THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 24, Issue of June 16, pp. 18054–18060, 2000

Printed in U.S.A.

Stepwise Assembly of a Glucocorticoid Receptor·hsp90 Heterocomplex Resolves Two Sequential ATP-dependent Events Involving First hsp70 and Then hsp90 in Opening of the Steroid Binding Pocket*

Received for publication, January 18, 2000, and in revised form, March 13, 2000
Published, JBC Papers in Press, April 7, 2000, DOI 10.1074/jbc.M000434200

Yoshihiro Morishima‡, Patrick J. M. Murphy‡, Da-Pei Li§, Edwin R. Sanchez§, and William B. Pratt‡¶

From the ‡Department of Pharmacology, The University of Michigan Medical School, Ann Arbor, Michigan 48109 and the §Department of Pharmacology, Medical College of Ohio, Toledo, Ohio 43699

A system of five purified proteins that assembles stable glucocorticoid receptor (GR)-hsp90 heterocomplexes has been reconstituted from reticulocyte lysate. Two proteins, hsp90 and hsp70, are required for the activation of steroid binding activity that occurs with heterocomplex assembly, and three proteins, Hop, hsp40, p23, act as co-chaperones that enhance activation and assembly (Morishima, Y., Kanelakis, K. C., Silverstein, A. M., Dittmar, K. D., Estrada, L., and Pratt, W. B. (2000) *J. Biol. Chem.* 275, 6894–6900). Here we demonstrate that the first step in assembly is the ATP-dependent and hsp40 (YDJ-1)-dependent binding of hsp70 to the GR. After elimination of free hsp70, these preformed GR·hsp70 complexes can be activated to the steroid binding state by the hsp70 free assembly system in a second ATP-dependent step. hsp90 is required for opening of the steroid binding pocket and is converted to its ATP-dependent conformation during this second step. We predict that hsp70 in its ATP-dependent conformation binds initially to the folded receptor and 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 the hydrophobic binding form of hsp90, which in turn must be converted to its ATP-dependent conformation for the pocket to be accessible by steroid.

Unliganded steroid receptors exist in cytosols in heterocomplexes with the abundant, ubiquitous, and essential heat shock protein hsp90[1] (for review, see Ref. 1). The glucocorticoid receptor (GR) must be in heterocomplex with hsp90 for it to have steroid binding activity (2, 3). The ligand binding domain (LBD) is the region of the receptor that interacts with hsp90 (1), and biochemical data (4) coupled with data from GR mutants (5, 6) support the notion (3) that formation of a complex with hsp90 opens up a hydrophobic pocket in the LBD to access by steroid. Steroid receptor·hsp90 heterocomplexes are formed in an ATP-dependent process by a multiprotein chaperone system that has been studied most extensively in reticulocyte lysate (7, 8) but is present in lysates of both animal and plant cells (9).

The receptor·hsp90 heterocomplex assembly system has now been reconstituted (10–14), and five purified proteins, including hsp90, hsp70,[2] Hop (hsp organizer protein), hsp40, and p23, are required for optimally efficient assembly (for review of heterocomplex assembly, see Refs. 15 and 16). Only two of these proteins, hsp70 and hsp90, are absolutely required for opening the steroid binding cleft in the GR LBD, and the other three proteins act as co-chaperones that increase the overall efficiency of GR·hsp90 heterocomplex assembly (17).

Hop binds independently to hsp90 and hsp70 to form an hsp90·Hop·hsp70 complex (18). Although Hop is not required for opening of the steroid binding cleft in the GR LBD, it increases the rate of the process (17). The peptide binding activity of hsp70 is coupled to the binding of ADP *versus* ATP (for review, see Ref. 19), and hsp70 possesses an intrinsic ATPase activity that is stimulated by hsp40, the vertebrate homolog of the bacterial DnaJ protein. The ADP-bound conformation of hsp70 has a high affinity for hydrophobic substrates, and hsp40 (provided as the purified yeast homolog YDJ-1) increases GR·hsp90 heterocomplex assembly (13) but it is not required for assembly (17). The p23 component of the system binds to and stabilizes the ATP-dependent conformation of hsp90 (20). Like hsp70, hsp90 possesses a nucleotide binding site that acts as an ATP/ADP switch domain that regulates its conformation, with the ADP-bound conformation possessing high affinity for hydrophobic substrate (20, 21). To have an open steroid binding cleft, the receptor-bound hsp90 must assume its ATP-dependent conformation (22), which is then stabilized by p23 (12).

When all five proteins of the chaperone system are present, the first step in GR·hsp90 heterocomplex assembly appears to be the formation of an hsp90·Hop·hsp70·hsp40 complex that acts as a machinery for opening the steroid binding cleft (11, 13, 23). This complex can be immunoadsorbed from reticulocyte lysate or prepared simply by mixing purified proteins, and when mixed with the immunoadsorbed GR, it converts the GR to its steroid binding form (11, 13).

