The glucocorticoid receptor (GR) is recovered from hormone-free cells in a heterocomplex with the molecular chaperone hsp90, which is required to produce the proper folding state for steroid binding. GR-hsp90 heterocomplexes are formed by a multiprotein system that appears to exist in all eukaryotic cells. Recently, we have reconstituted a receptor-hsp90 heterocomplex assembly system with purified rabbit hsp90 and hsp70 and bacterially expressed human p23 and p60. We have shown that hsp90, p60, and hsp70 form an hsp90-p60-hsp70 complex that converts the GR from a non-steroid binding to a steroid binding form (Dittmar, K. D., and Pratt, W. B. (1997) J. Biol. Chem. 272, 13047–13054). The resulting GR-hsp90 heterocomplex rapidly disassembles unless p23 is present to bind to the ATP-dependent conformation of hsp90 and stabilize its association with the receptor (Dittmar, K. D., Demady, D. R., Stancato, L. F., Krishna, P., and Pratt, W. B. (1997) J. Biol. Chem. 272, 21213–21220). In the current work, we show that the purified rabbit hsp70 utilized in prior studies is contaminated with a small amount of the rabbit DnaJ homolog hsp40. Elimination of the hsp40 from the purified GR-hsp90 assembly system reduces assembly activity, and the activity is restored by addition of the purified yeast DnaJ homolog YDJ-1. hsp40 is a component of the hsp90-p60-hsp70 heterocomplex isolated from reticulocyte lysate with antibody against p60. Under conditions that promote binding of p23 to hsp90 (elevated temperature, ATP, Nonidet P-40, molybdate), a five-membered (p23-hsp90-p60-hsp70-hsp40) complex of chaperone proteins is formed in reticulocyte lysate or from purified proteins. The hsp40-free, purified assembly system has a modest level of assembly activity that is maximally potentiated by YDJ-1 when it is present at about one-twentieth the concentration of hsp70. Although hsp40 is not in the final GR-hsp90 heterocomplex isolated from L cell cytosol, it is in the GR-hsp90 heterocomplex assembled in reticulocyte lysate. We conclude that hsp40 is a component of the multiprotein hsp90-based chaperone system where it potentiates GR-hsp90 heterocomplex assembly.

Several members of the nuclear receptor family, the dioxin receptor, and several protein kinases (e.g. Src and Raf) exist in multiprotein cytosolic complexes with the abundant heat shock protein (hsp)1 hsp90 (for review, see Refs. 1 and 2). These complexes can be formed under cell-free conditions by incubating the immunoadsorbed proteins with rabbit reticulocyte lysate (3–6) or with concentrated cytosols prepared from a variety of animal, insect, and plant cells (7). The formation of these protein-hsp90 heterocomplexes reflects a dynamic assembly/disassembly process (8), and regardless of the protein being complexed with hsp90, there appears to be a common mechanism of heterocomplex assembly (9). Receptor-hsp90 heterocomplex assembly by reticulocyte lysate requires ATP/Mg2+, a monovalent cation, such as K+ (10, 11), and at least three proteins, hsp70 (10, 12), p60 (13, 14), and p23 (15, 16).

Recently, we have reconstituted the heterocomplex assembly system of reticulocyte lysate (13). We have also developed a minimal heterocomplex assembly system in which incubation of a GR immune pellet with a mixture containing purified rabbit hsp90 and hsp70, purified human p23, and bacterial lysate containing human p60 yields GR-hsp90 heterocomplex assembly and restoration of the steroid binding conformation of the HBD (13). hsp90, hsp70, and p60 are present in a common complex in reticulocyte lysate (17), and Chen et al. (14) have suggested that p60 binds to hsp70 via an N-terminal TPR (tetratricopeptide repeat) region and to hsp90 via a central TPR region. Mixture of the three proteins results in spontaneous formation of an hsp90-p60-hsp70 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 (18). Although hsp90, hsp70, and p60 are sufficient to alter the folding state of the GR HBD to produce a binding site, the GR-hsp90 complexes that are formed rapidly disassemble unless p23 is present to stabilize it (19).

There is an ATP binding site in the N-terminal domain of hsp90 (20), and Sullivan et al. (21) have shown that hsp90 assumes two conformations depending upon whether or not this site is occupied by ATP. When hsp90 is bound by ADP, it has a high affinity for a hydrophobic resin, and when bound by ATP, it has a low affinity for the hydrophobic resin (21). In direct experiments, Sullivan et al. (21) have shown that p23 binds to the ATP-dependent state of hsp90 and stabilizes it in the conformation with low affinity for hydrophobic resin (21). This ATP-dependent conformation of hsp90 appears to be required for the GR HBD to have a steroid binding site, and binding of p23 to that state of GR-bound hsp90 stabilizes the GR-hsp90 heterocomplex to disassembly and loss of the binding site (19).

© 1998 by The American Society for Biochemistry and Molecular Biology, Inc.

Printed in U.S.A.

† Supported by Pharmacological Sciences Training Program Grant GM07767 from the National Institutes of Health.
§ To whom correspondence should be addressed: Dept. of Pharmacology, The University of Michigan Medical School, Medical Science Research Bldg. Ill, Ann Arbor, MI 48109-0632. Tel.: 313-764-5414; Fax: 313-763-4450.

