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Folding of the Glucocorticoid Receptor by the Reconstituted hsp90-based Chaperone Machinery

THE INITIAL hsp90·p60·hsp70-DEPENDENT STEP IS SUFFICIENT FOR CREATING THE STEROID BINDING CONFORMATION*

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Kurt D. Dittmar‡ and William B. Pratt§

From the Department of Pharmacology, The University of Michigan Medical School, Ann Arbor, Michigan 48109

Rabbit reticulocyte lysate contains a multiprotein chaperone system that assembles steroid receptors into a complex with hsp90. The glucocorticoid receptor (GR) is bound to hsp90 via its hormone binding domain (HBD), which must be associated with hsp90 to have a steroid binding conformation. Recently, we have reconstituted a receptor·hsp90 heterocomplex assembly system with purified rabbit hsp90 and hsp70 and bacterially expressed human p23 and p60 (Dittmar, K. D., Hutchison, K. A., Owens-Grillo, J. K., and Pratt, W. B. (1996) *J. Biol. Chem.* 271, 12833–12839). In this work we show that when the GR is incubated with hsp90, hsp70, and p60, steroid binding sites are generated despite the absence of p23. In this minimal reconstituted system, the GR is incubated with the chaperones in the presence of [^{3}H]triamcinolone acetonide ([^{3}H]TA), which binds to the receptor as GR·hsp90 complexes are formed. When molybdate or p23 is also present during the incubation with chaperones at 30 °C, the formation of steroid binding sites can be assayed by incubating the washed GR with [^{3}H]TA after heterocomplex assembly at 30 °C. However, in the absence of p23 or molybdate, rapid disassembly of GR·hsp90 complexes apparently occurs simultaneously with assembly, such that [^{3}H]TA must be present during the assembly process to trap evidence of conversion of the GR HBD from a non-steroid binding to a steroid binding conformation. Mixture of purified rabbit hsp90 and hsp70 with bacterial lysate containing human p60 results in spontaneous formation of an hsp90·p60·hsp70 complex that can be adsorbed with anti-p60 antibody, and the resulting immune complex converts the GR HBD to a steroid binding state in an ATP-dependent and K^+-dependent manner. When the GR is incubated with hsp90, hsp70, and p60 in the presence of the hsp90-binding antibiotic geldanamycin, GR·hsp90·p60·hsp70 complexes are formed, but they have no steroid binding activity. Our data suggest that hsp90, hsp70, and p60 work together as a chaperone complex that possesses all of the folding/unfolding activity necessary to generate the high affinity steroid binding conformation of the receptor.

Commercial preparations of rabbit reticulocyte lysate which are used for cell-free protein translation contain a system that assembles steroid receptors into multiprotein heterocomplexes that contain the 90-kDa heat shock protein, hsp90[1] (1, 2). Complex formation does not reflect a simple reversible binding of the hsp to the receptor; rather, the complexes are formed by a multiprotein chaperone machinery that appears to be ubiquitously present in eukaryotic cells (3). Because the hormone binding domain (HBD) of the glucocorticoid receptor (GR) must be bound to hsp90 for it to bind steroid (4), the conversion of receptors from a non-steroid binding state to a steroid binding state by the hsp90-based chaperone system can be used as a rapid folding[2] assay to detect the formation of GR·hsp90 complexes in which the HBD is in the high affinity steroid binding conformation (2, 5). Using both formation of steroid binding sites and direct measurement of the formation of receptor·hsp90 complexes, a number of details of heterocomplex assembly by reticulocyte lysate have been established.

Assembly requires ATP/Mg^{2+} and a monovalent cation, with K^+, NH_4^+, and Rb^+ permitting assembly and Na^+ and Li^+ being inactive (5, 6). The monovalent cation selectivity calls to mind a similar requirement for the action of hsp70 (7), which is often found in native steroid receptor heterocomplexes isolated from cytosols (for review of native heterocomplex composition, see Refs. 8 and 9) and is also found in complexes with hsp90 independent of steroid receptors (10, 11). Members of the hsp70 family chaperone protein folding (for review, see Ref. 12), and hsp70 is required for receptor·hsp90 heterocomplex assembly by reticulocyte lysate (6, 13).

Interestingly, purified hsp90 and hsp70 do not bind to each other unless a third factor in lysate and cytosols is present (14). Smith *et al.* (15) predicted that the combining factor was a 60-kDa protein (p60) that coimmunoadsorbed with hsp90 and hsp70. They had observed this p60 in progesterone receptor (PR) complexes formed in reticulocyte lysate when ATP was limiting (6) or at early times of heterocomplex assembly (16). The rabbit p60 (15) is the homolog of a human protein cloned by Honoré *et al.* (17) and the nonessential yeast heat shock protein Sti1 (18). Recently, Chen *et al.* (19) have shown that p60 binds independently to hsp70 via an NH_2-terminal TPR (tetratri-

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‡ Trainee under Pharmacological Sciences Training Program Training Grant GM07767 from the National Institutes of Health.

§ To whom correspondence should be addressed: Dept. of Pharmacology, The University of Michigan Medical School, 1301 Medical Science Research Bldg. III, Ann Arbor, MI 48109-0632. Tel.: 313-764-5414; Fax: 313-763-4450.

[1] The abbreviations used are: hsp, heat shock protein; HBD, hormone binding domain; GR, glucocorticoid receptor; PR, progesterone receptor; TPR, tetratricopeptide repeat; Hip, hsc70-interacting protein; FKBP, FK506-binding protein; CyP-40, the 40-kDa cyclosporin A-binding protein; TA, triamcinolone acetonide; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate.

[2] In this paper we will use the word *folding* to encompass a change in the folding state of the receptor HBD, which may be toward either a more folded state or a partially unfolded state, depending upon the absence or presence of hsp90.

copeptide repeat) region and to hsp90 via a central TPR region to form an hsp90·p60·hsp70 complex. Like hsp70, p60 is required for GR·hsp90 heterocomplex assembly by reticulocyte lysate (20).

Another component of the chaperone system is a conserved, widely distributed 23-kDa protein (21) that binds directly to hsp90 in an ATP-dependent manner (22). Depletion of p23 from reticulocyte lysate prevents receptor·hsp90 heterocomplex assembly, and addition of purified p23 restores the activity (23).

Recently, we have reconstituted a minimal heterocomplex assembly system with purified rabbit hsp90 and hsp70 and bacterially expressed p23 and p60. This reconstituted system produces GR·hsp90 heterocomplexes that are stable at 0 °C and that bind steroid (20). We call this a minimal system because it does not contain a 48-kDa protein recovered in PR heterocomplexes at early times of assembly (16) which was recently reported (24) to be the hsc70-interacting protein (Hip) reported by Höhfeld *et al.* (25). As yet, no role for Hip in a more complex receptor heterocomplex assembly system has been defined. Because mutations in the yeast DnaJ homolog Ydj1 affect steroid receptor function (26, 27), it is likely that mammalian DnaJ homologs (*i.e.* the hsp40 family) are important for optimal heterocomplex assembly in the intact cell. But, to date, hsp40 has not been found in heterocomplex intermediates formed by reticulocyte lysate.

