A variety of signaling proteins form heterocomplexes with and are regulated by the heat shock protein chaperone hsp90. These complexes are formed by a multiprotein machinery, including hsp90 and hsp70 as essential and abundant components and Hop, hsp40, and p23 as nonessential cochaperones that are present in much lower abundance in cells. Overexpression of signaling proteins can overwhelm the capacity of this machinery to properly assemble heterocomplexes with hsp90. Here, we show that the limiting component of this assembly machinery in vitro in reticulocyte lysate and in vivo in Sf9 cells is p23. Only a fraction of glucocorticoid receptors (GR) overexpressed in Sf9 cells are in heterocomplex with hsp90 and have steroid binding activity, with the majority of the receptors present as both insoluble and cytosolic GR aggregates. Coexpression of p23 with the GR increases the proportion of cytosolic receptors that are in stable GR-hsp90 heterocomplexes with steroid binding activity, a strictly hsp90-dependent activity. Coexpression of p23 with the GR increases the proportion of cytosolic receptors that are in stable GR-hsp90 heterocomplexes with steroid binding activity, a strictly hsp90-dependent activity. Coexpression of p23 with the GR increases the proportion of cytosolic receptors that are in stable GR-hsp90 heterocomplexes with steroid binding activity, a strictly hsp90-dependent activity. Coexpression of p23 with the GR increases the proportion of cytosolic receptors that are in stable GR-hsp90 heterocomplexes with steroid binding activity, a strictly hsp90-dependent activity. Coexpression of p23 with the GR increases the proportion of cytosolic receptors that are in stable GR-hsp90 heterocomplexes with steroid binding activity, a strictly hsp90-dependent activity.

Several transcription factors and a variety of protein kinases involved in signal transduction are regulated by the abundant, ubiquitous and essential protein chaperone hsp90 (1). Complexes between these “client” proteins and hsp90 are formed by a multiprotein chaperone machinery that is functionally conserved among eukaryotes (1). This chaperone machinery, which was originally identified in reticulocyte lysate (2, 3), has been reconstituted (4) and a mixture of five purified proteins, hsp90, hsp70, Hop, hsp40, and p23, is now used to achieve efficient receptor-hsp90 heterocomplex assembly (5, 6). It is important to realize that this purified five-protein system is a minimum system for achieving stable heterocomplex assembly. In various cell compartments or under conditions of stress, other proteins, such as the hsp70 cochaperones BAG-1 (Bcl-2-associated gene product-1) (7, 8) and Hip (Hsc70-interacting protein) (9) and the hsp90 cochaperone Aha (activator of hsp90 ATPase) (10), may also be involved in hsp90 heterocomplex assembly.

We have used glucocorticoid receptor (GR)-hsp90 heterocomplex assembly as a model system for studying the assembly process. Hsp90 binds to the ligand binding domain (LBD) of the GR, which must be in heterocomplex with hsp90 for it to bind steroid (1). The steroids bind deep in a hydrophobic cleft that appears to be collapsed in the absence of ligand (11), and the hsp90/hsp70-based chaperone machinery opens the binding cleft of the GR LBD such that it can be accessed by steroid (Ref. 12 and references therein). Because hsp90 is required for high affinity steroid binding in vivo, the generation of steroid binding activity by the five-protein system demonstrates that a physiologically appropriate folding change has occurred upon heterocomplex assembly in vitro. Among the five chaperones in the purified assembly system, we have determined that hsp90 and hsp70 are both essential for opening the steroid binding cleft in the GR LBD, and hsp40, Hop (hsp70/hsp90 organizing protein), and p23 act as cochaperones to increase the rate or extent of GR-hsp90 heterocomplex assembly (13). The cochaperones are much less abundant in cell lysates than the essential chaperones, and both Hop and hsp40 (we use the yeast ortholog YDJ-1) are optimally active in the five-protein assembly system when present at less than one-tenth the concentration of hsp70 and hsp90. In contrast, roughly stoichiometric levels of p23 are required for peak activity in the five-protein assembly system. Here, we investigate the consequences of increasing the concentration of each of the cochaperones on GR-hsp90 heterocomplex assembly in vivo in Sf9 cells.