The Journal of Biological Chemistry Vol. 273, No. 13, Issue of March 27, pp. 7358–7366, 1998

This paper is available on line at http://www.jbc.org

7358

The Role of DnaJ-like Proteins in Glucocorticoid Receptor-hsp90 Heterocomplex Assembly by the Reconstituted hsp90-p60-hsp70 Foldosome Complex*

(Received for publication, September 25, 1997, and in revised form, January 15, 1998)

Kurt D. Dittmar‡, Maria Banach, Mario D. Galigniana, and William B. Pratt§

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

* This investigation was supported in part by National Institutes of Health Grant DK31573. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by Pharmacological Sciences Training Program Grant GM07767 from the National Institutes of Health.
§ To whom correspondence should be addressed: Dept. of Pharmacology, The University of Michigan Medical School, Medical Science Research Bldg. Ill, Ann Arbor, MI 48109-0632. Tel.: 313-764-5414; Fax: 313-763-4450.

The abbreviations used are: hsp, heat shock protein; GR, glucocorticoid receptor; HBD, hormone binding domain; TA, triamcinolone acetonide; TPR, tetratricopeptide repeat; Src, pp60v-src; TES, 2-[(2-hydroxyethyl)methyl]amino]ethanesulfonic acid.
Mammalian homologs of the bacterial DnaJ protein are often contaminants of purified hsp70 preparations, and DnaJ-like proteins are possible components of the reconstituted assembly system. In Escherichia coli, DnaJ interacts with DnaK, the bacterial hsp70 homolog, to stimulate its ATPase activity (22). The resulting transition of DnaK to the ADP-bound state stabilizes the interaction of DnaK with protein substrates (23). A variety of DnaJ-like proteins in eukaryotes also stimulate the ATPase activity of various members of the hsp70 protein family (for review, see Ref. 24). One of the DnaJ-like proteins cloned from humans is hsp40 (25), and we use the term hsp40 here to refer to mammalian members of the DnaJ-like protein family.

To date, hsp40 has not been identified either in native receptor-hsp90 heterocomplexes recovered from mammalian cells or in heterocomplexes assembled in reticulocyte lysate. However, the yeast DnaJ homolog YDJ-1 has been recovered in GR-hsp90 (26) and v-Src-hsp90 heterocomplexes (27) isolated from yeast lysates. Both genetic and biochemical observations suggest that hsp40 may play a role in heterocomplex assembly by the hsp90-based chaperone system.

Studies in yeast involving the genetic manipulation of chaperone activity have shown that hsp90 plays a critical role in determining the ligand responsiveness of steroid and dioxin receptors (Refs. 28–33 and see Ref. 34 for review) and also affect both steroid receptor and v-Src function in yeast (27, 35, 36). Inasmuch as mutations in the gene for YDJ-1 also affect both steroid receptor and v-Src function in yeast (27, 35, 36), it is possible that hsp40 is a component of the hsp90 heterocomplex assembly system.

In biochemical studies, reticulocyte lysate has been utilized to promote chaperone-mediated refolding of denatured proteins. For example, Schumacher et al. (37) examined ATP-dependent refolding of thermally denatured firefly luciferase and found that reactivation of the enzyme activity correlated with the concentration of both hsp70 and hsp90. Additionally, both hsp70 and hsp90 immune pellets, as well as a mixture of purified hsp90 and hsp70, yielded partial luciferase reactivation (37). Subsequently, it was found that the purified preparations of hsp70 and hsp90 were contaminated with low levels of hsp40, and when hsp70 and hsp90 that were free of hsp40 were used to facilitate luciferase activation, the system had to be supplemented with a DnaJ-like protein (YDJ-1) to obtain renaturation activity (38). As DnaJ-like proteins have been found to operate in conjunction with eukaryotic hsp70s in several in vitro refolding systems (e.g. Refs. 39–41), it is reasonable to ask whether they play a role in GR-hsp90 heterocomplex assembly by the minimal (hsp90, hsp70, p60, and p23) assembly system.

In this work, we use a new commercial antibody against hsp40 to detect contamination of our purified rabbit hsp70 and hsp90 preparations with small amounts of hsp40. We also find that hsp40 is a component of the hsp90+p60+hsp70 foldosome complex isolated from reticulocyte lysate with antibody against p60. Under conditions that promote binding of p23 to hsp90 (elevated temperature, ATP, Nonidet P-40, and molybdate) a five-protein p23-hsp90+p60+hsp70+hsp40 heterocomplex is formed in reticulocyte lysate or from purified proteins. Elimination of rabbit hsp40 reduces the GR-hsp90 assembly activity of the purified system, and activity is restored by addition of the purified yeast homolog YDJ-1. We show that GR-hsp90 heterocomplexes assembled in reticulocyte lysate contain hsp40, but hsp40 is not a component of native GR-hsp90 heterocomplexes isolated from L cell cytosol.