Some high molecular weight immunophilins, such as FKBP52 and CyP-40, are present in native receptor heterocomplexes (8, 9). The immunophilins are protein chaperones with peptidylprolyl isomerase activity (28). These high molecular weight immunophilins possess TPR domains, and, like p60, they bind to hsp90 via their TPR domains (29–32), but they cannot bind to hsp90 as long as p60 is bound to it (32). The immunophilins are present in receptor heterocomplexes assembled in reticulocyte lysate (33). Because assembly of functional GR·hsp90 complexes that bind steroid is normal when peptidylprolyl isomerase activity is blocked by FK506 and cyclosporin A (33) and because complexes are assembled by a reconstituted system in which there are no immunophilins (20), we do not think that the immunophilins are involved in the binding of receptors to hsp90 and proper folding of the HBD.

In this work we focus on the earliest steps in the assembly process to define the components of the chaperone system required for forming the steroid binding conformation of the GR HBD. Using an assay in which evidence for conversion of receptors to the steroid binding state is trapped by binding of [^{3}H]triamcinolone acetonide (TA) during heterocomplex assembly, we demonstrate that hsp90, p60, and hsp70 are sufficient for forming a steroid binding site in an ATP/Mg^{2+}-dependent and K^+-dependent reaction. As purified proteins, these three components combine spontaneously to form an hsp90·p60·hsp70 complex that can be immunoadsorbed with an anti-p60 antibody, and this p60 immune pellet reactivates the GR HBD to the steroid binding state. Reactivation of GR steroid binding activity by hsp90, hsp70, and p60 is blocked by the hsp90-binding antibiotic geldanamycin, even though GR·hsp90·p60·hsp70 complexes are formed. It has been thought that p23 is required for receptor folding (22, 34), but we show here that is not the case. However, the addition of molybdate or p23 yields higher steroid binding activity at the end of the assembly process than that achieved in the presence of only hsp90, p60, and hsp70. We propose that p23 may act to stabilize the receptor·hsp90 complex to rapid disassembly while the GR is incubated at 30 °C with the hsp90·p60·hsp70 chaperone complex.

EXPERIMENTAL PROCEDURES

Materials

[6,7-^{3}H]TA (42.8 Ci/mmol) and ^{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 and anti-rabbit IgG horseradish peroxidase conjugates were from Sigma. The BuGR2 monoclonal IgG antibody against the GR and the 3G3 monoclonal anti-hsp90 IgM 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). The JJ3 monoclonal IgG against p23 and *Escherichia coli* expressing human p23 were gifts from Dr. David Toft (Mayo Clinic). The DS14F5 monoclonal IgG against p60 and *E. coli* expressing p60 were kindly provided by Dr. David Smith (University of Nebraska Medical School). Actigel-ALD (activated aldehyde agarose) affinity support for protein immobilization was from Sterogene Biochemicals (San Gabriel, CA). Hybridoma cells producing FiGR monoclonal IgG against the GR were generously provided by Dr. Jack Bodwell (Dartmouth Medical School).

Methods

Cell Fractionation and Immunoadsorption—L929 mouse fibroblasts (L cells) were grown in monolayer in Dulbecco's modified Eagle's medium supplemented with 10% bovine serum. Cells were harvested by scraping into Earle's balanced saline, suspended in 1.5 volumes of HE buffer (10 mM Hepes, 1 mM EDTA, pH 7.4), and ruptured by Dounce homogenization. Homogenates were centrifuged for 1 h at 100,000 × *g*, and the supernatant is referred to as *cytosol*.

Immunoadsorption of GR, hsp90, and p60—Receptors were immunoadsorbed from 100-μl aliquots of L cell cytosol by rotation for 2 h at 4 °C with 8 μl of Actigel-ALD precoupled to 80 μl of FiGR ascites suspended in 300 μl of TEG (10 mM TES, pH 7.6, 50 mM NaCl, 4 mM EDTA, 10% glycerol). Prior to incubation with reticulocyte lysate or with other additions as noted, immunoadsorbed receptors were stripped of associated hsp90 by incubating the immunopellet an additional 2 h at 4 °C with 0.5 M NaCl followed by one wash with 1 ml of TEG and a second wash with 1 ml of Hepes buffer (10 mM Hepes, pH 7.4). For immunoadsorption of hsp90, 400-μl aliquots of reticulocyte lysate or various mixtures of purified proteins as noted were immunoadsorbed to 8-μl pellets of Actigel-ALD precoupled with either nonimmune mouse ascites or 3G3 anti-hsp90 IgM. The samples were rotated at 4 °C for 2 h, and the immunopellets were washed three times with 1 ml of Hepes buffer. For immunoadsorption of p60, 400-μl aliquots of reticulocyte lysate or various mixtures of purified proteins as noted were immunoadsorbed to 8 μl of protein A-agarose prebound with DS14F5 antibody against p60 (5%) or nonimmune mouse IgG (5%). The samples were rotated at 4 °C for 2 h, and immunopellets were washed twice with 1 ml of Hepes buffer.

Glucocorticoid Receptor Heterocomplex Reconstitution—FiGR immunopellets containing GR stripped of hsp90 were incubated with 50 μl of rabbit reticulocyte lysate or with combinations of proteins (12 μg of purified hsp90, 20 μg of purified hsp70, 3 μl of lysate from bacteria expressing p60) and adjusted to 50 μl with HKD buffer (10 mM Hepes, 100 mM KCl, 5 mM dithiothreitol, pH 7.35). For reconstitution of GR by the immunoadsorbed hsp90 or p60 heterocomplex, stripped receptors were suspended in 50 μl of an assay mix consisting of HKD buffer, and then the whole GR immunopellet suspension was pipetted onto either the 3G3 or DS14F5 immunopellet containing the immunoadsorbed hsp90 or p60, respectively, and their associated protein complexes. Dithiothreitol (1 μl) was added to each incubation to a final concentration of 5 mM, and 5 μl of an ATP-regenerating system (50 mM ATP, 250 mM creatine phosphate, 20 mM MgOAc, and 100 units/ml creatine phosphokinase) were added to all assays to yield a final assay volume of 56 μl. The assay mixtures were incubated for 20 min at 30 °C with suspension of the pellets by shaking the tubes every 5 min for soluble protein conditions or every min for immunoadsorbed p60 and hsp90 conditions. At the end of the incubation, the pellets were washed twice with 1 ml of ice-cold TEGM buffer (TEG buffer with 20 mM sodium molybdate) and assayed for steroid binding capacity and, in some experiments, receptor-associated proteins. To conserve the purified components of the reconstitution system, each experimental condition represents a single sample. The experimental observations have been replicated, and in most cases, the key observation from an experiment appears again as one of the conditions presented in another panel in the same figure or in one of the subsequent figures.