When a protein is overexpressed, there is always the possibility that so much of it is produced that it overwhelms the cellular protein folding systems required to generate the physiologically active state. This is the case with baculovirus-mediated expression of the GR in Sf9 cells. Alnemri and Litwack (14) showed that only a small fraction of the total expressed GR was present in the cytosolic fraction as GR-hsp90 heterocomplexes, and the majority of the receptor was present as insoluble aggregates, which did not form when a truncated GR lacking the LBD was expressed. Coexpression of the full-length GR with hsp90 or hsp70 did not increase formation of the steroid binding receptor heterocomplex, but incubation of GR partially purified from the aggregates with reticulocyte lysate resulted in reconstitution of GR-hsp90 heterocomplexes and restoration of steroid binding activity.

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of full steroid binding activity (14). This led to the conclusion that factors other than hsp90 and hsp70 are limiting in insect cells but can be supplied in vitro by reticulocyte lysate.

Here we coexpress the GR in Sf9 cells with Hop, YDJ-1, or p23 and show that p23 is the limiting component of the hsp90/hsp70-based chaperone system. Increasing p23 increases the fraction of cytosolic receptors that are stably bound to hsp90, and p23 coexpression also diminishes the formation of receptor aggregates. These observations argue strongly that the effects of p23 expression on a client protein function must be interpreted in terms of the ability of p23 to enhance the activity of hsp90 as opposed to direct chaperone effects in which p23 interacts with a partially denatured protein to affect its folding state. From a protein engineering viewpoint, coexpression of p23 may prove to be very useful in the production of the physiologically regulated state of client proteins that normally are present in persistent heterocomplexes with hsp90.

EXPERIMENTAL PROCEDURES

Materials

Untreated rabbit reticulocyte lysate was purchased from Green Hectors (Oregon, WI). [6,7-3H]Dexamethasone (40 Ci/mmol) and 125I-conjugated goat anti-mouse and goat anti-rabbit IgGs were obtained from PerkinElmer Life Sciences. Sephacryl S-300 was from Amersham Biosciences. Goat anti-mouse and goat anti-rabbit horseradish peroxidase conjugates were purchased from Sigma. The BmGR monoclonal IgG antibody against the GR was from Affinity Bioreagents (Golden, CO). The AC88 monoclonal IgG against hsp90, the NTT27-F-4 anti-T27/3-KDA hsp monoclonal IgG (anti-hsp70), and anti-hsp40 polyclonal IgG were from Stratagene (La Jolla, CA). The J33 monoclonal IgG against p23 and human p23 cDNA were provided by Dr. David Toft (The Mayo Clinic, Rochester, MN), and the DS14F6 monoclonal IgG against Hop and human Hop cDNA were provided by Dr. David Smith (Mayo Clinic, Scottsdale, AZ). Yeast YDJ-1 cDNA was a gift from Dr. Avrom Caplan (Mount Sinai School of Medicine). The rabbit antiserum against hsp70 and hsp90 used to immunoblot the insect chaperones was a gift from Dr. Ettore Appella (National Cancer Institute). Hybridoma cells producing the F1GR monoclonal antibody to hsp90 and hsp70 were a gift from Dr. Jack Bodwell (Dartmouth Medical School, Lebanon, NH). Construction of the baculovirus for expression of p23 and Glucocorticoid Receptor Folding

Construction of Baculoviruses for p23, Hop, and YDJ-1—Human p23 cDNA was excised from the pET23a plasmid (16) with XbaI and XhoI and a linker fragment was inserted into the multiple cloning site of the Baculovirus expression vector pFASTBAC to make pFB-p23. Human Hop cDNA was excised from the pET23 plasmid with XbaI and XhoI and inserted into the multiple cloning site of the pFASTBAC vector to make pFB-YDJ-1. Yeast YDJ-1 cDNA was excised from pET9d plasmid (18) with NcoI and inserted into the multiple cloning site of the pFASTBAC vector to make pFB-Hop. Human Hop cDNA was excised from the pET23 plasmid (16) with XbaI and XhoI and inserted into the multiple cloning site of the pFASTBAC vector to make pFB-Hop.
hydroxylapatite as described previously (5). The bacterial expression of YDJ-1 has been described (18) as has the expression of human Hop (4). 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 (10 mM Hepes, 100 mM KCl, 5 mM dithiothreitol, pH 7.35), flash frozen, and stored at −70 °C.

