Folding of the Glucocorticoid Receptor by the Heat Shock Protein (hsp) 90-based Chaperone Machinery

THE ROLE OF p23 IS TO STABILIZE RECEPTOR-hsp90 HETEROCOMPLEXES FORMED BY hsp90-p60-hsp70*

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Steroid receptors are recovered from hormone-free mammalian cells in multiprotein complexes that contain hsp90,1 a 23-kDa protein (p23), one of several immunophilins, and, often, substoichiometric amounts of hsp70 (for review, see Refs. 1 and 2). Several protein kinases involved in signal transduction (e.g. Src, Raf) exist in similar cytosolic heterocomplexes with hsp90 (1, 2), and genetic experiments in yeast show that hsp90 is an integral component of both steroid receptor and protein kinase signaling pathways (see Nathan and Lindquist (3) and references therein). Steroid receptor-hsp90 heterocomplexes can be created in vitro by incubating immunoadsorbed, hormone-free receptors with rabbit reticulocyte lysate (4, 5). This cell-free heterocomplex assembly system also forms heterocomplexes between hsp90 and protein kinases, such as Src, Raf, and Mek (6–8). In that concentrated lysates from mammalian, insect, and even plant cells are able to assemble the glucocorticoid receptor (GR) into a complex with hsp90 (9), it is likely the heterocomplex assembly system performs an ubiquitous and basic cellular function.

The hormone binding domain (HBD) of the GR must be bound to hsp90 for it to bind steroid (10), and the heterocomplex assembly system converts the GR HBD from a non-steroid-binding to a steroid-binding form (5, 11). Physical studies suggest that the HBD of the hsp90-free GR is in a folded conformation in which the steroid-binding pocket is not accessible to ligand and that the hsp90-bound HBD may be partially unfolded, such that a hydrophobic binding pocket is accessible to the ligand (12). Using formation of steroid binding sites as a rapid assay of “folding”1 as well as direct assay of receptor-hsp90 complex assembly, several details of the assembly mechanism have been established. Assembly of receptor-hsp90 complexes requires ATP/Mg2+, a monovalent cation, such as K+, and at least three proteins, hsp70 (13, 14), p60 (15), and p23 (16, 17). Although the mammalian DnaJ homolog, hsp40, has not been demonstrated in receptor heterocomplexes, it is possible that it also is required for heterocomplex assembly.

The p60 component of the assembly system was first identified by Smith et al. (13) in progesterone receptor heterocomplexes formed in reticulocyte lysates when ATP was limiting. The rabbit p60 (18) was then shown to be the homolog of a human protein that had been cloned by Honore et al. (19) and a homolog of the nonessential yeast heat shock protein Sti1

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1 The abbreviations used are: hsp, heat shock protein; GR, glucocorticoid receptor; HBD, hormone binding domain; TES, 2-(2-hydroxy-1,1-bis(hydroxymethyl)ethylamino)ethanesulfonic acid.

2 In this report, 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.

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In cytosols from animal and plant cells, the abundant heat shock protein hsp90 is associated with several proteins that act together to assemble steroid receptors into receptor-hsp90 heterocomplexes. We have reconstituted a minimal receptor-hsp90 assembly system containing four required components, hsp90, hsp70, p60, and p23 (Dittmar, K. D., Hutchison, K. A., Owens-Grillo, J. K., and Pratt, W. B. (1996) J. Biol. Chem. 271, 12833–12839). We have shown that hsp90, p60, and hsp70 are sufficient for carrying out the folding change that converts the glucocorticoid receptor (GR) hormone binding domain (HBD) from a non-steroid binding to a steroid binding conformation, but to form stable GR-hsp90 heterocomplexes, p23 must also be present in the incubation mix (Dittmar, K. D., and Pratt, W. B. (1997) J. Biol. Chem. 272, 13047–13054). In this work, we show that addition of p23 to native GR-hsp90 heterocomplexes immunoadsorbed from L cell cytosol or to GR-hsp90 heterocomplexes prepared with the minimal (hsp90-p60-hsp70) assembly system inhibits both receptor heterocomplex disassembly and loss of steroid binding activity. p23 stabilizes the GR-hsp90 heterocomplex in a dynamic and ATP-independent manner. In contrast to hsp90 that is bound to the GR, free hsp90 binds p23 in an ATP-dependent manner, and hsp90 in the hsp90-p60-hsp70 heterocomplex is in a conformation that does not bind p23 at all. The effect of p23 in the minimal GR heterocomplex assembly system is to stabilize GR-hsp90 heterocomplexes once they are formed and it does not appear to affect the rate of heterocomplex assembly. Molybdate has the same effect that hsp90 stabilizes the GR-hsp90 heterocomplex to inactivation and disassembly.
p23 and Receptor Heterocomplex Assembly