Assay of Steroid Binding Capacity—Immune pellets to be assayed for steroid binding were incubated overnight in 100 μl of TEGM buffer plus

4 mM dithiothreitol and 50 nM [^{3}H]TA. Samples were then washed twice with 1 ml TEGM and counted by liquid scintillation spectrometry as described previously. The steroid binding is expressed as cpm of [^{3}H]TA bound/FiGR immunopellet prepared from 100 μl of cytosol. As noted previously (5), 100 μl of L cell cytosol contains 60,000 cpm of [^{3}H]TA binding capacity, and we immunoadsorb about 50% of the GR. Thus ~30,000 cpm represents 100% of receptors reactivated to the steroid binding form.

Gel Electrophoresis and Western Blotting—For assay of GR and associated proteins or p60 and associated proteins, immune pellets were boiled in SDS sample buffer with 10% β-mercaptoethanol, and proteins were resolved on 7% SDS-polyacrylamide gels (12% for resolving p23). Proteins were then transferred to Immobilon-P membranes and probed with 2 μg/ml BuGR monoclonal antibody for the GR, 1 μg/ml AC88 for hsp90, 1 μg/ml N27F3-4 for hsp70, 0.1% DS14F5 anti-p60 mouse ascites for p60, or 0.1% JJ3 mouse ascites for p23. The immunoblots were then incubated a second time with the appropriate ^{125}I-conjugated counter-antibody to visualize the immunoreactive bands.

Protein Purification—The bacterial expression of human p23 and its purification have been described (23). Briefly, p23 is soluble in bacterial lysates, and its abundance and high affinity for DEAE-cellulose allowed purification to 90% purity by chromatography on DEAE-cellulose. The protein was concentrated by precipitation with ammonium sulfate at 80% of saturation. It was dissolved and dialyzed into 10 mM Tris, 100 mM KCl, and 10% glycerol, pH 7.4, and stored at −70 °C.

Hsp70 and hsp90 were purified from rabbit reticulocyte lysate as described previously (13). Briefly, reticulocyte lysate was chromatographed on a DE52 column exactly as described by Dittmar *et al.* (20). Fractions containing hsp70 were chromatographed on an ATP-agarose column and eluted with ATP followed by ammonium sulfate precipitation, and DE52 fractions containing hsp90 were chromatographed on hydroxylapatite followed by chromatography over ATP-agarose exactly as described by Hutchison *et al.* (13). The purified hsp70 and hsp90 were dialyzed against HKD buffer, flash frozen, and stored at −70 °C.

Expression of p60—The bacterial expression of p60 has been described previously (20). Control *E. coli* and bacteria expressing p60 were grown to an A_{600} of 0.6, induced with isopropyl-1-thio-β-D-galactopyranoside for 3 h at 25 °C, and harvested. Bacterial lysates were prepared by sonication in phosphate-buffered saline, and aliquots were flash frozen and stored at −70 °C.

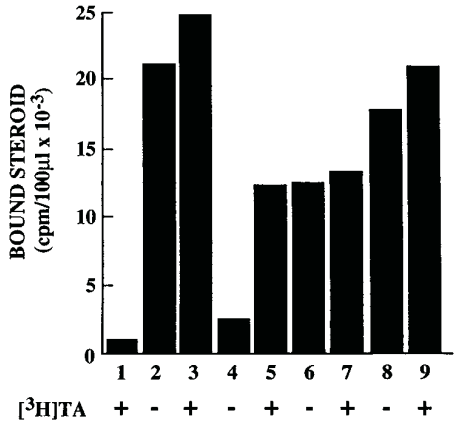

FIG. 1. **A mixture of hsp90, p60, and hsp70 is sufficient to convert the GR HBD to the steroid binding conformation.** GR was immunoadsorbed to FiGR-Actigel from replicate 100-μl aliquots of L cell cytosol, receptor-associated proteins were stripped from the immune pellets with 0.5 M NaCl, and the immune pellets were then incubated for 20 min at 30 °C in the presence of an ATP-regenerating system and the indicated additions, as described under "Methods." At the end of the incubation, the immune pellets were washed, and [^{3}H]triamcinolone acetonide binding was assayed. The *graph* presents reconstitution of steroid binding activity assayed with 50 nM [^{3}H]TA present during the incubation at 30 °C (+) or by incubating with [^{3}H]TA after the 30 °C incubation and washing of the immune pellets (−). Conditions: *lane 1*, stripped GR incubated with buffer and [^{3}H]TA; *lanes 2* and *3*, stripped GR incubated with reticulocyte lysate in the absence (*lane 2*) or presence (*lane 3*) of [^{3}H]TA; *lanes 4* and *5*, stripped GR incubated with hsp90, hsp70, and p60 in the absence (*lane 4*) or presence (*lane 5*) of [^{3}H]TA; *lanes 6* and *7*, GR incubated with hsp90, hsp70, p60, and 20 mM sodium molybdate in the absence (*lane 6*) or presence (*lane 7*) of [^{3}H]TA; *lanes 8* and *9*, GR incubated with hsp90, hsp70, p60, and p23 in the absence (*lane 8*) or presence (*lane 9*) of [^{3}H]TA.

RESULTS

Reactivation of GR Steroid Binding Activity with hsp90, hsp70, and p60—Using purified proteins, we have shown that four proteins, hsp90, hsp70, p60, and p23, are required to form a GR·hsp90 complex and reactivate GR steroid binding activity (20). This was determined by incubating the immunoadsorbed GR (stripped free of associated proteins) with rabbit hsp90 and hsp70 and bacterially expressed human p60 and p23 at 30 °C, followed by washing the immune pellet and binding [^{3}H]TA to the immune pellet at 0 °C. Thus, this method requires the assembly of a stable GR·hsp90 heterocomplex that does not disassemble during washing and subsequent incubation with ligand. In experiments using wheat germ extract instead of reticulocyte lysate to reconstitute GR·hsp90 heterocomplexes, we found that GR·plant hsp90 complexes were very unstable, but formation of steroid binding sites could be detected by having [^{3}H]TA present while the GR was incubated with the wheat germ extract (3). Thus, as soon as a GR·plant hsp90 complex was formed, the receptor was bound by steroid, and evidence of proper folding of the HBD to the steroid binding conformation was preserved. Here, we use this technique of heterocomplex assembly in the presence of ligand to show that a mixture of hsp90, hsp70, and p60 is sufficient to generate the steroid binding conformation of the GR HBD, but a stable heterocomplex is formed only when p23 or molybdate is present during assembly.