Relative Abundance of Proteins in Reticulocyte Lysate—To determine the concentrations of the five proteins of the chaperone system in reticulocyte lysate, aliquots of reticulocyte lysate were electrophoresed on SDS-polyacrylamide gels that also contained various amounts of purified hsp90, hsp70, Hop, YDJ-1, and p23 to provide a standard curve for each protein. Immunoblots were prepared and probed with monoclonal IgGs against each protein, followed by incubation with 125I-labeled anti-IgG counterantibody. Samples and purified standards were then excised and counted to permit calculation of the concentration of each protein.

Fractionation of Sf9 Cytosol—Sf9 cytosol (500 μl) was incubated overnight with 50 nM [3H]dexamethasone and then applied to a column (1.5 × 113 cm) of Sephacryl S-300. The column was eluted with HEM buffer, and 0.5-ml aliquots of each 2.75-ml fraction were assayed for radioactivity. Aliquots of 100 μl were assayed for GR and p23 by SDS-polyacrylamide gel electrophoresis and immunoblotting.

RESULTS

Relative Concentrations of the Assembly Proteins in Reticulocyte Lysate—Because some of our antibodies do not recognize...
the corresponding insect chaperones in Sf9 cytosol, the relative abundance of the five proteins of the chaperone machinery was assayed in rabbit reticulocyte lysate. Table I and Fig. 1A show the concentrations of each protein in reticulocyte lysate, and Table I presents a comparison with the optimal concentration of the protein in the purified five-protein assembly system. In reticulocyte lysate, the cochaperones Hop, hsp40, and p23 are present at only a fraction of the concentration of the essential chaperones. In the purified system, Hop and hsp40 are optimally active at similarly low molar ratios with respect to hsp90 and hsp70, but p23 is optimally active when it is present at roughly the same concentration as hsp90. Addition of purified hsp90, hsp70, Hop, or YDJ-1 to reticulocyte lysate does not enhance its ability to activate the GR to the steroid binding state, but addition of purified p23 yields a substantial increase in GR-hsp90 heterocomplexes with steroid binding activity (Fig. 1B). Maximum activation of GR steroid binding activity is achieved when 4–8 μg of p23 are added to the 50-μl incubation mixture (Fig. 1C), a concentration of p23 that is 10-fold higher than that in reticulocyte lysate.

Effect of Coexpression of Each Cochaperone on GR Steroid Binding Activity in Sf9 Cytosols—Before beginning coexpression experiments, we expressed each of the cochaperones in Sf9 cells, purified them, and demonstrated that they had the same activity in the purified five-protein GR-hsp90 assembly assay as the bacterially expressed proteins (data not shown). Also, to minimize toxicity related to infection with too much baculovirus, the baculovirus for GR expression was titrated to determine the amount of virus just sufficient to produce the maximum amount of cytosolic GR in 48 h of infection. An m.o.i. of 0.1 was sufficient, and that amount of baculovirus was used in all of the experiments in this work.

The effect of coexpression of each of the cochaperones on GR steroid binding activity in Sf9 cytosol is shown in Fig. 2. In Fig. 2A, it can be seen that p23 can be expressed in quite high levels without affecting the amount of GR, hsp90, or hsp70 in cytosol and without affecting steroid binding activity. Within the range of m.o.i. shown for YDJ-1 expression, there is a slight increase in steroid binding activity that is consistently seen at the lowest level of expression followed by a decrease in binding activity at higher levels of expression (Fig. 2B). Coexpression of Hop does not affect the amount of cytosolic GR, hsp90, or hsp70 or the level of steroid binding activity (Fig. 2C).

