Evidence for Iterative Ratcheting of Receptor-Bound hsp70 between Its ATP and ADP Conformations during Assembly of Glucocorticoid Receptor·hsp90 Heterocomplexes†

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ABSTRACT: hsp90 and hsp70 are essential components of a five-protein system, including also the nonessential cochaperones Hop, hsp40, and p23, that assembles glucocorticoid receptor (GR)·hsp90 heterocomplexes and causes the simultaneous opening of the steroid binding pocket to access by steroid. The first event in assembly is the ATP-dependent and hsp40 (YDJ-1)-dependent binding of hsp70 to the GR, which primes the receptor for subsequent ATP-dependent activation by hsp90 [Morishima, Y., Murphy, P. J. M., Li, D. P., Sanchez, E. R., and Pratt, W. B. (2000) *J. Biol. Chem. 275*, 18054−18060]. Here, we demonstrate that, during the priming step, ATP-bound hsp70 is converted to GR-bound hsp70 that is $\sim^1/_3$ in the ADP- and $\sim^2/_3$ in the ATP-dependent conformation. In the second step, hsp90, which is provided in the non-nucleotide-bound state, is converted to GR-bound hsp90 in the ATP-dependent conformation. The ATPase activity of hsp70 is K^+-dependent, and the priming step is K^+-dependent. Surprisingly, the subsequent hsp90-dependent step, which is rate-limiting for receptor activation, is also potassium-dependent. This suggests that GR-bound hsp70 is also converted from the ATP-dependent to the ADP-dependent conformation while it cooperates with hsp90 to activate steroid binding activity. Because the priming step requires both sustained high levels of ATP and YDJ-1 for optimal activity and because both steps require potassium, we predict that receptor-bound hsp70 undergoes iterative ratcheting between its ATP- and ADP-dependent conformations in opening the hydrophobic steroid binding pocket.

Unliganded steroid receptors are bound via their ligand binding domains (LBDs)[1] to the ubiquitous molecular chaperone hsp90 (for a review, see ref *1*). The glucocorticoid receptor (GR) must be bound to hsp90 for the hydrophobic ligand binding pocket to be open to access by steroid (*2*, *3*). A multichaperone system that was first studied in reticulocyte lysate (*4*, *5*) assembles receptor·hsp90 heterocomplexes and converts the unliganded GR LBD from a nonbinding state to a state with normal high-affinity steroid binding activity (*2*). This assembly system has been reconstituted (*6*), and a mixture of five purified proteins is now used to achieve optimal receptor·hsp90 heterocomplex assembly (*7*, *8*). The five proteins are hsp90, hsp70,[2] Hop (hsp organizer protein), hsp40, and p23. hsp70 and hsp90 are both required to open the steroid binding cleft in the GR LBD (*9*, *10*), and p23 stabilizes GR·hsp90 complexes once they are assembled (*11*).

The two key components of the system, hsp70 (for a review, see ref *12*) and hsp90 (for a review, see ref *13*), both possess nucleotide binding sites and have intrinsic ATPase activity, with the peptide binding activity of the chaperones being coupled to the binding of ATP versus ADP. For both of these chaperones, it is the ADP-bound conformation that possesses high affinity for hydrophobic substrates (*12*, *14*, *15*). The ATPase activity of hsp70 is stimulated by hsp40, the vertebrate homologue of the bacterial DnaJ protein, and hsp40 (provided as the purified yeast homologue YDJ-1) increases the level of receptor·hsp90 heterocomplex assembly (*7*, *8*), but is not required for assembly (*9*). Hop is a protein that binds independently to hsp90 and hsp70 to form an hsp90·Hop·hsp70 complex (*16*). Although Hop is not required for opening the steroid binding cleft in the GR LBD, it increases the rate of the process (*9*), consistent with the notion (*17*, *18*) that hsp90 and hsp70 function together more effectively when they are brought together in a machinery than when they are present as separate units.

Recently, we have carried out two-stage GR·hsp90 assembly experiments demonstrating sequential ATP-dependent events involving first hsp70 and then hsp90 in opening of the steroid binding pocket (*19*). In the first step of assembly, the immunoadsorbed GR is incubated with purified hsp70, YDJ-1, and an ATP-regenerating system, producing a GR·hsp70 complex that can be washed free of unbound hsp70 and then incubated with purified hsp90, Hop, and p23. As illustrated in the diagram in Figure 8, during the first

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[1] Abbreviations: hsp, heat shock protein; GR, glucocorticoid receptor; LBD, ligand binding domain; Hop, 60 kDa hsp organizer protein; EGS, energy (ATP)-generating system.

[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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reaction, the GR is "primed" to bind hsp90 during the second incubation. In the second step, the GR undergoes ATP-dependent conversion to the steroid binding form and the GR-bound hsp90 is converted to its ATP-dependent conformation. This is inferred from observations of Toft and his collaborators, who have shown that receptor-bound hsp90 must be in its ATP-dependent conformation for receptor to bind steroid (*20*) and that it is the ATP-dependent conformation of hsp90 that binds p23 (*14*), which is present in the GR·hsp90 complex formed in the second step (*19*). In the model of Figure 8, we predict (*19*) that hsp70 in its ATP-dependent conformation binds initially to the folded GR and the GR-bound hsp70 is then converted to the ADP-dependent form with high affinity for hydrophobic substrate. This conversion initiates the opening of the hydrophobic steroid binding pocket such that it can now accept a hydrophobic binding form of hsp90, which in turn must be converted to its ATP-dependent conformation for the pocket to be accessible to steroid.