(20). Smith (21) showed that p60 associates with progesterone receptors incubated with reticulocyte lysate early in the heterocomplex assembly process and then exits the complex. Chen et al. (22) have shown that p60 binds to hsph70 via an N-terminal tetratricopeptide repeat region, and it binds to hsph90 via a central tetratricopeptide repeat region to form an hsph90+p60*hsph70 complex. Recently, we have demonstrated that the mixture of purified rabbit hsph90 and hsph70 with bacterial lysate containing human p60 results in spontaneous formation of an hsph90+p60*hsph70 complex that can be adsorbed with an 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 (23). In this minimal reconstituted system, conversion of the GR HBD to the steroid binding state was detected by incubating the GR with the chaperones in the presence of \(^{3}H\)triamcinolone acetonide, which binds to the receptor as soon as GR-hsph90 complexes are formed. Thus, despite the fact that the heterocomplexes formed by this minimal system undergo rapid inactivation and/or disassembly, evidence of proper folding of the GR HBD to the steroid binding conformation could be preserved.

To demonstrate maximum formation of GR-hsph90 heterocomplexes directly by immunoblotting, p23 must be present in the heterocomplex assembly mixture along with hsph90, hsph70, and p60 (23). p23 is a 23-kDa acidic protein that was first coimmunoadsorbed from cell lysates with the chicken progesterone receptor (24) and the murine GR (25). Toft and his co-workers prepared the human cDNA and showed that p23 engages in a dynamic association with hsph90, not only when hsph90 is free as shown by Toft and his co-workers (27, 30) but also not known at what stage in the assembly process p23 can convert the GR HBD to a steroid binding state in an ATP-dependent state of hsph90, we would propose that the ATP-dependent conformation of hsph90 is required for the HBD to have a high affinity steroid binding site and that binding of p23 to that state of hsph90 stabilizes the GR-hsph90 heterocomplex against inactivation and disassembly.

EXPERIMENTAL PROCEDURES

Materials

\[6,7-^{3}H\]Tiamcinolone acetonide (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), and wheat germ extract was from Promega. Protein A-Sepharose and Protein G-Sepharose 4B were obtained from Amersham. 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 \(\mu\)l. The assay mixtures were incubated at 20 min at 30 °C with suspension of the pellets by shaking the tubes every 5 min for soluble protein conditions or every minute for the immunoadsorbed p60 condition. At the end of the incubation, the pellets were washed twice with 1 ml of ice-cold TEGM buffer (TEGM buffer with 50 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 5 mM dithiothreitol and 50 mM [3H]triamcinolone acetonide. Samples were then washed twice with 1 ml of TEGM and counted by liquid scintillation spectrometry as described previously. The steroid binding is expressed as counts/min [3H]triamcinolone acetonide bound/GR immunopellet prepared from 100 μl of cytosol. As noted previously (11), 100 μl of L cell cytosol contains 60,000–80,000 cpm [3H]triamcinolone acetonide binding capacity and we immunoadsorb about 50% of the GR. Thus, reactivation of 100% of receptors to the steroid binding form represents 30,000–40,000 cpm of binding activity.

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 AC58 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 125I-conjugated antibody to visualize the immunoreactive bands.

Protein Purification—The bacterial expression of human p23 and its purification have been described by Johnson and Toft (16). Briefly, p23 is soluble in bacterial lysates, and its abundance and high affinity for DE52 column allowed purification 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.

Rabbit hsp70 and hsp90 were purified from brain cytosol as described previously (14). Briefly, reticulocyte lysate was chromatographed on a DE52 column exactly as described by Dittmar et al. (15). 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. (14). The purified hsp70 and hsp90 were dialyzed against HKD buffer, flash frozen, and stored at −70 °C.

For expression and purification of plant hsp90, the Brassica napus hsp90-1 cDNA clone (31) was modified to add six histidines at the COOH terminus and expressed in Sf9 insect cells to prepare a recombinant, tagged hsp90. The recombinant protein was purified over a Ni2+-Ni tril triacetic acid-agarose column and its identity confirmed with the plant hsp90-specific rabbit R2 antisera (31). Expression of p60—The bacterial expression of p60 has been described previously by Johnson et al. (26). Control E. coli and bacteria expressing p60 were grown to an A600 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.