Coexpression of p23 Increases GR-Hsp90 Heterocomplex Stability in Vivo—We have shown previously that p23 stabilizes GR-hsp90 heterocomplexes formed in vitro (20). Receptor-hsp90 heterocomplex assembly in cells or reticulocyte lysate is a very dynamic process in which assembly and disassembly are occurring simultaneously (21). In the purified assembly system, it has been shown that the steroid binding cleft is opened to access by steroid at the same rate with or without the presence of p23, but p23 is necessary to produce stable GR-hsp90 heterocomplexes that can be immunopurified, washed, and then bound with steroid (20). The receptor-bound hsp90 must achieve its ATP-bound conformation for the receptor to have steroid binding activity (22), and this is the conformation of
hsp90 that is bound by p23 (23). In binding to the ATP-dependent conformation of receptor-bound hsp90, p23 stabilizes the heterocomplex.

The data of Fig. 3 show that p23 is also stabilizing the GR·hsp90 heterocomplexes formed in vivo in SF9 cells. In these experiments, SF9 cells were cotransfected with the GR baculovirus and various amounts of p23 baculovirus. After 48 h of infection, cytosols were prepared and immunoadsorbed with antibody against the GR. Fig. 3A shows the insect hsp90 and human p23 coimmunoadsorbed with the native GR heterocomplexes, as well as duplicate GR immune pellets that have been stripped of insect hsp90. Fig. 3B shows the stripped GR immune pellets after they were incubated with reticulocyte lysate, washed, and immunoblotted for rabbit hsp90 and p23 in the reconstituted heterocomplexes. Fig. 3C shows the steroid binding activity of the immunoadsorbed native GR·hsp90 heterocomplexes (solid bars) and the GR-hsp90 heterocomplexes reconstituted in reticulocyte lysate (open bars). It can be seen that the steroid binding activity of the immunoadsorbed native GR·hsp90 heterocomplexes increases with increasing expression of p23 in the SF9 cells until it reaches the level of binding achieved by GR·hsp90 heterocomplex reconstitution in reticulocyte lysate.

**Fig. 3.** Coexpression of p23 increases GR·hsp90 heterocomplex stability in vivo. SF9 cells were cotransfected with the GR baculovirus and with p23 baculovirus at the indicated m.o.i.s. After 48 h of infection, cytosols were prepared, and the GR was immunoadsorbed from 50-μl aliquots. A, GR, insect hsp90 and hsp70, and human p23 in native (N) heterocomplexes and after salt stripping (S) the chaperones from the immunoadsorbed GR. B, GR and rabbit hsp90, hsp70, and p23 after stripped GR samples were reactivated by incubation with reticulocyte lysate. C, steroid binding activity of immunoadsorbed native GR·hsp90 heterocomplexes (solid bars) and reticulocyte lysate-reconstituted GR·hsp90 heterocomplexes (open bars). D, comparison of the relative amounts of hsp90 in and steroid binding activity of native GR·hsp90 heterocomplexes immunoadsorbed from cytosol. Native GR·hsp90 immune pellets were electrophoresed and immunoblotted as in A and bound with steroid as in C. The GR-bound hsp90 bands were counterblotted with 125I-labeled anti-rabbit IgG, and the bands were excised and counted in a γ-counter to determine the relative amount of hsp90. The data show the average amount of GR-bound hsp90 (open bars) and steroid binding activity (solid bars) for three experiments ± S.E.
The increase in steroid binding activity in native heterocomplexes (Fig. 3C) is accompanied by an increase in GR-bound hsp90 (Fig. 3A). The relationship between GR-bound hsp90 and steroid binding activity with increasing coexpression of p23 is shown in the graph of Fig. 3D.