Fig. 1 shows reactivation of GR steroid binding activity by the reconstituted hsp90-based chaperone system. In this experiment, steroid binding was assayed in two ways. The samples indicated with a (+) have [^{3}H]TA present during the incubation of GR with chaperones at 30 °C; the samples indicated by the (−) were incubated with chaperones in the absence of steroid, and the washed immune pellets were subsequently incubated with [^{3}H]TA according to our usual assay procedure. When GR that is stripped of associated proteins (*lane 1*) is incubated with a mixture of hsp90, hsp70, and p60 in the absence of steroid, little or no steroid binding activity is seen on subsequent incubation of the immune pellet with [^{3}H]TA (*lane 4*). But when [^{3}H]TA is present while the receptor is incubated with the chaperones, it can be seen that hormone binding sites were formed (*lane 5*). If molybdate is present during the 30 °C incubation to stabilize receptor heterocomplexes as they are formed by hsp90, hsp70, and p60, then the immune pellet binds [^{3}H]TA when the ligand is added after the incubation (*lane 6*) as well as when it is present during the incubation (*lane 7*). If p23 is present during the incubation with hsp90, hsp70, and p60, then more steroid binding sites are detected with both assays (*lanes 8* and *9*), and reactivation is nearly to the level obtained with reticulocyte lysate (*lanes 2* and *3*). Molybdate stabilizes GR·hsp90 complexes formed by the hsp90, hsp70, p60 mixture with maximal effect at 20 mM (data not shown), which is the concentration used throughout this work.

Formation of GR·hsp90 Complexes by hsp90, hsp70, and p60 Is ATP-dependent—It is known that assembly of receptor·hsp90 complexes and activation of steroid binding activity by whole reticulocyte lysate requires ATP/Mg^{2+} (5, 6). Under the assay conditions used in those experiments, p23 is also required for receptor heterocomplex formation (20, 23), and the binding of p23 to hsp90 is itself ATP-dependent (22, 35). It is likely that other events in the receptor folding/heterocomplex assembly process are ATP-dependent in addition to p23 binding to hsp90. Thus, we wanted to ask if GR·hsp90 heterocomplex assembly by hsp90, hsp70, and p60 in the absence of p23 was ATP-dependent. Fig. 2 shows that incubation

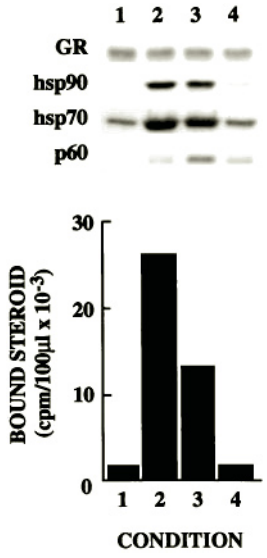

FIG. 2. **Formation of the GR·hsp90 heterocomplex and activation of steroid binding activity by hsp90, hsp70, and p60 are ATP-dependent.** Receptor was immunoadsorbed from 400-μl (Western blot) or 100-μl (steroid assay) aliquots of mouse L cell cytosol, and receptor-associated proteins were stripped from the immunopellets with 0.5 M NaCl. The stripped immune pellets were incubated for 20 min at 30 °C with 20 mM molybdate and the indicated additions. GR, hsp90, hsp70, and p60 were assayed in each sample by Western blotting. Immunopellets for the steroid binding assay were reconstituted for 20 min at 30 °C in the presence of [^{3}H]triamcinolone acetonide as well. *Lane 1*, stripped receptor incubated with buffer; *lane 2*, stripped receptor incubated with reticulocyte lysate and an ATP-regenerating system; *lanes 3* and *4*, stripped receptor incubated with hsp90, hsp70, p60, and an ATP-regenerating system (*lane 3*) or an ATP-regenerating system with 5 mM AMP-PNP instead of 5 mM ATP (*lane 4*).

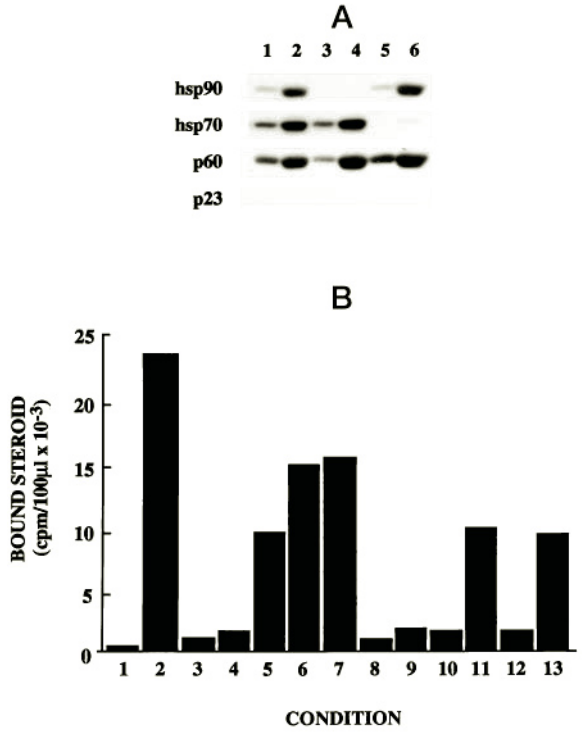

FIG. 3. **Reconstitution of an hsp90·p60·p70 complex that reconstitutes GR steroid binding activity.** *Panel A*, formation of the hsp90·p60·hsp70 complex. Aliquots (4 μl) of lysate from bacteria expressing p60 were incubated for 15 min with 30 μg of hsp70 or 20 μg of hsp90 or both hsps. The mixtures were then diluted with an equal volume of TEG buffer and immunoadsorbed with nonimmune IgG or anti-p60. The immune pellets were washed twice, and proteins were resolved by SDS-polyacrylamide gel electrophoresis and Western blotting. Conditions: *lanes 1* and *2*, nonimmune (*lane 1*) and anti-p60 (*lane 2*) immunoadsorption of p60, hsp90, hsp70 mixture; *lanes 3* and *4*, nonimmune (*lane 3*) and anti-p60 (*lane 4*) immunoadsorption of p60, hsp70 mixture; *lanes 5* and *6*, nonimmune (*lane 5*) and anti-p60 (*lane 6*) immunoadsorption of p60, hsp90 mixture. *Panel B*, reactivation of GR steroid binding activity with the immunoadsorbed p60 complex. Washed p60 immune pellets prepared from a mixture of p60, hsp90, and hsp70 prepared as in *panel A* were incubated for 20 min at 30 °C with immunoadsorbed, stripped GR, an ATP-regenerating system, and additions as indicated. For *conditions 1–11*, the combined immune pellets were washed at the end of the 30 °C incubation and then incubated overnight with [^{3}H]TA. Conditions: *lane 1*, stripped GR; *lane 2*, stripped GR incubated with reticulocyte lysate; *lane 3* and *4*, stripped GR incubated with a nonimmune (*lane 3*) or anti-p60 (*lane 4*) immunopellet prepared from reticulocyte lysate as in Fig. 2; *lanes 5–7*, stripped GR incubated with the p60 immunopellet from reticulocyte lysate and 20 mM molybdate (*lane 5*), 4 μg of p23 (*lane 6*), or both molybdate and p23 (*lane 7*); *lanes 8* and *9*, stripped GR incubated with a nonimmune (*lane 8*) or anti-p60 (*lane 9*) immunopellet from the p60, hsp90, hsp70 mixture; *lanes 10* and *11*, stripped GR incubated with a nonimmune (*lane 10*) or anti-p60 (*lane 11*) immunopellet from the p60, hsp90, hsp70 mixture and molybdate and p23; *lanes 12* and *13*, stripped GR incubated with a nonimmune (*lane 12*) or anti-p60 (*lane 13*) immunopellet from the p60, hsp90, hsp70 mixture, without molybdate or p23 but with [^{3}H]TA being present during the 30 °C incubation.

of stripped receptors (*lane 1*) with the mixture of hsp90, hsp70, and p60 in the presence of an ATP-regenerating system and molybdate produced a GR·hsp90 heterocomplex with steroid binding activity (*lane 3*), but in the presence of the nonhydrolyzable analog AMP-PNP, no heterocomplexes or steroid binding activity was generated. Thus, GR·hsp90 heterocomplex assembly in the absence of p23 seems to require ATP hydrolysis, and it is likely that there are at least two ATP-dependent steps when reconstitution is carried out in reticulocyte lysate where all components of the chaperone system (hsp90, hsp70, p60, p23, and the potential participants Hip and hsp40) are present.