RESULTS

p23 Potentiates Reactivation of Steroid Binding Activity by hsp90/p60/hsp70—In the experiment of Fig. 1, p60 was immunoadsorbed from rabbit reticulocyte lysate, and the immune pellet was washed and incubated with GR immune pellets that had been stripped of associated proteins by washing them with salt. Fig. 1A shows the composition of the washed p60 immune pellet. This native p60 heterocomplex contains hsp90 and hsp70, but not p23. In Fig. 1B it can be seen that the immune pellet containing p60 and its coadsorbed proteins generates only a modest number of steroid binding sites (lane 4) and that their formation is ATP-dependent (cf. lanes 3 and 4). In this experiment, steroid binding activity was assayed in the usual manner by incubating the immunoadsorbed GR with [3H]triamcinolone acetonide after heterocomplex assembly at 30 °C. When molybdate is present to stabilize the GR-hsp90 complexes as they are formed, then there are more steroid binding sites at the end of the incubation (cf. lane 6 with lane 4). In the presence of p23 (lane 8), GR reactivation is nearly to the level achieved with reticulocyte lysate (lane 2). It is interesting that

addition of p23 to reticulocyte lysate increases the extent of GR reactivation by 20–50% (data not shown), suggesting that p23 may be limiting in the lysate.

To examine the relationship between p23 potentiation of steroid binding activity and hsp90 association with the receptor, stripped GR immune pellets were incubated with the minimal assembly system consisting of purified rabbit hsp90 and hsp70 and bacterial lysate containing expressed human p60. As shown in Fig. 2, when stripped receptors (lane 1) are incubated with rabbit reticulocyte lysate (lane 2), they become associated with hsp90 and hsp70 and they are activated to the steroid binding state (it will be shown later (Fig. 7A) that GR-hsp90 complexes assembled in reticulocyte lysate also contain p23). Incubation with the minimal assembly system produces a GR-hsp90/p60/hsp70 complex (lane 3) with low steroid binding activity. It is not known why p60 is present in GR-hsp90 heterocomplexes formed with purified hsp90/p60/hsp70 and not in complexes formed by reticulocyte lysate, but we have previously suggested that reticulocyte lysate must contain an as yet unidentified activity that facilitates the exit of p60 from the receptor heterocomplex and is not present in the reconstituted system (23).

It is interesting that, in this experiment, the amount of GR-associated hsp90 is roughly the same in complexes made in reticulocyte lysate (Fig. 2, lane 2) as in complexes made by the minimal assembly system (lane 3), yet the steroid binding activity of the two complexes is very different. This is consistent with the possibility (which will be developed later) that the hsp90-bound GR HBD can be in a nonbinding conformation or in a steroid binding conformation. When the stripped GR was incubated with purified hsp90, p60, hsp70, and p23, the p23 caused about a doubling in the GR-associated hsp90 and an increase in steroid binding activity that is greater if p23 is present for the entire incubation (lane 5) than if p23 is added after 10 min of the 20-min incubation (lane 4). Thus, the pres-
ence of p23 increases the number of GR-hsp90 complexes that are recovered, and it seems to stabilize the steroid binding state of the GR-hsp90 complex.

p23 Inhibits GR-hsp90 Complex Disassembly in a Dynamic Heterocomplex Assembly/Disassembly System—It has been shown previously that receptor-hsp90 heterocomplex assembly in reticulocyte lysate is very dynamic in the sense that both heterocomplex assembly and disassembly are occurring simultaneously (9, 21). To determine the effect of p23 in a dynamic system, the mouse GR-hsp90 heterocomplex was incubated with wheat germ extract, a system where both heterocomplex assembly and disassembly can be assayed because the wheat hsp90 migrates faster than mouse hsp90 on gel electrophoresis. As shown in Fig. 3 (lane 2), the native GR-hsp90 heterocomplex immunoadsorbed from L cell cytosol contains hsp90 and p23. When the complex is incubated in buffer at 30 °C, much of the hsp90 and the p23 dissociates from the GR (lane 4), and dissociation of both components is inhibited by molybdate (lane 5).

As shown in the data of Fig. 3 that p23 can associate with native GR-hsp90 complexes formed in the intact L cells to inhibit disassembly of these native heterocomplexes in wheat germ extract. The ability of p23 to increase recovery of GR-wheat hsp90 heterocomplexes could reflect the ability of p23 to inhibit their disassembly in this dynamic assembly/disassembly system. To determine if p23 alone was sufficient to stabilize the native heterocomplex, we asked whether immunopurified, native GR-hsp90 complexes suspended in buffer could be stabilized.

ATP-dependent Manner—In the experiment of Fig. 4A, immunoadsorbed native GR-hsp90 heterocomplexes were incubated in the HKD buffer we use for heterocomplex assembly with or without an ATP-generating system. Because ATP is required for binding of p23 to free hsp90 (27-30), we expected p23 stabilization of GR-hsp90 heterocomplexes to be ATP-dependent. However, as shown in Fig. 4A, p23 stabilized the steroid binding activity of native GR-hsp90 complexes better in the absence of ATP than in the presence of an ATP-generating system, and in the absence of ATP, the stabilization by p23 was concentration-dependent.