In all previous studies of receptor·hsp90 heterocomplex assembly by the purified chaperone system, the receptor has been

* This work was supported by National Institutes of Health Grants DK31573 (to W. B. P.) and DK43867 (to E. R. S.). 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 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Pharmacology, The University of Michigan Medical School, 1301 MSRB III, Ann Arbor, Michigan 48109-0632. Tel.: 734-764-5414; Fax: 734-763-4450.

[1] The abbreviations used are: hsp, heat shock protein; GR, glucocorticoid receptor; Hop, 60-kDa hsp organizer protein; LBD, ligand binding domain; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-ethanesulfonic acid.

[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.

exposed to all components of the system simultaneously (10–14). In this work we have assembled the chaperone machinery on the receptor in stepwise fashion. Preincubation of the GR with hsp70, YDJ-1 (hsp40), and ATP results in a GR·hsp70 complex. After elimination of free hsp70, the GR·hsp70 complex will bind Hop, and the resulting GR·hsp70·Hop complex will bind hsp90. In a second ATP-dependent step, hsp90 is converted to its ATP-dependent conformation, and the receptor acquires steroid binding activity.

EXPERIMENTAL PROCEDURES

Materials

[6,7-^{3}H]Triamcinolone acetonide (38 Ci/mmol) and ^{125}I-conjugated goat anti-mouse and anti-rabbit IgGs were obtained from NEN Life Science Products. 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. 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, Canada). The JJ3 monoclonal IgG against p23 and *Escherichia coli* expressing human p23 were gifts from Dr. David Toft (The Mayo Clinic). *E. coli* expressing YDJ-1 was a gift from Dr. Avrom Caplan (Mount Sinai School of Medicine). The DS14F5 monoclonal IgG against Hop and *E. coli*-expressing Hop were kindly provided by Dr. David F. Smith (University of Nebraska Medical School). Hybridoma cells producing the FiGR monoclonal IgG against the GR were generously provided by Dr. Jack Bodwell (Dartmouth Medical School).

Methods

Expression of Mouse GR in Sf9 Cells—Overexpression of mouse GR in Sf9 cells was achieved according to the Bac-N-Blue transfection kit protocol of Invitrogen Corp. Briefly, mouse GR cDNA was excised from the pSV_2Wrec plasmid (24) as a 2.67-kilobase fragment. This fragment was inserted into the multiple cloning site of the p2Bac transfer vector to make the recombinant transfer vector p2Bac-mGR. The p2Bac-mGR and Bac-N-Blue DNA were then cotransfected into Sf9 cells to make recombinant baculovirus, followed by plaque purification. 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 post-infection as described by Srinivasan *et al.* (25). Cells were harvested, washed in Hanks' buffered saline solution, resuspended in 1.5 volumes of buffer (10 mM Hepes, pH 7.5, 1 mM EDTA, 20 mM molybdate, 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, divided into aliquots, 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, 10% glycerol). Before 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 KCl 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 various mixtures of proteins (20 μg of purified hsp90, 15 μg of purified hsp70, 0.6 μg of purified human Hop, 6 μg of purified p23, 0.4 μg of purified YDJ-1) and adjusted to 50 μl with HKD buffer (10 mM Hepes, 100 mM KCl, 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 proteins.

Assay of Steroid Binding Capacity—Immune pellets to be assayed for steroid binding were incubated overnight at 4 °C in 50 μl of 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. The amount of GR immunoadsorbed from 50 μl of Sf9 cytosol has been measured using an in-gel assay with a bovine albumin standard. 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.

Western Blotting—To assay GR and associated proteins, immune pellets were resolved on 10% 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, or 0.1% JJ3 mouse ascites for p23. 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 (10). Human p23 (26) was purified from 10 ml of bacterial lysate by chromatography on DE52, followed by hydroxylapatite chromatography (13). 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 (13). The bacterial expression of YDJ-1 has been described previously (27, 28) as has the expression of human Hop (10). 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.

RESULTS

Conversion of hsp70-prebound Receptors to the Steroid Binding State—It has not been determined which of the two essential chaperones, hsp70 or hsp90, first contacts the receptor. The three laboratories studying this receptor·hsp90 heterocomplex assembly system have speculated that hsp70 binds initially (11, 14, 29), and we wanted to find appropriate conditions for prebinding hsp70 to the GR before incubating the GR·hsp70 complex with the other components of the GR·hsp90 heterocomplex assembly system. One limitation to such an approach is shown in Fig. 1*A*. In this experiment, GR immune pellets stripped of insect hsp90 were incubated at 30 °C under the buffer conditions required for heterocomplex assembly. After various times of preincubation, the immune pellets were washed and incubated with reticulocyte lysate to reactivate steroid binding activity. It can be seen that preincubation of the stripped GR results in a time-dependent loss in its ability to be reactivated. As shown by the Western blot in the inset to Fig. 1*A*, the loss is not due to receptor proteolysis, and the reason for the loss is not known. As indicated in Fig. 1*B*, the GR is inactivated in the absence of ATP (*solid bars*), but inactivation is faster in the presence of the ATP-regenerating system (*open bars*) that we use for heterocomplex assembly. Because of this inactivation, all of the subsequent preincubations of GR with hsp70 will be limited to 5 min.

