experimental Procedures. After 48 h of transfection, cells were harvested, cytosol was prepared, and steroid binding capacity (black bars) was determined by charcoal assay. An aliquot of each cytosol was immunoadsorbed with the BuGR2 antibody and GFP-GR was visualized by Western blotting. Aliquots of the cytosol were resolved by SDS−PAGE and immunoblotted for hsp70, Hip, and BAG-1. Conditions were as follows: lane 1, no transfection; lane 2, transfected with GFP-GR, pcDNA3, and pCI vectors; lane 3, transfected with GFP-GR, pcDNA3 vector, and pCI-Hip; lane 4, transfected with GFP-GR, pCI vector, and pcDNA3-hu-BAG-1; lane 5, transfected with GFP-GR, pcDNA3-hu-BAG-1, and pCI-Hip. Steroid binding represents the means ± SE from three experiments. The steroid binding activity in lane 4 is significantly different from lanes 2 and 5 at $p < 0.01$.

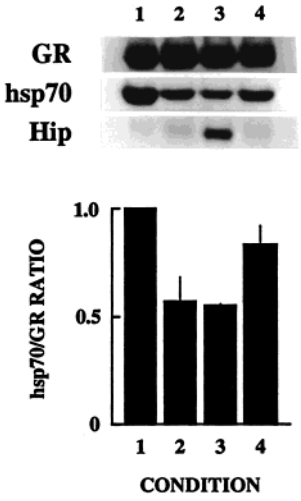

FIGURE 5: Hip does not inhibit hsp70 dissociation from GR·hsp70 complexes. Stripped GR immune pellets were incubated for 5 min at 30 °C with hsp70, YDJ-1, and the ATP-regenerating system. The GR·hsp70 complexes were washed and incubated for 20 min at 30 °C in HKD buffer with additions as indicated. The pellets were washed again, and proteins were resolved by gel electrophoresis and Western blotting followed by incubation with ^{125}I-labeled counterantibody. Lane 1, no 20 min incubation; lane 2, incubated with the ATP-regenerating system alone; lane 3, incubated with the ATP-regenerating system and purified Hip at a molar ratio of 3:1 with respect to hsp70; lane 4, incubated with HKD buffer alone. The hsp70/GR ratios in the bar graphs were determined by scanning autoradiograms of the iodine labeled bands such as those shown at the top, and they are expressed as a fraction of the lane 1 (no incubation) control. The values represent the average of two experiments with the range indicated by the vertical lines.

In the experiment of Figure 5, stripped GR immunopellets were incubated for 5 min with hsp70, YDJ-1, and ATP, and then the pellets were washed. The GR·hsp70 complexes in these washed immune pellets can be activated to the steroid binding state by a subsequent 20 min incubation at 30 °C with hsp90, Hop, p23, and molybdate (*20*). During the second incubation of 20 min, a portion of the receptor-bound hsp70 dissociates even when the GR·hsp70 complex is incubated in buffer alone, and this dissociation is increased by ATP (*20*). Because YDJ-1 markedly promotes the production of GR·hsp70 complexes that can be activated on subsequent incubation with the other proteins of the assembly system (*20*), it is reasonable to conclude that the hsp70 that is prebound to the GR has been converted to its ADP state. In the experiment of Figure 5, we have asked if Hip stabilizes the binding of hsp70 that is prebound to the GR. As shown in Figure 5, hsp70 dissociates from preformed GR·hsp70 complexes that are incubated in buffer alone (lane 4), and there is more dissociation when ATP is present (lane 2), but the presence of Hip does not inhibit this dissociation (lane 3). Thus, Hip does not appear to stabilize the association of hsp70 with its GR substrate (Figure 5).

Hip Is Not Enriched in GR Heterocomplexes Prepared with Geldanamycin. In a study of the time course of progesterone receptor·hsp90 heterocomplex assembly in reticulocyte lysate, Hip was found in intermediate complexes but not in mature complexes (*36*). Subsequently, it was shown that intermediate progesterone receptor heterocomplexes formed by reticulocyte lysate in the presence of geldanamycin differ from mature complexes formed in the absence of the hsp90 inhibitor in that they contain a substantially increased amount of Hip as well as increased Hop (*40*). Because the observations we make in this paper indicate that Hip is not an intrinsic component of the hsp90-based chaperone machinery, we have reexamined the effect of geldanamycin, on the composition of GR·hsp90 heterocomplexes prepared in the presence and absence of geldanamycin. As shown in Figure 6, GR·hsp90 heterocomplexes assembled in reticulocyte lysate in the presence of geldanamycin (lane 3) contain decreased amounts of hsp90 and increased amounts of Hop, as has been reported previously for progesterone receptor complexes assembled in reticulocyte lysate (*40*). The same pattern was seen when geldanamycin was added to the purified five-protein system, either without (lane 5) or with purified Hip (lane 7). However, the amount of Hip that enters the heterocomplex does not change with geldanamycin treatment of reticulocyte lysate (cf. lanes 2 and 3) or with geldanamycin treatment of the five-protein assembly system (cf. lanes 6 and 7). Thus, we do not find that Hip is uniquely present in or enriched in intermediate heterocomplexes.