In this work, we test the prediction by assaying the chaperone-bound nucleotide before and after the priming step in two-step assembly of GR·hsp90 heterocomplexes. We show that our purified hsp70 is bound by ATP and that GR-bound hsp70 at the end of the priming step is bound by ADP. For optimal activity, the priming step by hsp70 requires YDJ-1, K^+, and a sustained high level of ATP, showing that both ATP binding and ATPase activity are required to produce GR·hsp70 complexes that can bind hsp90 in a manner that leads to opening of the steroid binding cleft in the LBD. Interestingly, K^+ and YDJ-1 also optimize activity during the second step, suggesting continued ATP binding and ATPase activity of the GR-bound hsp70. These observations lead us to a model in which GR-bound hsp70 ratchets back and forth between ATP- and ADP-dependent conformations during GR·hsp90 heterocomplex assembly and opening of the steroid binding cleft. As discussed later, it is likely that hsp90 undergoes a similar iterative ratcheting between ATP and ADP conformations after it binds to the hsp70-primed GR.

EXPERIMENTAL PROCEDURES

Materials. [6,7-^{3}H]Triamcinolone acetonide (38 Ci/mmol) and ^{125}I-conjugated goat anti-mouse IgG were obtained from DuPont NEN. Untreated rabbit reticulocyte lysate was from Green Hectares (Oregon, WI). Protein A−Sepharose and goat anti-mouse horseradish peroxidase conjugate were from Sigma. The BuGR2 monoclonal IgG antibody against the GR was 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). *Escherichia coli* expressing human p23, YDJ-1, and Hop were kindly provided by D. Toft (The Mayo Clinic, Rochester, MN), A. Caplan (Mount Sinai School of Medicine, New York, NY), and D. Smith (Mayo Clinic, Scottsdale, AZ), respectively. Hybridoma cells producing the FiGR monoclonal IgG against the GR were generously provided by J. Bodwell (Dartmouth Medical School, Hanover, NH).

Expression of the 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 the 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. (*21*). Cells were harvested, washed in Hank's buffered saline solution, resuspended in 1.5 volumes of buffer [10 mM Hepes (pH 7.35), 1 mM EDTA, 20 mM molybdate, and 1 mM phenylmethyl sulfonyl fluoride] 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. 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 various mixtures of purified proteins as noted, immunoadsorbed receptors were stripped of associated hsp90 by incubating the immunopellet for 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 7.35)]. For single-step assembly of GR·hsp90 heterocomplexes, FiGR immunopellets containing the GR stripped of chaperones were incubated with the five-protein assembly system (20 μg of purified hsp90, 15 μg of purified hsp70, 0.6 μg of purified human Hop, 6 μg of purified 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 and 5 μL of an ATP-regenerating system (50 mM ATP, 250 mM creatine phosphate, 20 mM magnesium acetate, 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 some experiments, for receptor-associated hsp70. In the experiments whose results are depicted in Figures 1 and 3, the autoradiogram of the GR immunoblot was scanned and the steroid binding capacity was normalized according to the relative amount of receptor. For two-step assembly, stripped GR immune pellets were mixed with hsp70, YDJ-1, and the ATP-regenerating system in a final volume of 55 μL adjusted with HKD buffer. After incubation for 5 min at 30 °C, the pellets were washed twice with TEG buffer and once with Hepes buffer and then incubated for 20 min at 30 °C with the ATP-regenerating system, 20 mM molybdate, and the purified heterocomplex assembly system without hsp70. At the end of the second incubation, steroid binding was assayed.

Assay of Steroid Binding Capacity. Immune pellets to be assayed for steroid binding were incubated overnight at 4 °C in 50 μL of HEM buffer [10 mM Hepes (pH 7.35), 1 mM EDTA, and 20 mM molybdate] with 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 per minute of [^{3}H]triamcinolone acetonide bound per FiGR immunopellet prepared from 50 μL of Sf9 cytosol.

Using the Sf9-overexpressed mouse GR saves a lot of time and expense compared to reconstituting heterocomplexes with the GR immunoadsorbed from mouse L cell cytosol, but the extent of reconstitution is lower. With the L cell GR, we reactivated 75−100% of the steroid binding activity with reticulocyte lysate (*22*), but with the mouse GR from Sf9 cells, we reactivate 13−15% of the receptors (*9*). 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 per GR immunopellet from 50 μL of Sf9 cytosol represents ~0.13 mol of steroid bound per mole of GR (*19*).

Western Blotting. To assay the GR and associated hsp70, 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 the GR or 1 μg/mL N27F3-4 for hsp70. The immunoblots were then incubated a second time with the appropriate ^{125}I-conjugated 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 (*23*). As we have noted before, hsp70 purified in this manner contains a small amount of hsp40 and trace amounts of Hop (identified by immunoblotting) that can be eliminated by a more fastidious purification (*9*). However, the yield of purified, hsp40-free, and Hop-free hsp70 is very low. Thus, the hsp70 used in this work contains small amounts of hsp40. Nevertheless, it still responds to addition of YDJ-1 as shown in Figure 1. Human p23 was purified from 10 mL of bacterial lysate by chromatography on DE52 followed by hydroxyapatite chromatography as described previously (*24*). For purification of YDJ-1, bacterial sonicates were cleared by centrifugation, and YDJ-1 was purified by sequential chromatography on DE52 and hydroxyapatite as described previously (*7*). The bacterial expression of YDJ-1 has been described (*25*) as has the expression of human Hop (*6*). Purification of human Hop was carried out in a similar manner by sequential chromatography on DE52 and hydroxyapatite. 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.