In the experiment of Fig. 2, stripped GR immune pellets were preincubated for 5 min at 30 °C with hsp70, the ATP-regenerating system, and YDJ-1. The immune pellets were then washed and immunoblotted for the GR and hsp70. As shown in the Western blot at the top of Fig. 2, both ATP and YDJ-1 were required for a high level of hsp70 binding to the GR (*lane 8*). The *bars* in Fig. 2 show the steroid binding activity that is achieved when the washed hsp70-bound GR pellets were incubated for 20 min at 30 °C with the purified heterocomplex assembly system without hsp70. Preincubation of the GR with hsp70 in the presence of ATP and YDJ-1 (*lane 8*) was required

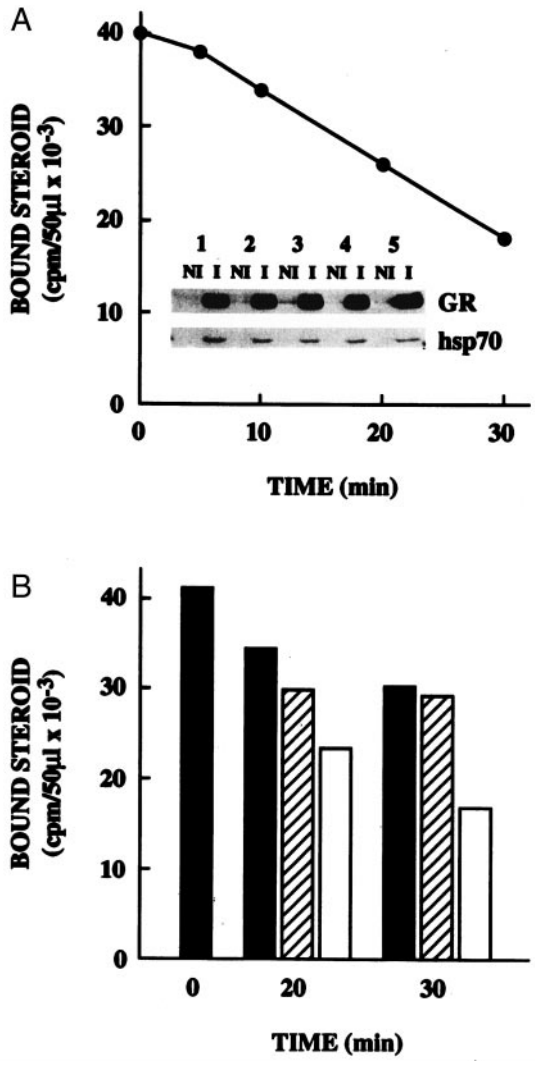

FIG. 1. **Preincubation of stripped GR with buffer containing the ATP-regenerating system reduces its ability to be reactivated upon incubation with reticulocyte lysate.** *A*, inactivation of the GR. Immune pellets with stripped GR were incubated at 30 °C with HKD buffer containing the ATP-regenerating system, and the pellets were then washed twice with TEG buffer and once with 10 mM Hepes. The washed pellets were then incubated for 20 min at 30 °C with reticulocyte lysate and the ATP-regenerating system, and the pellets were washed and incubated with 50 nM [^{3}H]triamcinolone acetonide to determine steroid binding activity. The *inset* shows parallel nonimmune (*NI*) and immune (*I*) pellets incubated for the same times with the ATP-regenerating system and then immunoblotted for GR and hsp70 (detected with a rabbit antiserum that reacts with insect hsp70). *B*, the energy-regenerating system yields faster receptor inactivation than ATP or buffer alone. Stripped GR immune pellets were incubated for 0, 20, or 30 min at 30 °C with HKD buffer alone (*solid bars*), buffer plus 10 mM ATP (*hatched bars*), or buffer with the ATP-regenerating system (*open bars*). Pellets were then washed and incubated with reticulocyte lysate as above.

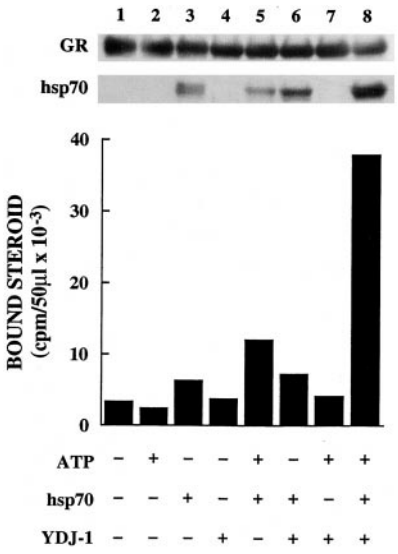

FIG. 2. **Reactivation of GR prebound with hsp70.** Immune pellets with stripped receptors were incubated for 5 min at 30 °C with various combinations of purified hsp70, YDJ-1, and the ATP-regenerating system as indicated. The pellets were washed four times with TEG buffer and immunoblotted for GR and hsp70. Duplicate pellets were washed twice with TEG buffer and once with Hepes and then incubated with the purified heterocomplex assembly system without hsp70, and steroid binding activity was assayed.