The Three Chaperones Act as an hsp90·p60·hsp70 Complex—The fact that hsp90, hsp70, and p60 are sufficient for folding the HBD to the steroid binding conformation is useful in deriving a model of receptor heterocomplex assembly. We proposed earlier that the proteins required for this folding event are preassociated as a protein folding machine that we have called a *foldosome* (36). This notion was based on the observation that immunoadsorption of hsp90 from reticulocyte lysate with a monoclonal antibody yielded an immune pellet containing all of the factors (including hsp90, hsp70, and p60) required for GR·hsp90 heterocomplex assembly and activation of steroid binding activity. A weakly bound component that could be washed off this hsp90 immune pellet was required for heterocomplex assembly and was later identified as p23 (34). It is now clear, however, that hsp90 has several protein binding sites and that multiple complexes of hsp90 and other proteins can be detected in reticulocyte lysate depending upon the antibody (anti-p60, anti-p23, etc.) used for immunoadsorption (32). Immune adsorption of p60 from reticulocyte lysate yields coadsorption of hsp90 and hsp70 (15, 32), and Chen *et al.* (19) have shown that p60 binds at independent sites to hsp90 and hsp70 to form an hsp90·p60·hsp70 complex. It is important to determine whether this complex has GR folding activity.

In the experiment of Fig. 3*A*, p60 was immunoadsorbed from a mixture of p60, hsp90, and hsp70. As shown in *lane 2* (Fig. 3*A*), both hsp90 and hsp70 are coimmunoadsorbed with p60. Fig. 3*B* shows the ability of this reconstituted hsp90·p60·hsp70 complex to reactivate GR steroid binding activity compared with a p60 immune pellet prepared from reticulocyte lysate. Although incubation of stripped GR (Fig. 3*B*, *lane 1*) with the reconstituted complex does not yield steroid binding activity when assayed in the usual manner by adding [^{3}H]TA to the washed immunopellet mixture after the 30 °C incubation (*lane 9*), generation of steroid binding activity can be detected when

[^{3}H]TA is present during the incubation (*lane 13*). Steroid binding sites are detected with the usual postincubation binding assay if p23 is present during the incubation of GR with the reconstituted hsp90·p60·hsp70 complex (*lane 11*). It is clear that p23 is not required for forming steroid binding sites because the presence of only the hsp90·p60·hsp70 complex is sufficient for reconstitution (*lane 13*), and it should be noted that there is no p23 contaminant in the reconstituted hsp90·p60·hsp70 complex (Fig. 3*A*).

In these experiments where anti-p60 antibody is used to immunoadsorb a reconstituted hsp90·p60·hsp70 complex, more activation of steroid binding capacity can be obtained if more p60 is immunoadsorbed, but we have not increased the amounts of antibody to spare the reagent. It should be mentioned that there could be a low amount of the hsp40 family of proteins (rabbit DnaJ homologs) which copurifies with hsp70 and is not detected in stained gels of our purified product. Unfortunately, we do not have access to an antibody to test for low levels of hsp40 contamination by immunoblotting.

Our interpretation of the data thus far is that the hsp90·p60·hsp70 complex is sufficient for forming a GR·hsp90 heterocomplex in which the HBD is properly folded into the steroid binding conformation. But the GR·hsp90 complex is disassembled rapidly during the 30 °C incubation unless p23 is present to yield stable complexes that can be assayed at the end of the incubation by adding [^{3}H]TA to the washed immunopellet. Even though the complexes formed by hsp90·p60·hsp70 undergo dynamic assembly/disassembly, if [^{3}H]TA is present during the 30 °C incubation when the complexes are formed, the steroid binds to the receptor and remains bound despite subsequent disassembly of the GR heterocomplex.

In the case of GR activation caused by hsp90 immune pellets from reticulocyte lysate, we have shown that the receptor-activating system dissociates from the protein A-Sepharose-bound antibody during the incubation with the GR (36). Thus, the immunoadsorbed complex can be acting as a solubilized protein complex or as solubilized proteins, not as a particulate-bound complex. This is also the case when the reconstituted hsp90·p60·hsp70 complex is immunoadsorbed with the anti-p60 antibody, and the immune pellet is incubated with the GR. As shown in Fig. 4*A* (*condition 3*), incubation of a p60 immune pellet (prepared from purified hsp90, hsp70, and p60) under the conditions that are used for receptor heterocomplex assembly is accompanied by release of hsp90, hsp70, and p60 from the immune pellet. At least some of the dissociated protein is present in the supernatant as an hsp90·p60·hsp70 complex that can be immunoadsorbed again with anti-p60 (Fig. 4*A*, *condition 4*). The proteins released from the p60 immune pellet are able to reactivate GR steroid binding activity (Fig. 4*B*, *lanes 6* and *8*).

Geldanamycin Blocks Generation of Steroid Binding Activity—The GR·hsp90 heterocomplexes formed by the reconstituted system contain a lot of p60, whereas those formed by reticulocyte lysate contain little (Fig. 2, compare *lane 3* with *lane 2*) or, sometimes, none. On the basis of experiments with the hsp90-binding antibiotic geldanamycin, it has been thought that a receptor·hsp90 heterocomplex containing p60 does not have steroid binding activity. It has been shown that treatment of cells with geldanamycin causes a rapid loss in steroid binding activity (37, 38); and when PR heterocomplexes are formed in reticulocyte lysate in the presence of geldanamycin, the PR does not bind steroid, and the PR·hsp90 complexes contain increased p60 and no p23 (22, 37). Because geldanamycin also blocks the binding of p23 to hsp90 in reticulocyte lysate independent of the presence of PR (22), it has been proposed that the ability of geldanamycin to arrest PR heterocomplex assembly is due to its ability to directly block binding of p23 to hsp90, thus preventing formation of a mature (p23-containing, p60-free) steroid binding receptor heterocomplex from a non-steroid-binding intermediate (p60-containing, p23-free) complex (22, 37). With the minimal reconstitution system, we can test the effect of geldanamycin on GR·hsp90 heterocomplex assembly independent of the presence of p23.