Determination of Nucleotide-Bound States of hsp70 and hsp90. Nucleotides were extracted with ice-cold 0.4 N perchloric acid from purified hsp70 and hsp90, as well as from GR·hsp70 pellets formed in the priming step and washed twice with 10 mM Hepes buffer with 2 mM $MgCl_2$. The ATP and ADP were then separated and quantitated by strong anion-exchange, high-performance liquid chromatography using a Waters (Milford, MA) gradient system controlled by Millenium software as previously described (*26*). Briefly, samples were loaded onto a 5 μm Partisphere 4.6 mm × 250 mm SAX column (Whatman, Hillsboro, OR), and nucleotides were eluted with a linear gradient of ammonium phosphate buffer ranging from 0.15 (pH 3.6) to 0.60 M (pH 3.8) at a flow rate of 2 mL/min. Retention times

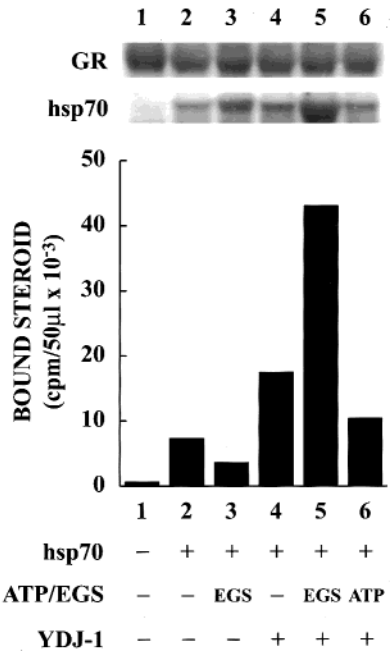

FIGURE 1: Optimal priming of the GR by hsp70 requires YDJ-1 and a sustained high level of ATP. Immune pellets with stripped receptors were incubated for 5 min at 30 °C with hsp70, YDJ-1, 5 mM ATP, and the 5 mM ATP-regenerating system (EGS) as indicated. The pellets were washed four times with TEG buffer and immunoblotted for GR and hsp70. Duplicate pellets were washed twice with TEG buffer and once with Hepes, and then incubated for 20 min at 30 °C with the purified heterocomplex assembly system without hsp70, and the pellets were washed and incubated with [^{3}H]triamcinolone acetonide to determine steroid binding activity.

were 7.7 and 23.3 min for ADP and ATP, respectively. Nucleotides were identified by their absorbance between 240 and 350 nm using a Waters photodiode array detector. ADP and ATP were quantitated by comparison of their peak areas with that of a known amount of appropriate standard at wavelengths of 254 and 281 nm. The concentration of purified hsp70 and hsp90 was determined by Bradford assay, and 20 μg of each was assayed for bound nucleotide. The amount of hsp70 in the primed GR·hsp70 complex was assayed by electrophoresing the immune pellet and standard amounts of purified hsp70 on the same gel and comparing the intensity of bands developed with the ^{125}I-labeled counterantibody in immunoblots. Approximately 2 μg of hsp70 in the primed GR·hsp70 complex was assayed for bound nucleotide. All perchloric acid extracts were filtered prior to the HPLC step, and after correction for the amount of nucleotide removed during filtration, the percent of the protein that could be accounted for in the nucleotide-bound form was calculated. In the samples of Figure 2, 88% of the hsp70 was accounted for in the ATP-bound form, <5% of the hsp90 was in the nucleotide-bound form, and 44% of the hsp70 in the primed GR·hsp70 complex was in the nucleotide (both ATP and ADP)-bound form. For the experiment whose results are depicted in Figure 5B where hsp90 was converted to its ATP- and ADP-bound forms, 17 μg of purified rabbit hsp90 was incubated with 5 mM ATP, with 5 mM ADP, or with HKD buffer in the presence of 2 mM $MgCl_2$ at 30 °C for 10 min. The free nucleotide was removed by four rounds of dextran-coated charcoal adsorption [1% (w/v) charcoal and 0.2% (w/v) dextran]. The bound