It has been shown for many years that ATP can promote steroid receptor transformation (see Pratt and Toft (1) and references therein), and it can be seen in Fig. 4B that dissociation of hsp90 from immunoadsorbed native GR-hsp90 heterocomplexes is complete when receptors are incubated in the presence of ATP (lane 4) but dissociation of hsp90 is not complete in the absence of ATP (lane 3). The basis for this modest ATP enhancement of dissociation of the native GR-hsp90 heterocomplex is unknown, but it may explain why complete dissociation of mouse hsp90 is observed in Fig. 3 when GR heterocomplexes are incubated in wheat germ extract (Fig. 3, lane 7), which contains an ATP-generating system, whereas dissociation of heterocomplexes incubated in buffer (Fig. 3, lane 4) is incomplete. In Fig. 4B, it can be seen that p23 stabilizes both native GR-hsp90 heterocomplexes and steroid binding activity much better in the absence of ATP (lane 6) than in the presence of ATP (lane 7). In contrast, we have consistently observed that the stabilization produced by molybdate is essentially the same in the presence or absence of ATP (cf. lanes 8 and 9 of Fig. 4B).

As shown in Fig. 5, GR-hsp90 heterocomplexes formed by the minimal hsp90-p60/hsp70 assembly system are also stabilized better by p23 in the absence of ATP (lane 4) than in the presence of ATP (lane 5). The data of Figs. 4 and 5 show that ATP is not required for p23 to bind to hsp90 that is bound to the GR, at least when the HBD is in the steroid binding conformation. The interaction of p23 with the GR-hsp90 complex in its steroid binding form must be dynamic and p23 alone is sufficient for producing stabilization. Also, we have observed that p23 usually stabilizes GR-hsp90 heterocomplexes somewhat more than the maximally effective concentration of molybdate.

p23 Does Not Appear to Accelerate GR-hsp90 Heterocomplex Assembly—Unlike p23 inhibition of GR-hsp90 heterocomplex disassembly, any potential effect of p23 on heterocomplex assembly must be detected in the dynamic system where both
and 7 was incubated with \(^{3}\text{H}\)triamcinolone acetonide to determine steroid in each sample by Western blotting, and a portion of the immunopellet in the absence or presence of an EGS. Receptor and hsp90 were assayed for 30 min at 30 °C, and steroid binding was assayed. Conditions are: lane 1, control GR pellet that was not incubated; lanes 2 and 3, GR pellet incubated in the presence (lane 2) or absence (lane 3) of the EGS; lanes 4–9, GR pellets incubated with or without the EGS and 5 μg (lanes 4 and 5), 12.5 μg (lanes 6 and 7), or 25 μg (lanes 8 and 9) of purified p23. B, stabilization of native GR-hsp90 heterocomplex with p23 and molybdate. GR-hsp90 complexes were immunoadsorbed from 400-μl aliquots of L cell cytosol, and immune pellets were suspended in HKD buffer and incubated 30 min at 4 °C (conditions 1 and 2) or 30 °C (conditions 3–9) in the presence or absence of an ATP-generating system as indicated. Conditions are: lanes 1 and 2 nonimmune and GR immune pellets maintained on ice; lanes 3 and 4, GR incubated at 30 °C in the absence or presence of the EGS; lanes 5–7, nonimmune (lane 5) and GR (lanes 6 and 7) immune pellets incubated with 12.5 μg of purified p23 in the absence or presence of EGS as indicated; lanes 8 and 9, GR incubated with 20 mM molybdate in the absence or presence of EGS.

Fig. 4. Stabilization of the native L cell GR-hsp90 complex by p23. A, concentration dependence of p23 stabilization. GR-hsp90 complexes were immunoadsorbed from replicate aliquots (100 μl) of L cell cytosol, immune pellets were suspended in HKD buffer with (+) or without (−) an ATP-generating system (energy-generating system, EGS), samples were incubated for 30 min at 30 °C, and steroid binding was assayed. Conditions are: lane 1, control GR pellet that was not incubated; lanes 2 and 3, GR pellet incubated in the presence (lane 2) or absence (lane 3) of the EGS; lanes 4–9, GR pellets incubated with or without the EGS and 5 μg (lanes 4 and 5), 12.5 μg (lanes 6 and 7), or 25 μg (lanes 8 and 9) of purified p23. B, stabilization of native GR-hsp90 heterocomplex with p23 and molybdate. GR-hsp90 complexes were immunoadsorbed from 400-μl aliquots of L cell cytosol, and immune pellets were suspended in HKD buffer and incubated 30 min at 4 °C (conditions 1 and 2) or 30 °C (conditions 3–9) in the presence or absence of an ATP-generating system as indicated. Conditions are: lanes 1 and 2 nonimmune and GR immune pellets maintained on ice; lanes 3 and 4, GR incubated at 30 °C in the absence or presence of the EGS; lanes 5–7, nonimmune (lane 5) and GR (lanes 6 and 7) immune pellets incubated with 12.5 μg of purified p23 in the absence or presence of EGS as indicated; lanes 8 and 9, GR incubated with 20 mM molybdate in the absence or presence of EGS.