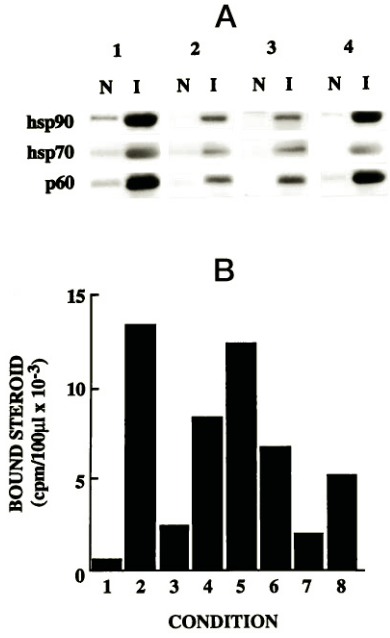

FIG. 4. **When the p60 immune pellet is incubated at 30 °C, hsp90, p60, and hsp70 dissociate from the immune pellet as a complex, and the soluble proteins can fold the HBD to the steroid binding conformation.** *Panel A*, release of p60, hsp90, and hsp70 from the immune pellet. Complexes of hsp90·p60·hsp70 were formed as described under Fig. 3 and immunoadsorbed with nonimmune IgG (*N*) or anti-p60 (*I*). The immune pellets were suspended in HKD buffer with an ATP-generating system, incubated for 20 min at 30 °C, and proteins remaining in the pellet and released from the pellet were assayed by Western blotting. *Condition 1*, proteins in an immune pellet that was not incubated at 30 °C; *condition 2*, proteins remaining in immune pellets after 30 °C incubation; *condition 3*, proteins released from immune pellets incubated at 30 °C; *condition 4*, released proteins from two immune pellets were readsorbed with anti-p60. *Panel B*, the proteins released from the p60 immune pellet convert the GR HBD to the steroid binding conformation. Stripped receptors were incubated for 20 min at 30 °C under the following conditions, and steroid binding activity was assayed: *lane 1*, stripped GR incubated with HKD buffer; *lane 2*, stripped GR incubated with hsp90, hsp70, p60, and p23; *lane 3*, stripped GR incubated with the nonimmune pellet shown in *condition 1* of *panel A* plus p23; *lanes 4* and *5*, stripped GR incubated with the immune pellet shown in *condition 1* of *panel A* plus 20 mM molybdate (*lane 4*) or p23 (*lane 5*); *lane 6*, stripped GR incubated with proteins released from the p60 immune pellet as shown in *condition 3* of *panel A* plus molybdate; *lanes 7* and *8*, stripped GR incubated with a nonimmune (*lane 7*) or anti-p60 (*lane 8*) immune pellet prepared by reimmunoadsorption of released proteins as shown in *condition 4* of *panel A* plus molybdate.

In the experiment of Fig. 5, stripped GR (*lane 1*) was incubated with whole reticulocyte lysate (*lanes 3* and *4*), a mixture of hsp90, hsp70, p60, and p23 (*lanes 6* and *7*), or a mixture of hsp90, hsp70, and p60 with 20 mM molybdate (*lanes 9* and *10*). Under each condition of heterocomplex assembly, geldanamycin increased the amount of p60 in the complex and inhibited or blocked generation of steroid binding activity (compare *lanes 3* and *4*, *6* and *7*, *9* and *10*). From *lane 10* of Fig. 5, it is clear that geldanamycin inhibits the generation of steroid binding activity in the absence of p23. Thus, we would suggest that geldanamycin blocks receptor heterocomplex assembly at a p60-containing intermediate and that it also blocks binding of p23 to hsp90 but that generation of steroid binding activity is not

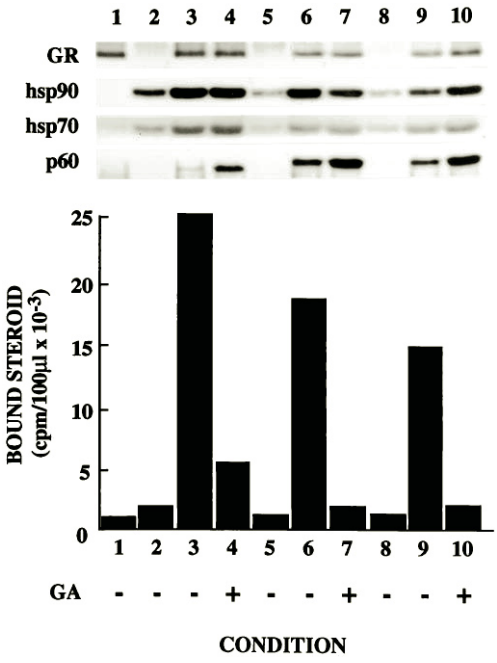

FIG. 5. **Geldanamycin blocks the activation of GR steroid binding activity by the mixture of hsp90, hsp70, and p60.** Samples of stripped GR were incubated for 20 min at 30 °C with the ATP-regenerating system, K^+, molybdate, and the indicated additions. GR, hsp90, hsp70, and p60 in the immune pellet were assayed by Western blotting, and a portion of the immune pellet was incubated with [^{3}H]TA to determine steroid binding activity. *Lane 1*, stripped GR; *lanes 2–4*, stripped nonimmune (*lane 2*) and GR (*lanes 3* and *4*) immunopellets incubated with reticulocyte lysate in the absence (*lane 3*) or presence (*lane 4*) of 10 µM geldanamycin (*GA*); *lanes 5–7*, stripped nonimmune (*lane 5*) and GR (*lanes 6* and *7*) immunopellets incubated with hsp90, hsp70, p60, and p23 in the absence (*lane 6*) or presence (*lane 7*) of geldanamycin; *lanes 8–10*, stripped nonimmune (*lane 8*) and GR (*lanes 9* and *10*) immunopellets incubated with hsp90, hsp70, p60, and 20 mM molybdate in the absence (*lane 9*) or presence (*lane 10*) of geldanamycin. Note that the human p60 in *lanes 6*, *7*, *9*, and *10* migrates slightly slower than the rabbit p60 in *lane 4*.