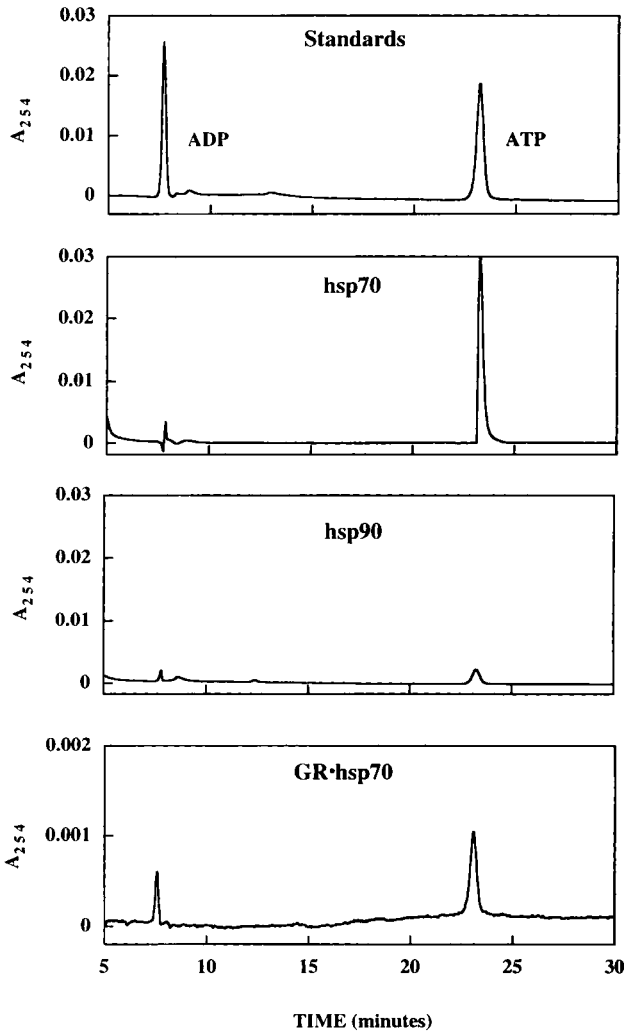

FIGURE 2: Nucleotide binding states of free and receptor-bound hsp70 and hsp90. Nucleotides were extracted from purified hsp70 and hsp90 and from primed GR·hsp70 complexes with perchloric acid, and ATP and ADP in the perchloric acid extract were assayed by high-performance liquid chromatography as described in Experimental Procedures.

nucleotide was then extracted with 0.4 N perchloric acid, filtered, and analyzed by HPLC as described above.

RESULTS

Priming of the GR by hsp70 Requires Sustained High Levels of ATP. It was shown previously that both YDJ-1 and ATP were needed for formation of GR·hsp70 complexes that could bind hsp90 and be assembled into steroid binding GR·hsp90 heterocomplexes in a second ATP-dependent step (*19*). In our previous work, the ATP level was maintained at ~5 mM by the routine inclusion of an ATP-regenerating system. In the experiment whose results are depicted in Figure 1, stripped GR immune pellets were preincubated for 5 min at 30 °C with hsp70, YDJ-1, and 5 mM ATP or 5 mM ATP with an ATP-regenerating system. The immune pellets were then washed and immunoblotted for the GR and hsp70. As shown in the Western blot at the top of Figure 1, both YDJ-1 and sustained high levels of ATP provided by the energy-generating system (EGS) were required for a high level of hsp70 binding to the GR (lane 5). ATP provided without the regenerating system yielded less hsp70 binding to the GR (lane 6). The bars in Figure 1 show the steroid binding activity that was achieved when the washed hsp70-bound GR pellets were incubated for 20 min at 30 °C with the purified heterocomplex assembly system without hsp70. It is clear that preincubation of the GR with hsp70, YDJ-1, and ATP in the presence of the regenerating system (lane 5) yielded a much greater activation of steroid binding activity during the second incubation with the hsp70-free assembly system (hsp90, Hop, YDJ-1, and p23) than preincubation without added ATP (lane 4) or with 5 mM ATP without the regenerating system (lane 6). It can be seen that the amount of GR-bound hsp70 obtained with the incomplete priming systems in lanes 2−4 does not correlate with the amount of hormone binding activity achieved in the second step, showing that hsp70 can be bound to the receptor without having fully primed it for assembly with hsp90.

Because the last step in purification of hsp70 is its elution from an ATP−Sepharose column with ATP, the hsp70 we purify should be predominantly in the ATP-dependent conformation. As shown by direct assay in Figure 2, the purified hsp70 that is added to the GR-priming incubation is bound by ATP. After the 5 min incubation with YDJ-1 and the ATP-regenerating system, $\sim^1/_3$ of the GR-bound hsp70 is bound by ADP and $\sim^2/_3$ is bound by ATP (Figure 2). If the priming event involved binding of hsp70 in its ATP-dependent conformation to the GR followed by YDJ-1-promoted hydrolysis of the ATP and single-step conversion of the GR-bound hsp70 to the ADP-dependent conformation, then one might expect that a sustained high level of ATP would not be as important as indicated in the experiment whose results are depicted in Figure 1. However, sustained high levels of ATP might be required if multiple molecules of hsp70 interacted serially with the GR during the priming step and the ATP-dependent conformation had to be regenerated upon hsp70 release. Another possibility is that the GR-bound hsp70 undergoes iterative ratcheting back and forth between its ADP- and ATP-dependent conformations during the priming process.

A way to differentiate between these two possibilities might be to prime the GR by incubating it with hsp70, YDJ-1, and the ATP-regenerating system, wash the immune pellet to eliminate free hsp70, and then incubate the GR·hsp70 complex with combinations of YDJ-1 and ATP without any free hsp70 being provided. We have shown previously that about 50% of the hsp70 dissociates from the GR·hsp70 complex during subsequent incubation for 20 min at 30 °C with either buffer or the hsp70-free assembly system of hsp90, Hop, YDJ-1, and p23 (*19*). In Figure 3, the GR was primed and the resulting GR·hsp70 complex was incubated for only 3 min at 30 °C. If hsp70 was constantly attaching and detaching from the GR in the primary step, then incubation in the absence of free hsp70 should be expected to result in essentially complete dissociation of hsp70 and loss of activity in the subsequent assembly assay with hsp90. Ideally, if GR-bound hsp70 were ratcheting back and forth between ADP- and ATP-dependent states, the hsp70 would remain bound to the GR, especially when YDJ-1 and the ATP-regenerating system were present to support oscillation between the states. As can be seen in Figure 3, the ability to activate the GR with hsp90 decreases when the primed GR·hsp70 complex is incubated in buffer alone (lane 3), and as we reported for the longer incubation time (*19*), the loss is greater with ATP (lane 4). YDJ-1 inhibits most of the