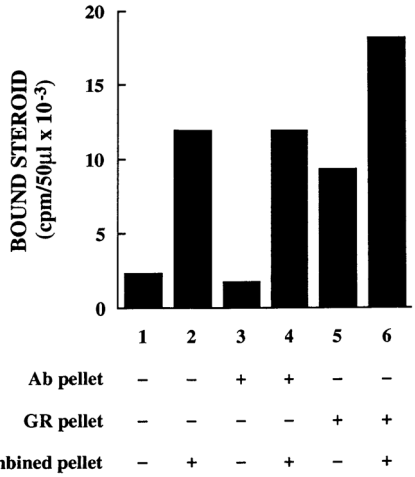

FIG. 3. **GR-prebound hsp70 is responsible for subsequent reactivation by the hsp70-free assembly system.** Immune pellets with stripped receptors (*GR pellet*) were preincubated for 5 min at 30 °C either with buffer alone or with hsp70, YDJ-1, and the ATP-regenerating system. GR-free antibody pellets (*Ab pellet*), which were prepared by adsorbing HEM buffer rather than Sf9 cytosol, were preincubated with or without hsp70 under the same conditions. All pellets were then washed twice with TEG buffer and once with 10 mM Hepes. The Ab pellets and GR pellets were then mixed and incubated for 20 min at 30 °C with hsp90, Hop, YDJ-1, p23, and 20 mM molybdate in the presence or absence of hsp70. At the end of the second incubation, the pellets were washed and assayed for steroid binding activity. The presence of hsp70 during the preincubation or during incubation of the combined pellets is indicated by a +.

to obtain substantial reactivation of steroid binding activity during the second incubation with the hsp70-free assembly system (hsp90, Hop, YDJ-1, p23).

hsp70 has the ability to bind to a wide variety of proteins, and it is possible that it binds to antibody and/or protein A-Sepharose in the GR immune pellet. The experiment of Fig. 3 was performed to demonstrate that it is the hsp70 that is prebound to the GR and not nonspecifically retained hsp70 that is responsible for subsequent activation of steroid binding activity by the hsp70-free assembly system. Antibody pellets with or without bound GR were preincubated with or without hsp70 in the presence of YDJ-1 and the ATP-regenerating system. After washing, the preincubated pellets were combined and incubated with the purified assembly system with or without hsp70. It can be seen that combining an antibody pellet preincubated with hsp70 and a GR pellet preincubated in the absence of hsp70 does not yield steroid binding activity (*lane 3*), whereas combining a GR pellet preincubated with hsp70 with an antibody pellet preincubated in the absence of hsp70 does yield steroid binding activity (*lane 5*). Thus, it is clear that it is receptor-bound hsp70 that is responsible for the subsequent

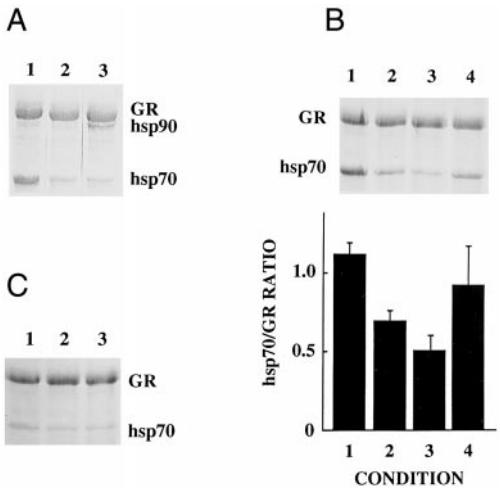

FIG. 4. **Dissociation of hsp70 from the GR·hsp70 complex.** *A*, dissociation of hsp70. Stripped GR immune pellets were incubated for 5 min at 30 °C with hsp70, YDJ-1, and the ATP-regenerating system. The GR·hsp70 complexes were washed and incubated for 20 min at 30 °C as indicated. The pellets were washed again, and proteins were resolved by gel electrophoresis and staining with Coomassie Blue. *Lane 1*, no 20-min incubation; *lane 2*, incubated with the ATP-regenerating system alone; *lane 3*, incubated with the ATP-regenerating system and the purified heterocomplex assembly system without hsp70. *B*, about 50% of the GR-bound hsp70 dissociates during the 20-min incubation at 30 °C. GR was bound to hsp70 as above and incubated for 20 min at 30 °C as follows: *lane 1*, no incubation; *lane 2*, incubated with the ATP-regenerating system; *lane 3*, incubated with 5 mM ATP; *lane 4*, incubated with 5 mM ADP. The hsp70/GR ratios in the *bar graphs* were determined by scanning multiple Coomassie Blue-stained bands like those shown at the top, with the *vertical lines* representing the standard error (from 3 experiments for *lane 4* to 10 experiments for *lane 1*). C, overexpressed GR is bound to some insect hsp70. Stripped GR (*lane 1*) was incubated for 5 min at 30 °C with the ATP-regenerating system (*lane 2*) followed by washing and a subsequent incubation for 20 min at 30 °C with the ATP-regenerating system (*lane 3*). The gel was stained with Coomassie Blue.