DISCUSSION

Because assembly of heterocomplexes with hsp90 is critical to a number of cellular functions and because the components of the heterocomplex machinery are widely (probably ubiquitously) distributed among eukaryotes (*1*), it is important to define the intrinsic components of the machinery so that mechanistic studies of heterocomplex assembly can be undertaken. Because a previous study has indicated that Hip is an intrinsic component of the multiprotein assembly system (*41*), we have directly studied the effect of purified Hip in two cell-free assembly systems. We found that immunodepletion of Hip from reticulocyte lysate

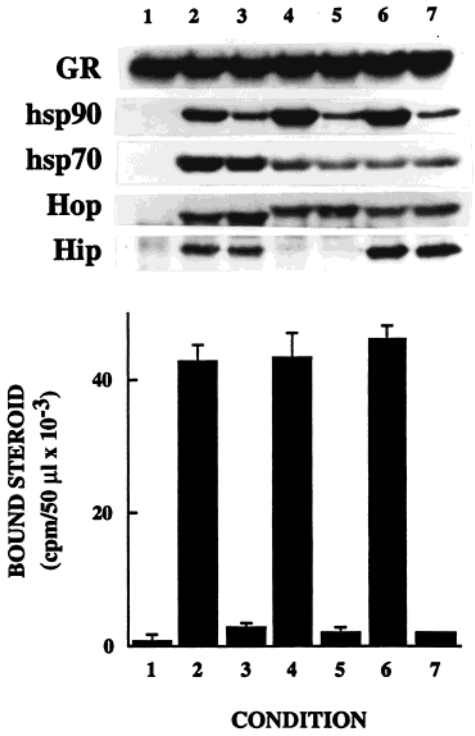

FIGURE 6: Hip is found in both intermediate GR heterocomplexes and steroid binding GR·hsp90 complexes. Stripped GR immune pellets were incubated for 20 min at 30 °C with rabbit reticulocyte lysate or with the five-protein system in the presence or absence of 10 μM geldanamycin. GR, hsp90, hsp70, Hop, and Hip in the washed immune pellets were assayed by SDS−PAGE and Western blotting, and a portion of each immune pellet was incubated with [^{3}H]triamcinolone acetonide to determine steroid-binding activity. Lane 1, stripped receptor; lane 2, GR reconstituted with reticulocyte lysate; lane 3, reconstituted with reticulocyte lysate in the presence of geldanamycin; lane 4, reconstituted with the five-protein system; lane 5, reconstituted with the five-protein system in the presence of geldanamycin; lane 6, reconstituted with the five-protein system in the presence of Hip at a molar ratio of 1.5:1 with respect to hsp70; lane 7, reconstituted with the five-protein system in the presence of geldanamycin and Hip. Steroid binding data are means ± SE from three experiments. Note that the rabbit Hop in lanes 2 and 3 migrates faster than the human Hop in lanes 4−7.

(Figure 1) or addition of Hip to the purified five-protein assembly system (Figures 2 and 3A) did not affect GR·hsp90 heterocomplex assembly or activation of steroid binding activity. These observations lead us to conclude that Hip is not required for heterocomplex assembly and that Hip does not act as an hsp70 co-chaperone that enhances assembly, as was shown for hsp40 (*16*, *19*).

Although Hip stabilizes hsp70 in the ADP state, which has a high affinity for nonnative substrate protein (*38*), it did not stabilize hsp70 binding to the GR in the assay shown in Figure 5. In assays of protein refolding where a purified enzyme, such as luciferase, is thermally denatured and then reactivated with the aid of reticulocyte lysate or combinations of purified chaperones (*53*−*55*), hsp70 may bind to extended segments of hydrophobic amino acids. This type of hsp70 interaction with non-native substrate may be more stable when hsp70 is in its ADP state. However, the GR that is bound by hsp70 in the presence of ATP and YDJ-1 may not be in a nonnative state that is similar to those of denatured enzymes that have been studied in refolding assays. Indeed, from stepwise assembly experiments, we have predicted that hsp70 binds initially in its ATP-dependent conformation to a properly folded GR and is then converted to the ADP-dependent form in preparing the GR to bind hsp90 (*20*). Whatever the nature of this critical GR·hsp70 interaction, the complex of hsp70 with its GR substrate is not stabilized by Hip (Figure 5).

High levels of Hip counteract the inhibition of GR unfolding caused by high levels of BAG-1, both in the purified assembly system (Figure 3B) and after transfection into COS-7 cells (Figure 4). At the low levels that exist in reticulocyte lysate, BAG-1 may have more subtle effects on heterocomplex assembly/disassembly cycling that are opposed by Hip. It is our impression that GR·hsp90 heterocomplexes formed by the purified assembly system are not subject to the same dynamic cycling as occurs in the more complex reticulocyte lysate system, and it is possible that subtle effects on cycling would not be observed in the much simpler five-protein system. Thus, in complex cellular systems, Hip opposition to BAG-1 might play a regulatory role in the dynamics of receptor heterocomplex assembly/disassembly.

The important conclusion from this work is that, despite the fact that Hip is present in both intermediate and steroid binding GR·hsp90 heterocomplexes (Figure 6), it does not by itself affect heterocomplex assembly. Like BAG-1, Hip is a ubiquitous hsp70 co-chaperone, and as proposed for BAG-1 (*34*, *35*), it may play a role in receptor action beyond heterocomplex assembly, possibly at the level of GR-mediated transactivation. Although, they are not intrinsic components of the heterocomplex assembly machinery, the hsp70 co-chaperones Hip and BAG-1/Hap46 might play opposing regulatory roles in dynamic GR·hsp90 assembly/disassembly cycling in cells.

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