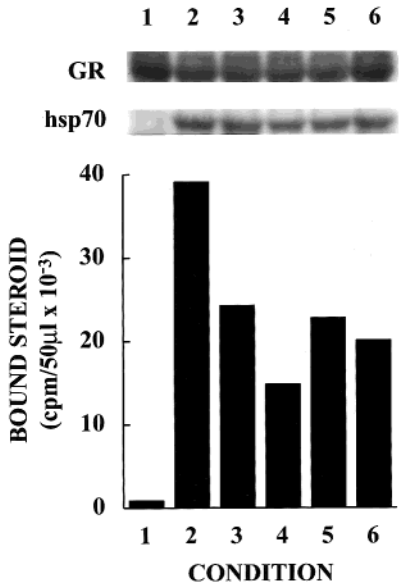

FIGURE 3: Dissociation of hsp70 from the GR·hsp70 complex. Stripped GR immune pellets were incubated for 5 min at 30 °C with hsp70, YDJ-1, and the ATP-regenerating system. The resulting GR·hsp70 complexes were washed and incubated for 3 min with buffer, the ATP-regenerating system, YDJ-1, or both the ATP-regenerating system and YDJ-1. The immune pellets were then washed once with TEG buffer and once with 10 mM Hepes prior to incubation for 20 min at 30 °C with the purified assembly system without hsp70. The blots at the top show the GR and hsp70 in the immune pellet after the 3 min incubation and washing, and the bar graphs show the steroid binding after incubation for 20 min with the assembly system without hsp70. Conditions were as follows: lane 1, stripped GR; lane 2, stripped GR reconstituted in two steps without the intervening 3 min incubation; and lanes 3−6, GR·hsp70 pellets incubated for 3 min with buffer alone (lane 3), the ATP-regenerating system alone (lane 4), YDJ-1 alone (lane 5), or both the ATP-regenerating system and YDJ-1 (lane 6).

ATP-dependent loss (lane 6), but the direct approach used in this experiment does not allow us to distinguish between the two mechanisms of ATP utilization by hsp70. Thus, to examine the ATPase activity of GR-bound hsp70 in the absence of a pool of free hsp70, we focused on the evidence of continued hsp70 ATPase activity during the assembly reaction with hsp90.

Activation by hsp90 Is Rate-Limiting in GR·hsp90 Heterocomplex Assembly. In Figure 4A, GR·hsp90 heterocomplexes were assembled in a single incubation with the five-protein system (●) or by the two-step method (○). In Figure 4B, the same data are expressed as a percentage of the 20 min value for each curve to show that the rates of generation of steroid binding activity are the same. Thus, the second step of two-step assembly is rate-limiting. Although we have routinely preincubated the GR with hsp70, YDJ-1, and the ATP-regenerating system for 5 min, the priming reaction is nearly complete in 1 min (Figure 5A). Less than 5% of the purified hsp90 that we add to the assembly system is bound by nucleotide (Figure 2), and preincubation of the hsp90 with either ADP or ATP does not change the rate of the second step in two-step assembly (Figure 5B). Conversion of hsp90 to the ADP- or ATP-bound form during the preincubation was confirmed by HPLC analysis as shown in Figure 2 (data not shown).

Evidence for Continued ATPase Activity of hsp70 during the Assembly Step with hsp90. Because YDJ-1 stimulates

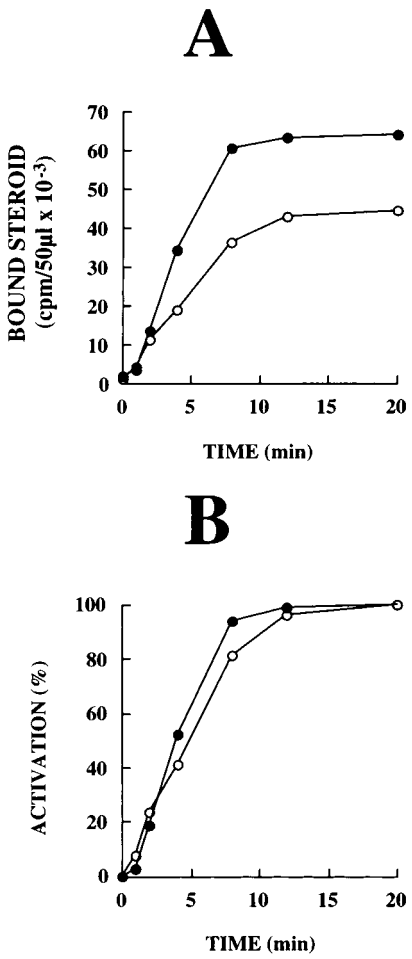

FIGURE 4: Activation by hsp90 is rate-limiting. Stripped GR immune pellets either were incubated in a single step at 30 °C with the five-protein assembly system (●) or were first primed by preincubation with hsp70, YDJ-1, and the ATP-regenerating system, and the washed pellets were then incubated at 30 °C with the assembly system without hsp70 (○). Panel A shows the time course of GR activation for the one-step assembly and the second incubation of two-step assembly. Panel B shows the same data replotted as a percentage of the highest value at 20 min.