Fig. 5. Stabilization by p23 of GR-hsp90 heterocomplexes formed by the purified minimal assembly system. Stripped GR immune pellets were incubated for 20 min at 30 °C with purified hsp90 and hsp70 and 3 μl of bacterial lysate with p60 in the presence of an ATP-generating system (energy-generating system, EGS) and 20 mM molybdate. The pellets were subsequently washed twice with 1 ml of Hepes buffer, suspended in HKD buffer with the indicated additions, and incubated at 4 °C (condition 1) or 30 °C (conditions 2–7) for 30 min in the absence or presence of an EGS. Receptor and hsp90 were assayed in each sample by Western blotting, and a portion of the immunopellet was incubated with \(^{3}\text{H}\)triamcinolone acetonide to determine steroid binding activity. Conditions are: lane 1, reconstituted GR-hsp90 heterocomplex incubated on ice; lanes 2 and 3, reconstituted GR incubated at 30 °C with HKD buffer in the absence (lane 2) or presence (lane 3) of EGS; lanes 4 and 5, reconstituted GR incubated in HKD buffer with 12.5 μg of p23 in the absence (lane 4) or presence (lane 5) of EGS; lanes 6 and 7, reconstituted GR incubated in HKD buffer with 20 mM molybdate in the absence (lane 6) or presence (lane 7) of EGS.

assembly and disassembly are occurring simultaneously. Fig. 6 presents the time course of reactivation of steroid binding activity by hsp90, p60, and hsp70 with (open circles) and without (closed circles) p23. In this experiment, \(^{3}\text{H}\)triamcinolone acetonide was present during the incubation with chaperones at 30 °C, and steroid binding is used as an indirect assay of GR-hsp90 heterocomplex assembly. There is an initial lag of 2–3 min in reactivation of steroid binding activity, which likely reflects the interval required for samples kept in ice to warm to 30 °C. Both in the presence and absence of p23, the assembly reaches a plateau in about 20 min, although the plateau for the assembly mixture that contains p23 is about double that for the mixture without p23. We do not know why the time course of assembly turns over before all of the receptors are reactivated to the steroid binding form. But, as shown in the inset in Fig. 6, when the initial reactivation values are plotted as a fraction of the maximal steroid binding achieved in each condition, there is no apparent effect of p23 on reactivation rate. Thus, we suggest that p23 may not affect the rate of GR-hsp90 heterocomplex assembly; rather, it may act to stabilize hsp90 in the receptor-bound conformation that yields the steroid binding state of the HBD.

Binding of p23 to hsp90 Alone and in Heterocomplex with GR, p60, and hsp70—In Fig. 3 (lane 2), it was shown that native GR-hsp90 heterocomplexes isolated from L cell cytosol contain p23, and in Fig. 7A, it is shown that GR-hsp90 heterocomplexes contain p23 after assembly with either reticulocyte lysate (lane 2) or with the mixture of purified hsp90, hsp70, p60, and p23 (lane 4). Sullivan et al. (30) have shown that binding of purified p23 to purified hsp90 requires elevated temperature and ATP/Mg\(^{2+}\) and is strongly promoted by the nonionic detergent Nonidet P-40 (0.02%). We also find that binding of the purified p23 to purified hsp90 is ATP-dependent and temperature-dependent (data not shown) and that the binding is increased by 0.02% Nonidet P-40 (Fig. 7C, cf. lane 4 with lane 2). However, addition of 0.02% Nonidet P-40 to the assembly mixture containing hsp90, hsp70, p60, and p23 elimi-
inates GR-hsp90 heterocomplex assembly activity (data not shown), and we find that p23 stabilization of GR-hsp90 complexes formed by hsp90, p60, and hsp70 does not require ATP (Fig. 5). This suggests that free hsp90 and hsp90 that are in the GR-hsp90 heterocomplex are in different states.

We have also found that native hsp90-p60-hsp70 heterocomplexes immunoadsorbed from rabbit reticulocyte lysate with anti-p60 do not contain any p23 (Fig. 1A (32)). In the experiment of Fig. 7B (lane 2), we incubated hsp90, p60, hsp70, and p23 with an ATP-regenerating system under the same conditions used for GR-hsp90 heterocomplex assembly, and at the end of the incubation, p23 was immunoadsorbed. Some hsp90 was coimmunoadsorbed with p23, demonstrating the formation of p23-hsp90 complexes, but no p60 or hsp70 was coadsorbed. The number of p23-hsp90 complexes formed was increased by Nonidet P-40 (cf. lanes 2 and 4), but again, p23-hsp90-p60-hsp70 complexes were not formed. Thus, we suggest that hsp90 in the hsp90-p60-hsp70 complex is not in a p23-binding conformation, but that hsp90 in a GR-hsp90-p60-hsp70 complex formed under identical conditions with the same proteins is in a p23-binding conformation.