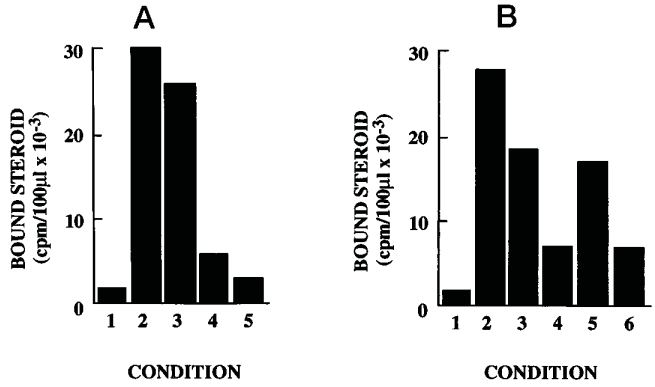

FIG. 6. **Activation of the GR to the steroid binding state with the hsp90·p60·hsp70 complex is potassium-dependent.** *Panel A*, immunoadsorbed p60 heterocomplex of reticulocyte lysate requires K^+ to reconstitute GR. Reticulocyte lysate was immunoadsorbed with anti-p60 antibody, and the p60 immunopellets were incubated with stripped GR immunopellets in the presence of the ATP-regenerating system, [^{3}H]TA, and the indicated buffer. *Lane 1*, stripped GR incubated with HKD buffer; *lane 2*, stripped GR incubated with reticulocyte lysate; *lanes 3–5*, stripped GR incubated with p60 immunopellet suspended in HKD buffer (*lane 3*), HND buffer (10 mM Hepes, 100 mM NaCl, 1 mM dithiothreitol) (*lane 4*), or HD buffer (10 mM Hepes, 1 mM dithiothreitol) (*lane 5*). *Panel B*, potassium is required only after formation of a functional hsp90·p60·hsp70 heterocomplex from purified proteins. hsp90, hsp70, and p60 were each dialyzed against HKD or HND buffer, combined, and immunoadsorbed with anti-p60. The p60 immunopellets were washed twice with 1 ml of Hepes, mixed with stripped GR immunopellets, and incubated at 30 °C in the presence of the ATP-regenerating system, [^{3}H]TA, and p23. *Lane 1*, stripped GR incubated with HKD; *lane 2*, stripped GR incubated with a mixture of hsp90, hsp70, p60, and p23 in HKD; *lanes 3* and *4*, stripped GR incubated in either HKD (*lane 3*) or HND (*lane 4*) with a p60 immunopellet created from purified proteins dialyzed against HKD plus p23; *lanes 5* and *6*, stripped GR incubated in either HKD (*lane 5*) or HND (*lane 6*) with a p60 immunopellet prepared from purified proteins dialyzed against HND plus p23.

blocked because p23 cannot bind to hsp90. Rather, geldanamycin binding must induce or stabilize an inactive state of hsp90 which does not carry out the folding change in the HBD required to produce a steroid binding site.

Folding of the HBD by hsp90·p60·hsp70 Is a K^+-dependent Step—It has been shown that folding of the GR HBD to the steroid binding conformation by rabbit reticulocyte lysate requires potassium (5), but the K^+-dependent step(s) has not been determined. Palleros *et al.* (7) have shown that K^+ is required for ATP-induced dissociation of hsp70 from proteins that are being chaperoned. However, the GR·hsp90 complex formed by the reconstituted system still contains hsp70 (*e.g.* Fig. 2, *lane 3*) as well as p60. Thus, we expected that generation of steroid binding activity by the hsp90·p60·hsp70 complex would not be K^+-dependent. In the experiment of Fig. 6*A*, stripped GR (*lane 1*) was incubated with the immunoadsorbed hsp90·p60·hsp70 complex (*i.e.* p60 immunopellet) of reticulocyte lysate in buffer containing KCl (*lane 3*) or NaCl (*lane 4*). In this minimal system there is extensive activation of steroid binding activity in the presence of K^+ (*lane 3*) but not in the presence of Na^+ (*lane 4*) or in the absence of monovalent cation (*lane 5*).

To determine if K^+ was required for formation of the hsp90·p60·hsp70 complex or for folding of the HBD we performed the experiment of Fig. 6*B*. In this experiment, hsp90, hsp70, and p60 were dialyzed against buffer with K^+ or Na^+, the proteins were then mixed, and after immunoadsorption of p60, the p60 immune pellets were incubated with the GR and p23 in either K^+ or Na^+ buffer. It can be seen that a functional hsp90·p60·hsp70 heterocomplex assembles in the presence of either K^+ (*lane 3*) or Na^+ (*lane 5*), but subsequent activation of steroid binding activity requires K^+ (compare *lanes 3* and *5* with *lanes 4* and *6*).

DISCUSSION

In our previous study with the reconstituted chaperone system, which utilized only the postincubation steroid binding assay, we found that all four components of the system, hsp90, hsp70, p60, and p23, had to be present for both GR·hsp90 heterocomplex assembly and reactivation of steroid binding activity to occur (20). The notion that p23 was required for proper folding of the HBD to the steroid binding conformation was supported by previous studies showing that depletion of p23 from reticulocyte lysate inactivates PR·hsp90 heterocomplex assembly (23) and that p23 is required to form steroid binding sites when GR is incubated with washed hsp90 immune pellets (34). However, with steroid present during heterocomplex assembly, we show here that hsp90, hsp70, and p60 are sufficient to obtain proper folding of the GR HBD such that it binds steroid. When only the three chaperones are present, the complexes that are formed are unstable unless molybdate or p23 is present to stabilize them. Interestingly, when human p23 is added to wheat germ lysate, it binds to the plant hsp90 in an ATP-dependent manner (35), and it has the same effect on GR incubated with wheat germ lysate that is shown in Fig. 1 for GR incubated with the mixture of hsp90, hsp70, and p60 (34).

A model of GR heterocomplex assembly that incorporates findings from this reconstitution work is presented in Fig. 7. Consistent with the results of physical studies on hsp90-free

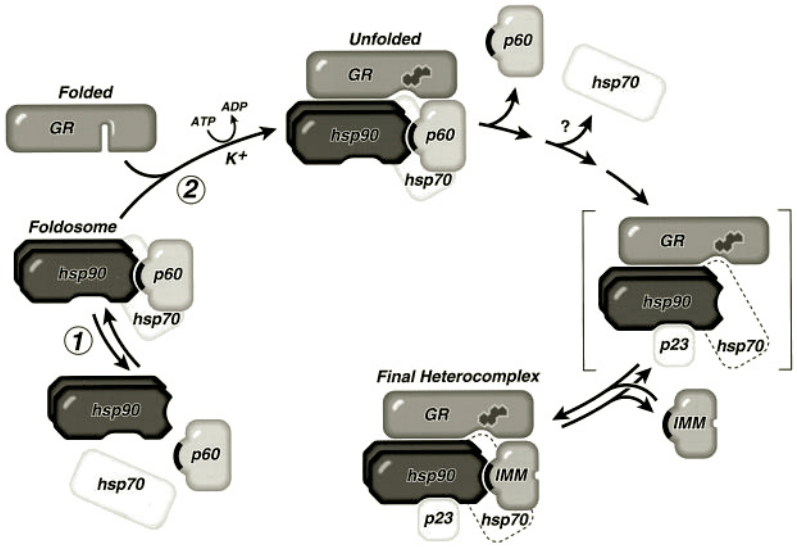

FIG. 7. **Model of the earliest steps in GR heterocomplex assembly by the minimal reconstitution system of hsp90, hsp70, and p60.** The HBD of the GR is viewed as proceeding from a folded conformation in which the hydrophobic steroid binding pocket is not accessible to bind hormone to a partially unfolded conformation with an accessible steroid binding site, indicated by the steroid structure. *IMM* stands for an immunophilin, such as FKBP52 or CyP-40. The immunophilins and p60 bind to hsp90 via TPR domains, which are indicated by the *solid black crescents* on these proteins. Native GR heterocomplexes isolated from cells usually do not contain hsp70; but native receptor heterocomplexes from some cells, as well as complexes generated by reticulocyte lysate, do contain hsp70. Thus, the hsp70 with the *dashed line* indicates its optional presence in the final product. Other details are summarized under "Results."

and hsp90-bound GR (39), the HBD of the hsp90-free receptor is pictured in a folded conformation in which the steroid binding pocket is not accessible to ligand. In this work we have shown that hsp90, hsp70, and p60 are sufficient to yield a steroid binding conformation of the HBD (indicated in Fig. 7 by the presence of the steroid structure within the GR). We envision the steroid binding conformation as a partially unfolded state of the HBD, with the unfolding process opening up a hydrophobic cavity that is internal and not accessible to the ligand in the folded, hsp90-free state (39).