the ATPase activity of hsp70, we wanted to see if its omission from the hsp70-free assembly system during the second assembly step would reduce the level of generation of steroid binding activity. In the experiment whose results are depicted in Figure 6, primed GR·hsp70 complexes were incubated with hsp90, Hop, YDJ-1, and p23 (lane 3) or with a three-protein mixture lacking one of these components (lanes 4−7). When hsp90 was omitted (lane 4) or when the stabilizers p23 and molybdate were not present to retard disassembly of GR·hsp90 complexes after they were assembled (lane 7), there was no steroid binding activity. Omission of Hop reduced the level of generation of steroid binding activity by ~50% (lane 5). Omission of YDJ-1 (lane 6) reduces the level of generation of steroid binding activity only to a small extent (compare lane 6 with lane 3 and lane 8 with lane 5).

We consistently see a modest reduction [17 ± 6%, mean ± standard error of the mean (SEM)] in steroid binding activity when YDJ-1 is omitted in the second step of two-step assembly. This is a small effect compared to omitting YDJ-1 from the priming step (Figure 1). However, the primed GR·hsp70 complexes contain YDJ-1 that is not

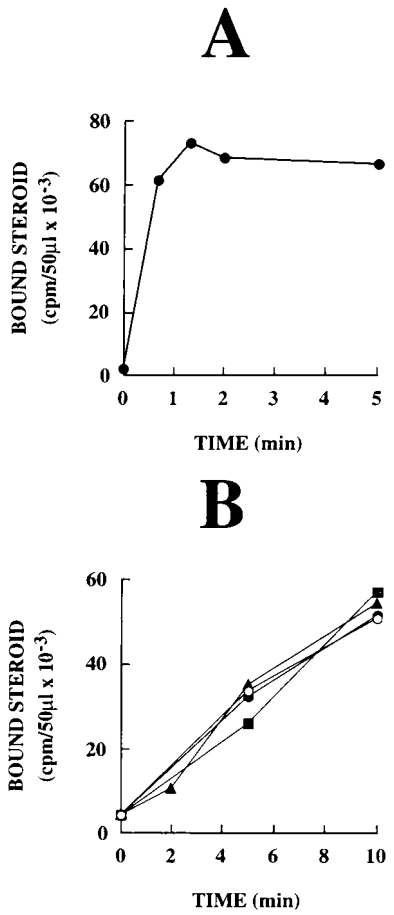

FIGURE 5: Priming step is rapid. (A) Time course of the priming step. Stripped GR immune pellets were incubated for various times at 30 °C with hsp70, YDJ-1, and the ATP-regenerating system. The resulting GR·hsp70 complexes were washed and incubated for 20 min at 30 °C with the purified assembly system without hsp70, and steroid binding activity was assayed. (B) The rate of activation by hsp90 is not affected by preincubation with nucleotide. hsp90 was preincubated for 10 min at 30 °C in HKD buffer with 2 mM $MgCl_2$ (●), buffer with $MgCl_2$ and 5 mM ATP (▲), or buffer with $MgCl_2$ and 5 mM ADP (■) prior to its addition to primed GR·hsp70 complexes along with the Hop, YDJ-1, and p23. (○) hsp90 that was not preincubated. After 20 min at 30 °C, steroid binding activity was assayed.

eliminated during the washing procedure. Thus, there is YDJ-1 present during the second incubation even when none is added as part of the hsp70-free assembly system. Therefore, we turned to another approach to determine if there was continued ATPase activity on the part of the GR-bound hsp70 during the second step of assembly with hsp90.

We have previously demonstrated that GR·hsp90 assembly and generation of steroid binding activity are dependent upon the presence of monovalent cation, with K^+ and NH_4^+ being active and Na^+ and Li^+ being inactive (*2*). Crystallographic analysis has revealed that K^+ binds specifically in the ATPase site of hsp70 (*27*). The ATPase activity of hsp70 is minimal in the absence of K^+ and reaches a maximum at about 100 mM K^+ (*28*). We also observed a maximum effect of K^+ on GR·hsp90 heterocomplex assembly at 100 mM (*2*), and that concentration of K^+ is always present in our assembly buffer. The ATPase activity of purified hsp90 has been readily assayed in the absence of K^+ (*29*) and in low-K^+ (*30*) buffers, and it appears not to belong to the class of enzymes (*31*), including a number of phosphotransferases

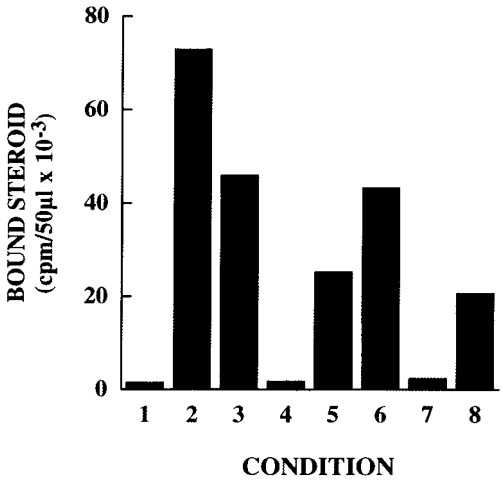

FIGURE 6: Cochaperone requirements for the second step of two-step GR activation. Stripped GR immune pellets were preincubated for 5 min at 30 °C with hsp70, YDJ-1, and the ATP-regenerating system. The washed GR·hsp70 complexes were then incubated for 20 min at 30 °C with hsp90, Hop, YDJ-1, and p23 or mixtures lacking one of these proteins. Conditions were as follows: lane 1, stripped GR; lane 2, stripped GR incubated with the five-protein system in one step; and lanes 3−8, primed GR·hsp70 immune pellets incubated with hsp90, Hop, YDJ-1, and p23 (lane 3), without hsp90 (lane 4), without Hop (lane 5), without YDJ-1 (lane 6), without p23 and molybdate (lane 7), and without Hop and YDJ-1 (lane 8).