Stabilization of GR-Plant hsp90 Heterocomplexes—In Fig. 8A, stripped GR immune pellets were incubated with purified rabbit hsp70, human p60 and p23, and various concentrations of either purified rabbit hsp90 (closed circles) or purified plant hsp90 from Brassica napus (open circles). It can be seen that the plant hsp90 is nearly equivalent to the rabbit hsp90 at generating steroid binding activity in a mixed folding system where all of the cochaperones are from mammalian sources. However, as shown in Fig. 8B, the Brassica hsp90 differs from the rabbit hsp90 in that the GR-plant hsp90 complex is stabilized by p23 (lane 9) but not by molybdate (lane 10). The ability of plant hsp90 to function with mammalian hsp70, p60, and p23 underlines the fundamental nature of the hsp90-based chaperone system. But the failure of molybdate to stabilize the GR heterocomplex with Brassica hsp90 as opposed to its ability to stabilize all heterocomplexes with hsp90s of animal and fungal origin may suggest a fundamental difference in plant versus animal and fungal hsp90s.

**DISCUSSION**

We previously showed that hsp90, hsp70, and p60 are sufficient to produce the folding change in the GR HBD that yields a high affinity steroid binding site (23), and in this study, we show that p23 must also be present to produce a stable GR-hsp90 heterocomplex. We suspect that p23 somehow locks hsp90 in a conformation that is required for proper folding of the HBD, a folding state that is achieved only transiently in the absence of p23. As we show (Figs. 1 and 5), the overall effect of p23 in yielding a stable steroid-binding GR-hsp90 complex can be replaced by having molybdate present during the incubation with purified hsp90, hsp70, and p60.

It is easiest to discuss the findings of this work within the context of an evolving model of heterocomplex assembly, the latest version of which is presented as Fig. 9. p23 is shown in the model as binding to the final heterocomplex as well as to intermediates in the assembly pathway. The final GR heterocomplex may be either the mammalian heterocomplex immunoadsorbed from L cell cytosol or the GR-hsp90 heterocomplex formed in rabbit reticulocyte lysate. These final GR-hsp90 heterocomplexes no longer contain p60 (23) but they contain an immunophilin, such as FKBP52 or CyP-40, which binds to hsp90 at the same site where p60 binds (32). A potential assembly intermediate that is p60-free but not yet bound by an
immunophilin is represented by the complex within brackets in the model (Fig. 9). We have shown here that p23 can bind to the native GR-hsp90 heterocomplex and stabilize it to both disassembly and loss of steroid binding activity (Figs. 3 and 4). p23 also stabilizes the intermediate GR-hsp90-p60-hsp70 complex assembled by purified hsp90, hsp70, and p60 (Fig. 5). In the case of both the intermediate and the final complex, stabilization by p23 does not require ATP (Figs. 4 and 5).

In previous work in which we showed that all of the components of reticulocyte lysate required to produce a stable GR-hsp90 heterocomplex were communoadsorbed with hsp90, we assumed that p23 was a weakly associated component of the foldosome complex (17). It is clear now that p23 was communoadsorbed with hsp90-p60-hsp70 complexes as a separate hsp90-p23 complex and that p23 is not itself a component of the foldosome (Figs. 1A and 7B). It is also clear that p23 alone is sufficient to stabilize the immunopurified GR-hsp90 complex (Fig. 4). Thus, it is not necessary that p23 enter an intermediate stage of receptor-hsp90 heterocomplex as a preformed p23-hsp90-immunophilin unit as suggested in earlier models of the assembly mechanism derived from studies in reticulocyte lysate (22, 33, 34).

When hsp90, hsp70, p60, and p23 are incubated together, we cannot create a complex that contains p60 and hsp70 as well as p23 and hsp90 (Fig. 7B). Also, p23 is not present in the native hsp90-p60-hsp70 foldosome heterocomplex isolated from reticulocyte lysate with anti-p60 (Fig. 1A). Thus, it seems that hsp90 in the foldosome complex (hsp90-p60-hsp70) is not in a configuration that binds p23. We have shown previously that the stripped GR combines with the proteins of the foldosome (step 2 in Fig. 9) in a process that is both ATP-dependent and K`-dependent to produce a GR-hsp90-p60-hsp70 complex with steroid binding activity (23). The hsp90 in the GR-hsp90-p60-hsp70 complex is in a configuration that can be bound and stabilized by p23 (Fig. 5). In so far as we can determine, the sole role of p23 in the minimal assembly system is to stabilize the GR-hsp90-p60-hsp70 complex once it is formed and not to accelerate the rate of heterocomplex assembly (Fig. 6). In their study of hsp90 binding to phenyl-Sepharose, Sullivan et al. (30) showed that hsp90 that is either bound by ADP or nucleotide-free has a high affinity for binding the hydrophobic resin. However, this hydrophobic binding state of hsp90 is altered by ATP such that the hsp assumes a conformation with low hydrophobic binding activity. This ATP-dependent conformation is the one that is bound by p23 (30). It is reasonable to suggest that hsp90 in the foldosome is in the hydrophobic binding conformation that does not bind p23 and that hsp90 in the steroid binding form of the GR-hsp90-p60-hsp70 complex is in the ATP-dependent conformation that binds and is stabilized by p23.