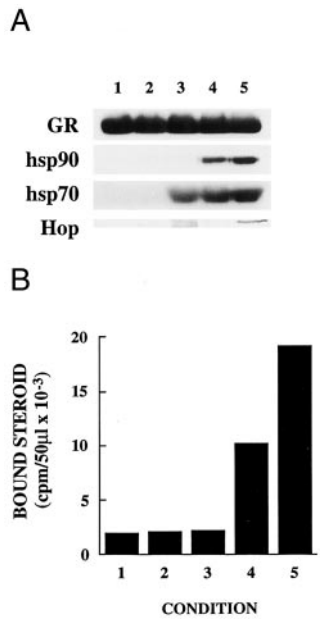

FIG. 5. **Stepwise assembly of a functional GR·hsp90 heterocomplex.** *A*, heterocomplex assembly. Stripped GR immune pellets were incubated for 5 min at 30 °C with YDJ-1 and the ATP-generating system in the presence (*lanes 3–5*) or absence (*lane 2*) of hsp70. The pellets were washed three times with TEG buffer and once with 10 mM Hepes and then incubated for 15 min on ice in the presence (*lanes 2*, *3*, and *5*) or absence (*lane 4*) of Hop. The pellets were then washed twice with 10 mM Hepes, and the pellets shown in *lanes 4* and *5* were incubated for 15 min on ice with hsp90 and washed with 10 mM Hepes. The proteins in the immune pellets were resolved by gel electrophoresis and immunoblotting. *Lane 1* is the unincubated, stripped GR. *B*, steroid binding activity. After proceeding through the step of incubating with Hop and washing, GR immune pellets treated as described above were incubated for 20 min at 30 °C in the presence (*lanes 4* and *5*) or absence (*lanes 1–3*) of the ATP-regenerating system, hsp90, p23, and 20 mM molybdate. Samples were washed again, and steroid binding was assayed.

generation of steroid binding sites by the rest of the assembly system.

Dissociation of hsp70 from the GR—As shown in Fig. 4*A*, hsp70 that is prebound to the GR dissociates during subsequent incubation at 30 °C with the ATP-regenerating system alone (*lane 2*) or with the hsp70-free assembly system (*lane 3*). As shown in Fig. 4*B*, about 50% of the hsp70 dissociates when the GR·hsp70 complexes are incubated for 20 min at 30 °C with the ATP-regenerating system (*lane 2*) or ATP alone (*lane 3*). Srinivasan *et al.* (30) note that human GR expressed in the baculovirus system is associated with some insect hsp70 that does not dissociate upon incubation with ATP. As shown by Western blot with an antibody that detects insect hsp70 (Fig. 1*A*, *inset*) and by staining with Coomassie Blue (Fig. 4*C*), the overexpressed mouse GR is associated with a small amount of Sf9 cell-derived hsp70 that does not dissociate during subsequent incubation with the ATP-regenerating system. The ratios of Fig. 4*B* have been corrected for the small contribution (<10%) by this insect hsp70.

Stepwise Assembly of GR·hsp90 Heterocomplex—In the experiment of Fig. 5*A* the GR·hsp70·Hop·hsp90 heterocomplex was assembled in stepwise fashion. Stripped GR immune pellets (*lane 1*) were first incubated at 30 °C with the ATP-regenerating system and YDJ-1 in the absence (*lane 2*) or presence (*lane 3*) of hsp70. After washing, the immune pellets were incubated on ice with Hop and washed again. Hop bound only to the GR·hsp70 complex (*lane 3*). GR·hsp70 complexes (*lane 4*) or GR·hsp70·Hop complexes (*lane 5*) were then incubated on ice with hsp90 and washed. Although more hsp90 was bound to GR·hsp70·Hop complexes (*lane 5*) than to GR·hsp70 complexes (*lane 4*), the GR·hsp70 complexes nevertheless bound some hsp90. In Fig. 5*B*, GR·hsp70 complexes (*lane 4*) or GR·hsp70·Hop complexes (*lane 5*) were prepared as above and then incubated at 30 °C with hsp90, p23, molybdate, and the ATP-regenerating system. About half the steroid binding activity was generated in the absence of Hop (*lane 4*) as in the presence of Hop (*lane 5*), consistent with the observation that hsp70 and hsp90 together are sufficient for generating some steroid binding activity (17).

ATP Is Required for Generating Steroid Binding Activity after Stepwise Receptor Heterocomplex Assembly—In Fig. 2, we showed that ATP was required during the 5-min preincubation with hsp70 to produce a GR·hsp70 complex that could be activated to the steroid binding state by a second incubation with the hsp70-free assembly system. This second incubation involves a second ATP-dependent process leading to steroid binding activity. In Fig. 6*A*, the GR·hsp70·Hop·hsp90 heterocomplex was assembled as in Fig. 5*A* (*lane 5*). These complexes were then incubated at 30 °C with p23 and molybdate in the absence (*lane 2*) or presence (*lane 3*) of the ATP-regenerating system. It is clear that steroid binding activity is generated only when ATP is present.