and a few ATPases, that require monovalent cations for activity. Thus, any potassium dependency of the second assembly step with the hsp70-free assembly system should indicate continued ATPase activity on the part of GR-bound hsp70.

In the experiment whose results are depicted in Figure 7A, the GR was incubated with hsp70, YDJ-1, and the ATP-regenerating system without K^+ (lanes 2 and 3) or with 100 mM KCl (lanes 4 and 5). The GR immune pellets were then washed and incubated with hsp90, Hop, YDJ-1, and p23 in the absence or presence of KCl. When there was no K^+ in either incubation, very little steroid binding activity was generated (lane 2). When K^+ was present in only the first incubation (lane 4) or only the second incubation (lane 3), an intermediate level of steroid binding activity was generated. However, when K^+ was present during both steps of the two-step assembly (lane 5), a normal amount of steroid binding activity was generated. As shown in Figure 7B, the second step is selective for K^+ versus Na^+, as expected for the monovalent cation selectivity of hsp70 ATPase activity. Taken together, the stimulation by potassium (Figure 7) and the modest stimulation by YDJ-1 (Figure 6) suggest a requirement for continued ATPase activity of GR-bound hsp70 during the second step when the other essential chaperone (hsp90) is also present.

DISCUSSION

It is clear that the GR is somehow primed by hsp70 so that it can then bind hsp90 (*19*). In our purified five-protein system, we provide hsp70 in its ATP-bound form, and in the primed GR·hsp70 complex, a significant portion of the GR·bound hsp70 is in the ADP-bound state (Figure 2). Regardless of whether one hsp70 or multiple hsp70s interact with the GR during the priming step, if the hsp70 underwent a single conversion from its ATP-dependent to its ADP-

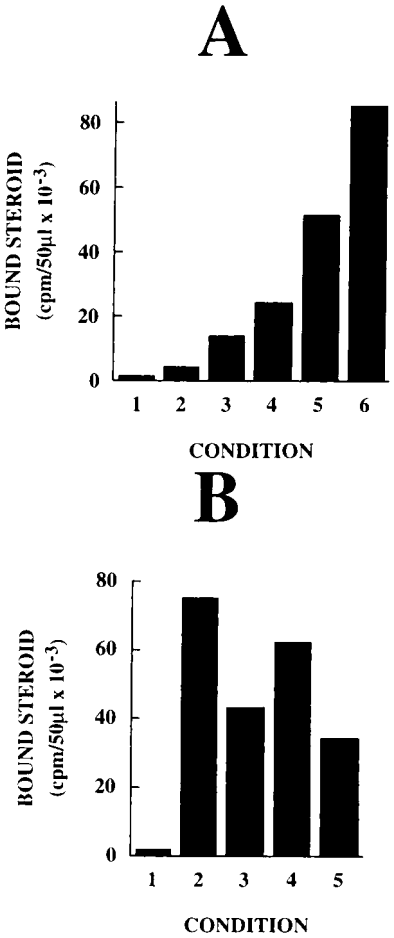

FIGURE 7: Potassium requirements for the first and second steps of GR activation. Both hsp90 and hsp70 were purified in potassium-free HD buffer [10 mM Hepes and 5 mM dithiothreitol (pH 7.35)]. (A) Potassium requirements for both steps of assembly. Stripped GR immune pellets were preincubated for 5 min at 30 °C with hsp70, YDJ-1, and the ATP-regenerating system in the presence or absence of 100 mM KCl. The GR·hsp70 complexes were washed and incubated for 20 min at 30 °C with hsp90, Hop, YDJ-1, and p23 in the presence or absence of 100 mM KCl, and steroid binding activity was assayed. Conditions were as follows: lane 1, stripped GR; lane 2 and 3, GR preincubated without K^+ and then incubated with the hsp70-free assembly system in the absence (lane 2) or presence (lane 3) of K^+; lanes 4 and 5, GR preincubated in the presence of K^+ and then incubated with the hsp70-free assembly system in the absence (lane 4) or presence (lane 5) of K^+; and lane 6, GR incubated for 20 min at 30 °C with the five-protein system with K^+. (B) The monovalent cation effect in the second step is selective for K^+ vs Na^+. Conditions were as follows: lane 1, stripped GR; lane 2, GR incubated in a single step with the five-protein system in the presence of K^+; and lanes 3−5, GR preincubated with hsp70, YDJ-1, and the ATP-regenerating system in the presence of K^+ and then incubated with the hsp70-free assembly system with no added monovalent cation (lane 3), 100 mM KCl (lane 4), or 100 mM NaCl (lane 5).

dependent conformation during the process, the priming should not require sustained high levels of ATP, as was shown in Figure 1. Thus, we considered two models for the priming event: one in which multiple molecules of hsp70 interact serially with the GR and a second in which the GR-bound hsp70 ratchets back and forth between its ADP- and ATP-dependent conformations during the priming process (Figure 8).