In that hsp90 is required for steroid binding activity of the GR and increasing p23 coexpression causes increased recovery of cytosolic GR-hsp90 heterocomplexes, the effect of p23 in vivo is to stabilize the client protein-hsp90 complex. p23 stabilizes the GR-hsp90 complex in a dynamic manner (20), and when cytosol is prepared and cytosolic steroid binding is assayed at 0–4 °C, there is the same binding in the absence of p23 coexpression as there is with various levels of coexpression (Fig. 2A). This suggests that there is sufficient endogenous insect p23 to maintain a limited amount of the overexpressed GR in cytosolic heterocomplex with hsp90, but at any instant, only
Coexpression of p23 reduces GR aggregate formation—Alnemri and Litwack (14) found the majority of the overexpressed GR in Sf9 cells to be present in self-aggregates that remain in the pellet when the cell lysate is centrifuged to prepare cytosol. Thus, we asked whether p23 coexpression would affect aggregate formation. As shown in Fig. 5, in the absence of human p23 coexpression, the majority of the GR is in the pellet fraction. However, at higher levels of human p23 expression, there is a marked decrease in the amount of GR in the pellet without substantial change in the amount of cytosolic GR.

Of the three cochaperones in the hsp90/hsp70-based chaperone system, only coexpression of p23 reduces GR aggregate formation. Although the production of aggregates is reduced, the amount of GR in the cytosolic fraction does not increase in a compensatory manner; thus, it is likely that GR degradation is taking place. The GR is known to be degraded by the ubiquitin-proteasome pathway of proteolysis (24), but treatment of Sf9 cells with the proteasome inhibitors lactacystin and MG132 did not change the amount of cytosolic GR or affect the loss of total GR that occurred with high levels of p23 coexpression (data not shown). However, the GR aggregates are formed over 48 h, and it is possible that inhibition of GR/hsp90 disassembly by p23 keeps the receptors from aggregating such that they can be shunted into alternative proteolytic pathways. We do not know what limits the amount of cytosolic GR, but we do not achieve higher levels by infection with more GR baculovirus or by coexpression of p23 (Fig. 2A).

Coexpression of YDJ-1 and Hop—At the lowest multiplicity of infection, coexpression of YDJ-1 modestly increases cytosolic steroid binding activity (Fig. 2B), the amount of GR in heterocomplex with hsp90 (Fig. 6A), and the steroid binding activity of immunoabsorbed receptor (Fig. 6C). At higher levels of YDJ-1 coexpression, there is a progressive decrease in steroid binding activity, and at the highest level, there is a decrease in the amount of receptor protein in cytosol (Fig. 2B). Coexpression of YDJ-1 does not affect the relative fraction of GR recovered in the cytosolic and particulate fractions (data not shown). The fact that the level of steroid binding activity decreases as cytosolic YDJ-1 increases suggests that the level of endogenous insect hsp40 in Sf9 cells is essentially optimal for the proper functioning of the multiprotein chaperone machinery in vivo.

Coexpression of Hop at the multiplicity of infections in Figs. 2C and 7 does not affect the steroid binding activity of GR in cytosol (Fig. 2C) or the proportion of cytosolic receptors in stable heterocomplexes with hsp90 that survive immunoabsorption and washing (Fig. 7). Coexpression of Hop also does not affect the relative amount of GR recovered in cytosolic and particulate fractions (data not shown). Thus, the level of endogenous insect Hop in Sf9 cells appears to be sufficient for the proper functioning of the multiprotein chaperone machinery in vivo.

**DISCUSSION**

Studies of the mechanism of p23 action on GR/hsp90 heterocomplexes derived from in vitro experiments have led to two opposing, but perhaps not exclusive, models. In Dittmar et al.
we showed that purified p23 stabilized immunopurified native and reconstituted GR-hsp90 heterocomplexes. Inhibition of hsp90 dissociation from the receptor was accompanied by stabilization of steroid binding activity. Young and Hartl (25) utilized a rat GR fragment (amino acids 518-795) comprising the LBD that was expressed in Escherichia coli inclusion bodies and solubilized in guanidine hydrochloride or detergent. The solubilized LBD fragment was partially refolded by slow removal of the denaturant, and LBD-hsp90 complexes were formed by incubating this non-native GR LBD with reticulocyte lysate. Addition of purified p23 to these complexes promoted ATP-dependent release of hsp90 from the non-native GR LBD.