The Toft laboratory has shown that receptor-bound hsp90 must be in its ATP-dependent conformation for the receptor to have steroid binding activity (22) and that p23 binds to purified hsp90 when hsp90 is in its ATP-dependent conformation (20). In the experiment of Fig. 6*B*, GR·hsp70 complexes were incubated at 30 °C with hsp90, Hop, p23, and molybdate in the presence (*lane 3*) or absence (*lane 2*) of the ATP-generating

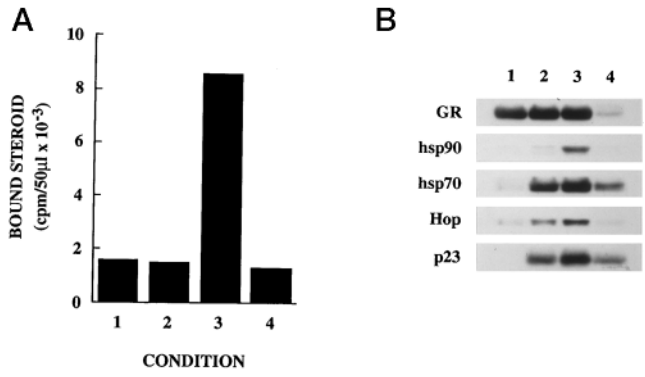

FIG. 6. **To generate steroid binding activity, ATP is required in the incubation with hsp90 at 30 °C.** *A*, GR·hsp90 heterocomplexes formed by three-step assembly requires ATP to generate steroid binding activity when they are incubated at 30 °C. Stripped GR immune pellets were incubated at 30 °C with hsp70, YDJ-1, and the ATP-regenerating system. The resulting GR·hsp70 complexes were washed and incubated on ice with Hop and then washed again and incubated with hsp90 as described in the legend of Fig. 5*A*. After washing two times with 10 mM Hepes, the GR·hsp70·Hop·hsp90 complexes were incubated for 20 min at 30 °C with p23 and molybdate in the absence (*lane 2*) or presence (*lanes 3* and *4*) of the ATP-regenerating system. Samples were washed twice with TEGM buffer, and steroid binding was assayed. *Lane 1*, stripped GR without treatment; *lane 2*, GR·hsp70·Hop·hsp90 complexes incubated without ATP; *lane 3*, GR·hsp70·Hop·hsp90 complexes incubated with ATP; *lane 4*, nonimmune pellet (no GR) treated the same as the sample of *lane 3*. B, GR·hsp90 heterocomplexes formed by two-step assembly in the presence of ATP bind p23. GR·hsp70 complexes were prepared as above, washed, and incubated for 20 min at 30 °C with hsp90, Hop, p23, and molybdate in the presence or absence of the ATP-regenerating system. After washing four times with TEGM buffer, the proteins in the immune pellets were resolved by gel electrophoresis and immunoblotting. *Lane 1*, untreated stripped GR; *lane 2*, GR·hsp70 complex incubated with hsp90, Hop, and p23 without ATP; *lane 3*, GR·hsp70 complex incubated with hsp90, Hop, and p23 with ATP; *lane 4*, nonimmune pellet (no GR) treated the same as the sample of *lane 3*.