We favor the model of iterative ratcheting between the ADP- and ATP-dependent states of hsp70 because of the continued requirement for hsp70 ATPase activity in the

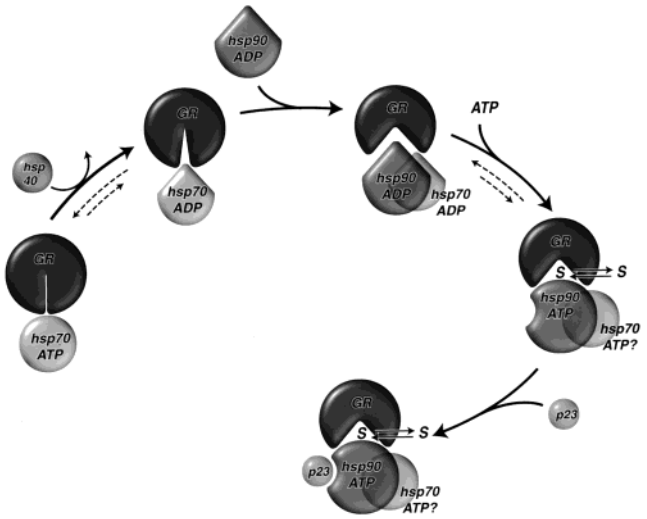

FIGURE 8: Model of GR unfolding predicted from sequential assembly data. The model is derived from published stepwise assembly data (*19*) and observations of this paper suggesting iterative ratcheting (indicated by dashed arrows) of GR-bound hsp70 between its ATP and ADP conformations both during the first step where the GR is primed to bind hsp90 and during the second step where the ligand binding cleft in the GR LBD is opened to access by steroid (S). As discussed in the text, it is likely that the GR-bound hsp90 undergoes a similar iterative ratcheting between its ATP and ADP conformations during this second step. p23 enters the receptor heterocomplex and stabilizes the ATP conformation of hsp90 when the ligand binding cleft is opened. The Hop component of the five-protein assembly system has been omitted for simplicity of presentation.

second reaction with hsp90. Continued hsp70 ATPase activity is inferred from the 10−15% stimulation of the second step provided by addition of purified YDJ-1 and from the K^+ dependency of the second step (Figure 7). Because we have eliminated the very large amount of free hsp70 by washing the primed GR·hsp70 immune pellet, the ATPase activity must reflect the activity of GR-bound hsp70. Thus, we arrive at a model illustrated by the dashed lines in Figure 8 in which the receptor-bound hsp70 ratchets back and forth between its ADP- and ATP-dependent conformations during the priming step and during the much slower, rate-limiting step with hsp90.

It seems likely that hsp90 also undergoes an iterative ratcheting between its ADP- and ATP-dependent conformations during the second step. Using a mutant of hsp90 that binds ATP but lacks ATPase activity, the Toft laboratory has shown that the mutant hsp90 binds to the progesterone receptor when it is substituted for hsp90 in the five-protein assembly system, but the receptor is not converted to the steroid binding state (*20*). This requirement for hsp90 to have ATPase activity in the five-protein system is consistent with at least one conversion of hsp90 between its ATP- and ADP-bound states during opening of the steroid binding cleft. It is also consistent with the requirement for hsp90 in other cell-free systems for completion of a full ATP reaction cycle to modulate the folding of its target protein (*32*, *33*).

In our case, the purified hsp90 we add is not bound by nucleotide and preincubation of the hsp90 with ADP or ATP did not affect the kinetics of the second step of assembly (Figure 5B). In the cell, the ATP binding site on hsp90 should be fully occupied (*34*), and in this sense, the purified system is clearly artifactual in providing hsp90 with an empty

nucleotide binding site. In the model shown in Figure 8, we proposed (*19*) that hsp70 priming reflected the partial opening of the steroid binding cleft to the point where hydrophobic amino acids were exposed to interaction with the ADP-dependent conformation of hsp90. However, at this time, it is not known whether it is the ADP- or ATP-dependent conformation of hsp90 that binds to the primed GR·hsp70 complex.

Mammalian hsp90s and hsp70s do not associate with each other such that hsp90·hsp70 complexes are formed that can be detected by the usual separation techniques used for complex detection. However, it seems that they must interact directly. Indeed, a major function of Hop in increasing the rate of assembly is probably to facilitate the interaction of the two essential chaperones. It has been shown that hsp80, an hsp90 homologue of *Neurospora crassa*, binds directly to hsp70 to form a complex that survives separation procedures (*35*). Because GR·hsp90 assembly can proceed, albeit more slowly, without Hop in the second incubation (Figure 6), one might consider the most elemental machinery for opening the steroid binding cleft to be a hsp90 dimer interacting with the primed LBD·hsp70 complex. During the process of cleft opening, the hsp90 and hsp70 may interact directly with each other, with both undergoing iterative ratcheting between their ADP- and ATP-dependent conformations. It is an attractive notion that the two chaperones may affect the conformations of each other such that they alternate in assuming the ADP- and ATP-dependent conformations during the cleft-opening process. In such a model, ATP-dependent transfer of substrate between chaperones might occur as suggested by Schnaider et al. (*36*) for hsp90 and hsp40 transfer of α-lactalbumiin to each other.

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