It seems clear that hsp90 in the GR-hsp90-p60-hsp70 complex can exist in either a steroid binding or nonbinding conformation. When GR-hsp90-p60-hsp70 complexes are assembled by hsp90-p60-hsp70 in the presence of the hsp90-binding antibiotic geldanamycin, they do not have steroid binding activity (23). Thus, hsp90 can be bound to the GR without the HBD being properly folded to the steroid binding state. It seems reasonable that the proteins of the foldosome may bind to the GR to yield first a nonsteroid-binding GR-hsp90-p60-hsp70 complex in which hsp90 is in the conformation with hydrophobic affinity and that an ATP-induced change in hsp90 conformation is required for the folding change in the GR HBD that yields the steroid binding site. In this conformation, hsp90 can now be bound by p23, which essentially stabilizes a conformation of hsp90 that is required for opening the steroid-binding pocket in the GR HBD.

Although p23 binds to free hsp90 in an ATP-dependent manner (27–30), we have not shown that bimolecular interaction in the model of GR-hsp90 heterocomplex assembly in Fig. 9, because we cannot demonstrate the presence of p23 in either native (Fig. 1A) or reconstituted (Fig. 7B) foldosome (hsp90-p60-hsp70) complexes. The study by Sullivan et al. (30) of p23-hsp90 complex formation utilizing purified proteins was extremely useful in proposing that the ATP requirement for forming the bimolecular complex reflects the ATP-dependent conversion of hsp90 from a conformation with hydrophobic...
affinity to a conformation with low hydrophobic affinity that can complex with p23. It is interesting that the ATP-dependent binding of p23 to free hsp90 is increased by the presence of the detergent Nonidet P-40 (30) (Fig. 7C). It is thought that the detergent promotes a change in the conformation of hsp90 that is stabilized by the binding of ATP (30). The effect of the detergent may mimic a similar change in the conformation of hsp90 that takes place with unfolding of the GR HBD by the proteins of the foldosome complex. It is reasonable to propose that the hsp90 in native GR-hsp90 heterocomplexes isolated from L cell cytosol is in this ATP-dependent conformation; thus, as shown in the model of Fig. 9, p23 binds to the receptor heterocomplex in a reversible manner that does not require ATP (Fig. 4).