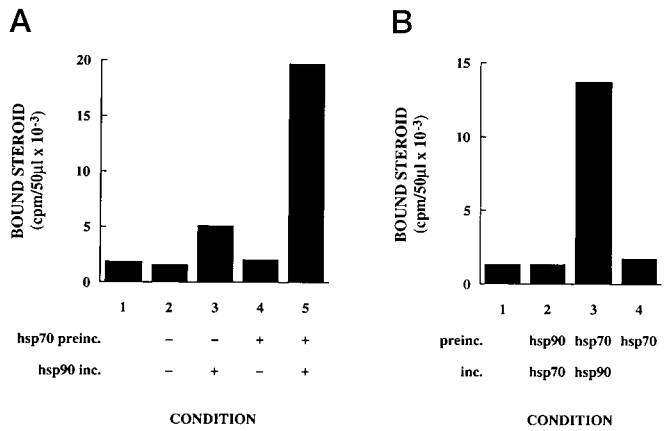

FIG. 7. **Two sequential ATP-dependent steps are required to generate steroid binding activity.** *A*, two ATP-dependent steps. Stripped GR immune pellets were incubated for 5 min at 30 °C with hsp70 and YDJ-1 in the presence (+) or absence (−) of the ATP-regenerating system. The pellets were washed and incubated for 20 min at 30 °C with hsp90, p23, molybdate, and 200 nM [^{3}H]triamcinolone acetonide in the presence (+) or absence (−) of the ATP-regenerating system. At the end of this second incubation the pellets were washed, and radioactivity was assayed. *Lane 1*, stripped GR that was incubated twice in the presence of the ATP-regenerating system but without any protein components of the assembly system. *B*, preincubation with hsp90 cannot prepare the receptor for activation by hsp70. Stripped GR immune pellets were preincubated for 5 min at 30 °C with the ATP-regenerating system alone (*lane 1*) or with hsp90 (*lane 2*) or with hsp70 and YDJ-1 (*lanes 3 and 4*). The pellets were washed and incubated for 20 min at 30 °C with the ATP-regenerating system and [^{3}H]triamcinolone acetonide, either alone (*lane 1*), with hsp70, YDJ-1, p23, and molybdate (*lane 2*), with hsp90, p23, and molybdate (*lane 3*), or with p23 and molybdate (*lane 4*). The pellets were washed, and radioactivity bound to the pellets was assayed.

system. The composition of the immune pellets was assayed by immunoblotting. The sample that had ATP present during the incubation with hsp90 (*lane 3*) contains more p23 than a similarly treated sample that did not contain GR (*lane 4*) or the GR·hsp70 sample that went through the second incubation in the absence of ATP (*lane 2*). Perhaps because of its high negative charge, nonspecific binding of p23 to immune pellets is high (*e.g. lane 4*), and the pellets must be washed at least four times with TEGM to reduce the nonspecific binding to this level. GR-bound hsp90 in its ATP-dependent conformation remains bound to the receptor under these wash conditions, but hsp90 that is not in its ATP-dependent conformation (*lane 2*) is eliminated. Nevertheless, the increased presence of p23 in the GR·hsp90 heterocomplex in *lane 3* is consistent with the notion that hsp90 has been converted to its ATP-dependent conformation during the second incubation.

Sequential ATP-dependent Steps—The experiments shown in Fig. 7 were carried out in the absence of Hop to demonstrate the sequential requirements for an initial ATP-dependent step mediated by hsp70 followed by a second ATP-dependent step mediated by hsp90. In the experiment of Fig. 7*A*, stripped GR immune pellets were preincubated with hsp70 and YDJ-1 in the presence or absence of ATP. These pellets were then washed and incubated for 20 min at 30 °C with hsp90, p23, and molybdate in the presence or absence of ATP. Because receptor heterocomplexes formed with a purified chaperone system lacking one or more of the co-chaperones can be less stable, [^{3}H]triamcinolone acetonide was present during this second incubation at 30 °C to permit steroid binding to GR·hsp90 heterocomplexes as soon as they are formed and before they disassemble. As shown in *lane 5*, substantial steroid binding activity is achieved when ATP is present during the preincubation with hsp70 and the second incubation with hsp90. Receptors preincubated with hsp70 in the presence of ATP and then with hsp90 in the absence of ATP do not have steroid binding activity (*lane 4*). Receptors preincubated with hsp70 in the absence of ATP and then with hsp90 in the presence of ATP (*lane 3*) have a low steroid binding activity. We have chromatographed the nucleotide in our purified hsp70 preparation by the method of Minami *et al.* (31) and determined that the hsp70 is bound by ATP (data not shown). We also know that some hsp70 binds to the GR in the absence of added ATP (Fig. 2, *lane 3*). Thus, it is not surprising that a low level of steroid binding activity is generated in the absence of added ATP during the preincubation with hsp70.

The experiment of Fig. 7*B* shows that the sequence cannot be reversed, with hsp90 being present in the first incubation and hsp70 in the second (*lane 2*). When hsp90 is not present in the second incubation with ATP, no steroid binding activity is generated (*lane 4*). In sum, all of the data support a model in which there is an initial ATP-dependent event mediated by hsp70, and this event is required for a second ATP-dependent event mediated by hsp90.

DISCUSSION

This study of GR·hsp90 heterocomplex assembly has been greatly facilitated by the use of GR expressed in the baculovirus system. This overexpressed mouse GR is somewhat different from mouse GR in immune pellets prepared from L cell cytosol. Essentially all of the receptors in L cell cytosol are bound to hsp90, and 75–100% of the stripped receptors are reactivated by reticulocyte lysate to the steroid binding state (23). In contrast, only about 12% of the GR immunoadsorbed from Sf9 cell cytosol is bound to insect hsp90, and about 15% of the total GR in stripped immune pellets is converted to the

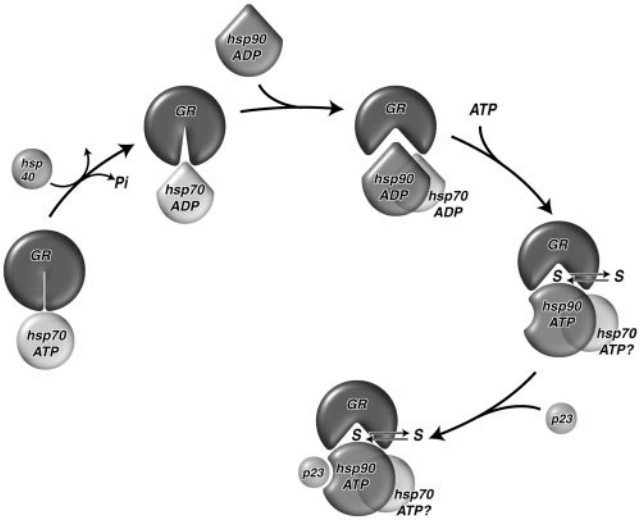

FIG. 8. **Model of GR unfolding by the hsp90-based chaperone machinery.** The model summarizes a series of events predicted from the sequential assembly data that are discussed in the text. The Hop component of the five-protein assembly system has been omitted for simplicity of presentation.