Thus, in one model (20), p23 binds to the GR-bound hsp90 in its ATP-dependent conformation and stabilizes the client protein-hsp90 complex, whereas in the other model (25), p23 functions as a client protein release factor for hsp90. The two models may not contradict each other but may reflect the study of p23 effects on hsp90 binding to native versus an extensively denatured LBD fragment (25). Here, we have seen that coexpression of p23 with the GR results in increased recovery of stable GR-hsp90 heterocomplexes with a concomitant increase in steroid binding activity (Fig. 3). Thus, in vivo in Sf9 cells, p23 stabilizes the client protein-hsp90 interaction.

In addition to its binding to hsp90 and affecting hsp90 function, p23 has been shown to directly interact with denatured protein to maintain a non-native folding-competent intermediate (26). Some effects of p23 in vivo have been attributed to such a direct passive chaperoning activity. In particular, p23 has been shown to promote the disassembly of transcription factors (including the GR) from their appropriate response elements (27, 28). Because overexpression of hsp90 yielded only a slight activity, the much stronger effect of p23 has been assumed to reflect its intrinsic chaperone activity, rather than its ability to affect hsp90 function (27, 28). However, with respect to the GR, there is good reason to focus on a p23 effect on hsp90. It was shown many years ago in in vitro experiments that the GR does not have DNA binding activity when it is in heterocomplex with hsp90 (29) and that the chaperone machinery in reticulocyte lysate acts on DNA-bound, hormone-free GR to convert the receptor to the non-DNA binding state and restore steroid binding activity (3). In permeabilized cells, Liu and DeFranco (31) have shown that, during hormone withdrawal, both release of GR from chromatin and generation of high affinity steroid binding activity in the nucleus are inhibited by geldanamycin, a specific inhibitor of the hsp90 family of chaperones.
Here, we have shown that expression of p23 results in increased stable hsp90 binding to the GR, as well as increased steroid binding activity, an hsp90-dependent activity for this receptor. Our conclusion is that p23 affects client protein function in vivo by stabilizing hsp90 association with the client protein. The notion that p23 effects in vivo reflect its action on hsp90 is supported in a recent study by Oxelmark et al. (32) on p23 effects on signal transduction by the estrogen receptor. A variety of mutations in the yeast ortholog of p23 were selected on the basis of their ability to affect estrogen receptor-dependent transcriptional activation. The ability of p23 mutants to increase or decrease estrogen receptor signal transduction correlated with their ability to affect estrogen receptor-dependent transcriptional activation. The ability of p23 mutants to increase or decrease estrogen receptor signal transduction correlated with their ability to bind to hsp90 (32). Taken together, the observations of this paper and those of Oxelmark et al. (32) argue strongly that p23 effects in vivo do not reflect a direct chaperoning interaction with the client protein of hsp90; rather, they reflect a direct interaction with hsp90 to stabilize its association with the client protein.

Genetic studies in yeast have shown that p23 is not essential for the action of hsp90 client proteins in vivo (30, 33). Here, we have shown that p23 is the component of the five-protein hsp90/hsp70-based chaperone system that is limiting for the production of stable client protein/hsp90 heterocomplexes in vivo. Although p23 is present at only ~1/6th the concentration of hsp90 (Table I), that level is probably optimal for the proper functioning of the multiprotein chaperone system in vivo. A dynamic cycle of heterocomplex assembly with hsp90 and dissociation from hsp90 is probably very important for hsp90 regulation of client protein function. Although expression of more p23 can decrease the rate of hsp90 heterocomplex disassembly that may be detrimental to hsp90-mediated regulation of signal transduction overall. Thus, when we say that p23 is the limiting component of the chaperone machinery, we mean that it is limiting for producing the stable client protein/hsp90 heterocomplexes and hsp90-dependent effects that we as investigators would like to study. From the viewpoint of the researcher it is important that p23 is limiting, because overexpression of p23 presents us with another way to study effects of

![Image](https://example.com/image.png)
the hsp90/hsp70-based chaperone machinery on biological processes in vivo.

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The Hsp90 Cochaperone p23 Is the Limiting Component of the Multiprotein Hsp90/Hsp70-based Chaperone System in Vivo Where It Acts to Stabilize the Client Protein·Hsp90 Complex

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