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REFERENCES

1. Pratt, W. B., and Toft, D. O. (1997) Endocr. Rev. 18, 306–360
2. Pratt, W. B. (1997) Annu. Rev. Pharmacol. Toxicol. 37, 297–326
3. Nathan, D. F., and Lindquist, S. (1995) Mol. Cell. Biol. 15, 3917–3925
4. Smith, D. F., Schueler, D. B., Kast, S. L., and Toft, D. O. (1990) Mol. Endocrinol. 4, 1794–1797
5. Scherrer, L. C., Dalman, F. C., Massa, E., Meshinchi, S., and Pratt, W. B. (1990) J. Biol. Chem. 265, 21397–21400
6. Hutchison, K. A., Brott, B. K., De Leon, J. H., Perdew, G. H., Jove, R., and Pratt, W. B. (1992) J. Biol. Chem. 267, 2902–2908
7. Stancato, L. F., Chow, Y-H., Hutchison, K. A., Perdew, G. H., Jove, R., and Pratt, W. B. (1993) J. Biol. Chem. 268, 21711–21716
8. Stancato, L. F., Silverstein, A. M., Owens-Grillo, J. K., Chow, Y-H., Jove, R., and Pratt, W. B. (1997) J. Biol. Chem. 272, 4013–4020
9. Stancato, L. F., Hutchison, K. A., Krishna, P., and Pratt, W. B. (1996) Biochemistry 35, 554–561
10. Bresnick, E. H., Dalman, F. C., Sanchez, E. R., and Pratt, W. B. (1989) J. Biol. Chem. 264, 4992–4997
11. Hutchison, K. A., Czar, M. J., Scherrer, L. C., and Pratt, W. B. (1992) J. Biol. Chem. 267, 14047–14053
12. Stancato, L. F., Silverstein, A. M., Gitler, C., Groner, B., and Pratt, W. B. (1996) J. Biol. Chem. 271, 8831–8836
13. Smith, D. F., Stensgard, B. A., Welch, W. J., and Toft, D. O. (1990) J. Biol. Chem. 265, 1350–1356
14. Hutchison, K. A., Dittmar, K. D., Czar, M. J., and Pratt, W. B. (1994) J. Biol. Chem. 269, 5043–5049
15. Dittmar, K. D., Hutchison, K. A., Owens-Grillo, J. K., and Pratt, W. B. (1996) J. Biol. Chem. 271, 12833–12839
16. Johnson, J. L., and Toft, D. O. (1994) J. Biol. Chem. 269, 24989–24993
17. Hutchison, K. A., Stancato, L. F., Owens-Grillo, J. K., Johnson, J. L., Krishna, P., Toft, D. O., and Pratt, W. B. (1995) J. Biol. Chem. 270, 18841–18847
18. Smith, D. F., Sullivan, W. P., Marion, T. N., Zaitsev, K., Madden, B., McCormick, D. J., and Toft, D. O. (1990) Mol. Cell. Biol. 10, 869–876
19. Honore, B., Leffers, H., Madsen, P., Rasmussen, H. H., Vanderkerckhove, J., and Celis, J. E. (1992) J. Biol. Chem. 267, 8485–8491
20. Nicolet, C. M., and Craig, E. A. (1989) Mol. Cell. Biol. 9, 3638–3646
21. Smith, D. F. (1990) Mol. Endocrinol. 4, 1418–1429
22. Chen, S., Prapapanich, V., Rimerman, R. A., Honore, B., and Smith, D. F. (1996) Mol. Endocrinol. 10, 682–693
23. Dittmar, K. D., and Pratt, W. B. (1997) J. Biol. Chem. 272, 13047–13054
24. Smith, D. F., Faber, L. E., and Toft, D. O. (1990) J. Biol. Chem. 265, 3996–4003
25. Bresnick, E. H., Dalman, F. C., and Pratt, W. B. (1990) Biochemistry 29, 520–527
26. Johnson, J. L., Beito, T. G., Kroo, C. J., and Toft, D. O. (1994) Mol. Cell. Biol. 14, 1956–1963
27. Johnson, J. L., and Toft, D. O. (1995) Mol. Endocrinol. 9, 670–678
28. Johnson, J., Corhisier, R., Stensgard, B., and Toft, D. O. (1996) J. Steroid Biochem. Mol. Biol. 56, 31–37
29. Owens-Grillo, J. K., Stancato, L. F., Hoffman, K., Pratt, W. B., and Krishna, P. (1990) Biochemistry 29, 15249–15255
30. Sullivan, W., Stensgard, B., Cauccutt, G., Bartha, B., McMahon, N., Alnemri, E. S., Litwack, G., and Toft, D. (1997) J. Biol. Chem. 272, 8007–8012
31. Krishna, P., Sacco, M., Cherutti, J. F., and Hill, S. (1995) Plant Physiol. 107, 915–923
32. Owens-Grillo, J. K., Czar, M. J., Hutchison, K. A., Hoffman, K., Perdew, G. H., and Pratt, W. B. (1996) J. Biol. Chem. 271, 13468–13475
33. Smith, D. F., Whitesell, L., Nair, S. C., Chen, S., Prapapanich, V., and Rimener, R. A. (1995) Mol. Cell. Biol. 15, 6804–6812
34. Prapapanich, V., Chen, S., Nair, S. C., Rimener, R. A., and Smith, D. A. (1996) Mol. Endocrinol. 10, 420–431

**FIG. 9.** The heterocomplex assembly pathway with sites of p23 interaction. The association of purified hsp90, p60, and hsp70 in step 1 to form an hsp90-p60-hsp70 complex (foldosome) and the ability of the proteins in this unit to associate in step 2 with the stripped, nonsteroid binding state of the GR to assemble a GR-hsp90-p60-hsp70 complex with a steroid binding site (indicated by the steroid structure) was reported by Dittmar and Pratt (23). IMM stands for an immunophilin, such as FKBP52 or CyrP-40, which is present in the final heterocomplex formed in reticulocyte lysate or isolated from cytosol. The immunophi- lins and p60 bind to hsp90 via tetratricopeptide repeat domains, which are indicated by the solid black crescents on these proteins. Native GR-hsp90 heterocomplexes isolated from cells usually do not contain hsp70, but native receptor complexes from some cells, as well as complexes assembled in reticulocyte lysate, do contain hsp70. Thus, the hsp70 with dashed lines indicates its optional presence in the final product. p23 associates with hsp90 in the GR-hsp90-p60-hsp70 assembly intermediate formed by the minimal assembly system as well as with native GR-hsp90 heterocomplexes isolated from cells. In both cases p23 binding is reversible, does not require ATP, and yields stabilization of the complex.