steroid binding state by reticulocyte lysate or by the purified assembly system (17). Because the overexpressed GR is further inactivated by incubation at 30 °C (Fig. 1), we have limited the time of preincubation with hsp70 to 5 min. During the second incubation with the hsp70-free assembly system, about 50% of the prebound hsp70 dissociates from the GR immune pellet (Fig. 4, *A* and *B*). Despite these limitations and losses, we are able to generate more steroid binding activity with two-step reactivation of the Sf9-expressed GR by the purified five protein assembly system (Fig. 2, *lane 8*) than we generate under the best of conditions with reticulocyte lysate and GR immune pellets prepared from L cell cytosol (10–13).

The abundance of baculovirus-expressed GR has permitted us for the first time to assemble in stepwise fashion a functional multiprotein hsp90 heterocomplex assembly system on the receptor. Our previous studies have led us to a model of assembly in reticulocyte lysate in which a preformed hsp90·Hop·hsp70·hsp40 complex associated as a unit with the GR to open the steroid binding pocket, with the co-chaperone p23 being a dynamic participant in the GR·hsp90 assembly mechanism (10–13). In deriving such a model, we have shown that immunoadsorbed preformed hsp90·Hop·hsp70·hsp40 complexes convert the GR to its steroid binding form (11, 13). It is clear that all of the Hop in reticulocyte lysate is bound to hsp90 (32) and that the presence of Hop in the purified assembly system accelerates the rate of formation of steroid binding sites (17). These observations are consistent with a model in which the hsp90·Hop·hsp70·hsp40 complex acts as a machine for heterocomplex assembly.

In the model of Toft and co-workers (14), receptor association with hsp70 and then hsp40-promoted conversion of hsp70 to the ADP-bound conformation are viewed as independent steps that are followed by attachment of an hsp90·Hop unit. Thus, the machinery is viewed as assembling on the receptor rather than being preassembled. It seems likely to us that both models apply, and the data of Figs. 5 and 6 show that a functional machinery can be formed on the receptor in stepwise fashion.

The stepwise assembly approach has allowed us to identify two separate ATP-dependent events that we had previously predicted (16) but had not been able to demonstrate. The model of Fig. 8 summarizes our current view of how hsp70 and hsp90 may interact to open the steroid binding pocket on the receptor. It has been established that hsp70 binds directly to the ligand binding domain of the receptor (1). The first ATP-dependent step involves hsp70 only, and binding of hsp70 to the GR in a manner that is productive for subsequent activation of steroid binding activity by the hsp70-free assembly system requires both ATP and YDJ-1 (Fig. 2). Thus, we predict that hsp70 binds to the GR in its ATP-bound form and undergoes an hsp40-enhanced conversion to its ADP-bound conformation. In Fig. 8, the hydrophobic steroid binding pocket in the GR is in the folded state when the ATP-dependent form of hsp70 binds. Upon ATP hydrolysis, the hsp70 undergoes a conformational change to a form with a high affinity for hydrophobic amino acids. We suggest that this conformational change in hsp70 may induce a conformational change in the GR LBD that begins the opening of the steroid binding pocket and brings hydrophobic residues in the interior of the pocket into contact with the substrate binding region of hsp70. It is possible that this initial ATP-dependent step really consists of multiple substeps in which the GR-bound hsp70 ratchets back and forth between ATP- and ADP-dependent conformations.

The second ATP-dependent step in generating steroid binding activity requires hsp90 (Fig. 7*B*). In the model of Fig. 8, hsp90 is pictured as being in the ADP-bound conformation when it binds to the GR·hsp70 complex, but it is possible that purified hsp90 without bound nucleotide can bind to GR·hsp70. The Toft laboratory has demonstrated that receptor-bound hsp90 must assume its ATP-dependent conformation (22) for the receptor to have steroid binding activity. Thus, the second ATP-dependent event must involve conversion of hsp90 to its ATP-dependent conformation. The Toft laboratory has also shown that p23 binds to the ATP-dependent conformation of hsp90 (20), and the data of Fig. 6*B* suggest that GR·hsp70 complexes that are converted to the steroid binding state by incubation with ATP, hsp90, and p23 contain p23. Thus, we predict that in the second ATP-dependent step, hsp90 is converted to its ATP-dependent conformation. As indicated in Fig. 8, we do not know if hsp70 is also converted to its ATP-dependent conformation during the second step of assembly.

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, and Jack Bodwell for providing FiGR-producing hybridoma cells.

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