This work has focused on the initial steps in the formation of a GR heterocomplex. We have shown that mixing purified hsp90 and hsp70 with bacterial lysate containing p60 results in spontaneous formation of an hsp90·p60·hsp70 complex that can be immunoadsorbed with anti-p60 antibody (Fig. 3*A*). The proteins in the immunoadsorbed complex are active in that they have the protein folding/unfolding activity required to convert the receptor HBD to a steroid binding state (Fig. 3*B*). Formation of the active hsp90·p60·hsp70 complex (*step 1* in the model) does not require K^+ or ATP/Mg^{2+}, but attachment to the receptor and/or accompanying conformational change in the HBD (*step 2*) is both ATP- and K^+-dependent (Figs. 2 and 6).

A major difference between the GR·hsp90 heterocomplexes assembled by reticulocyte lysate and those assembled by the minimal reconstitution system containing hsp90, hsp70, and p60 (plus or minus p23) is that the complexes formed by reticulocyte lysate contain little or, sometimes, no p60, whereas those formed by the reconstituted system do contain p60 (Figs. 2 and 5). It would seem that the reticulocyte lysate contains an activity that facilitates the exit of p60 from the receptor heterocomplex, and this activity is not present in the reconstituted system. What facilitates the exit of p60 from the receptor complex is not clear, but the data of Fig. 5 show that it is not p23.

Although p23 is clearly part of the final receptor heterocomplex, we do not know whether it can enter to stabilize at one stage or at several stages of assembly; and in Fig. 7, we have simply placed it in the complex that binds immunophilins. It may very well be that p23 engages in a very dynamic association, not only with free hsp90 as shown by Johnson and Toft (22), but with hsp90 in a complex with p60 and hsp70, and with hsp90 bound to the GR before and after the exit of p60 from the heterocomplex. Addition of purified p23 to the mixture of hsp90, hsp70, and p60 has a profound affect on GR heterocomplex assembly/disassembly at 30 °C, being required for production of stable complexes that can be assayed by Western blot and by steroid binding after the 30 °C incubation with the reconstituting system.

The final GR·hsp90 heterocomplexes formed in rabbit reticulocyte lysate, as well as native heterocomplexes recovered from cells, contain an immunophilin, such as FKBP52 or CyP-40, which is bound to hsp90 via its TPR domain (29–32). TPR domains are indicated by the *solid black crescents* in Fig. 7. p60 is also bound to hsp90 via a TPR domain (19), and immunophilins cannot bind to hsp90 when p60 is bound to it (32). Thus, immunophilins must bind as p60 dissociates from the complex, and we have placed a p60-free and immunophilin-free state of the GR·hsp90 heterocomplex in *brackets* to indicate that it is a potential assembly intermediate that has not yet been demonstrated.

The model of Fig. 7 is somewhat different from the model of Smith and his co-workers (16, 24, 37), which is based on the observation that certain components like hsp70, p60, and p48 are observed in PR immune pellets transiently at early times of heterocomplex assembly in reticulocyte lysate, whereas p23 and immunophilins are recovered in the immune pellets somewhat later. They have proposed an "ordered assembly pathway" in which the PR first binds to a complex containing hsp90, hsp70, and p60 (and perhaps p48); these proteins (including the PR-associated hsp90) then dissociate from the receptor and are replaced by a different hsp90 that is bound to p23 and an immunophilin to yield the "mature," steroid-binding form of the receptor heterocomplex. To us, it seems counterintuitive that the folding mechanism would involve binding of a receptor to an hsp90 that is then exchanged for a second hsp90 that is bound to p23. The fact that the immunoadsorbed reconstituted hsp90·p60·hsp70 complex is sufficient to generate GR steroid binding activity (Figs. 3 and 4) argues against a model of heterocomplex assembly in which there is ordered involvement of two separate hsp90-bound units with the receptor. Clearly, neither p23 nor molybdate, which can substitute for p23 in the reconstituted system, is required for folding of the HBD into a steroid binding conformation (Fig. 1).

Because ATP-dependent binding of hsp70 occurs very early in the time course of assembly in reticulocyte lysate, Prapapanich *et al.* (24) have suggested that hsp90 and p60 may assemble on hsp70-bound PR. That hsp70 binds to the receptor initially is a logical suggestion that can be tested with the reconstituted system. It should be noted that purified hsp70 and hsp70 homologs from bacteria (DnaK) and the endoplasmic reticulum (BiP) can bind to the stripped GR in a manner that is not productive for forming GR·hsp90 complexes (40). Thus, binding of hsp70 to receptor early in the time course of

receptor·hsp90 heterocomplex assembly does not have to reflect binding that is part of the receptor·hsp90 heterocomplex assembly process. Also, we have reported previously that incubation of hsp70-prebound GR with hsp70-free reticulocyte lysate does not yield GR·hsp90 complexes or activation of steroid binding activity (13).

Using the reconstituted system, we have found that incubation of the GR with hsp70 yields GR·hsp70 complexes, but if these complexes are washed and incubated with hsp90 and p60, almost no steroid binding activity is generated (data not shown). However, this does not exclude the possibility that an hsp90·p60·hsp70 complex may have to interact with an hsp70-prebound receptor. Because preformed, immune-isolated hsp90·p60·hsp70 complexes activate GR steroid binding activity (Fig. 3*B*), we suggest that the simplest explanation is that the receptor binds to a preassociated unit that acts as a self-sufficient protein folding machine. It is possible, however, that some of the hsp70 that dissociates from this preformed complex binds to the GR before p60 and hsp90 become associated with it. In a system such as reticulocyte lysate, where hsp70 is abundant and continuous binding of hsp70 is allowed, it may be possible for p60 and hsp90 to assemble with a GR·hsp70 complex and activate steroid binding activity. Even assuming, as in the model of Fig. 7, that the three chaperones act only as a preformed complex, it is likely that one of them is responsible for the initial interaction with the receptor; and given its ability to bind to many proteins regardless of their structure (41), hsp70 is probably a reasonable candidate for engaging in that initial interaction.

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