EXPERIMENTAL PROCEDURES

Materials

[6,7-3H]Triamcinolone acetonide (42.8 Ci/mmol) and [125I]-conjugated goat anti-mouse and anti-rabbit IgGs were obtained from NEN Life Science Products. Untreated rabbit reticulocyte lysate was from Green Hectares (Oregon, WI). Protein A-Sepharose and goat anti-mouse and anti-rabbit IgG horseradish peroxidase conjugates were from Sigma. The BuGHR2 monoclonal IgG antibody against the GR was from Affinity Bioreagents (Golden, CO). The 828 monoclonal IgG against the anti-hsp40 rabbit polyclonal antibody, the N27F3-4 antibody, and purified hsp90 monoclonal IgG (anti-hsp70), and purified DnaJ from E. coli were from StressGen (Victoria, British Columbia, Canada). The JJJ monoclonal IgG against p23, E. coli expressing human p23, and purified YDJ-1 were gifts from Dr. David Toft (The 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 FGFR 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% fetal bovine serum. Cells were harvested by scraping into Earl’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 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 acscites 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 TEGM and then the whole GR immunopellet suspension was pipetted onto the DS14F5 immunopellet containing the immunoadsorbed 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 μg of lysate from bacteria expressing p60, 4 μg of purified p23, 1.0 μg of purified YDJ-1, 1.0 μg of purified HDJ-1, or 0.5 μg of purified HDJ-2) and adjusted to 50 μl with HKD buffer (10 mM Hepes, 100 mM KCl, 5 mM dithiothreitol, pH 7.35). In some experiments p23 was substituted with 20 mM sodium molybdate, which has the same effect on GR-hsp90 heterocomplex assembly (19). For reconstitution of the GR by the immunoadsorbed 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 the DS14F5 immunopellet containing the immunoadsorbed p60 and the associated protein complex. 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 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 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 assay of renaturation 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 mM [3H]triamcinolone acetonide. Samples were then washed twice with 1 ml of TEGM and counted by liquid scintillation.
The dashed line represents the amount of steroid bound by stripped GR. Conditions are as follows: lane 1, stripped GR incubated with HKD buffer; lanes 2 and 3, nonimmune (lane 2) and GR (lane 3) pellets incubated with the assembly mix of hs90, hs70, p60, and p23; lanes 4 and 5, nonimmune and GR incubated with the assembly mix plus YDJ-1.

scintillation spectrometry as described previously. The steroid binding is expressed as counts/min of [3H]triamcinolone acetonide-bound/FIGR immunopellet prepared from 100 μl of cytosol. As noted previously (11), 100 μl of L cell cytosol contains 60,000 cpm of [3H]triamcinolone acetonide binding capacity, and we immunoadsorb about 50% of the GR. Thus, ~30,000 cpm represents 100% of receptors reactivated to the steroid binding form.

**Effect of purified YDJ-1 on GR-hs90 heterocomplex assembly by the purified system**—In the experiment of Fig. 1A, GR immune pellets were incubated for 20 min at 30 °C with hs90, hs70, p23, and lysate from bacteria expressing p60 in the presence of various amounts of purified YDJ-1. The immune pellets were then washed and incubated with [3H]TA to determine steroid binding activity. The abscissa represents the microgram of YDJ-1 protein added to 56 μl of incubation mixture. The dashed line represents the amount of steroid bound by stripped GR. B, effect of YDJ-1 on GR-hs90 heterocomplex assembly. Salt-stripped GR immune pellets were incubated as above in the presence of 1 μg of YDJ-1; GR and hs90 were assayed in each sample by Western blotting. Thus, 0.02% Nonidet P-40 was added to lysates and proteins yields substantial amounts of hsp40 in nonimmune as well as immune pellets. Thus, 0.02% Nonidet P-40 was added to lysates and protein solutions during immunoadsorption, and the resulting protein A pellets were washed once with 1 ml of detergent-containing (0.02% Nonidet P-40) buffer prior to two washes with detergent-free buffer. This markedly reduces or eliminates nonspecific hs40 and YDJ-1 binding.

**RESULTS**

Effect of YDJ-1 on GR-hs90 Heterocomplex Assembly by the Purified System—In the experiment of Fig. 1A, GR immune pellets that were stripped free of receptor-associated proteins with salt were incubated with the purified assembly system (hs90, hs70, p60, and p23) and various concentrations of the yeast DnaJ-like protein YDJ-1. Addition of the purified YDJ-1 produces a small increase in the level of steroid binding activity that is generated by the purified assembly system (Fig. 1A). The increase in steroid binding activity is accompanied by a small increase in the amount of GR-hs90 heterocomplexes that are assembled (Fig. 1B). The YDJ-1 preparation used here is very active in promoting the activation of thermally denatured luciferase by hs70 (38). Assuming a requirement for DnaJ-like protein in heterocomplex assembly, the low activity of YDJ-1 when added to the purified assembly system could be explained by contamination of the purified proteins with suf-
Arranged in combinations of subfractions A1, A2, and stripped GR incubated with the various steroid binding activity for samples of treated to 0.5 ml, and stored at dialyzed against HKD buffer, concentration of peak hsp90 fractions at each step in the purification protocol (lane 6). An additional purification step is required to prepare hsp40-free hsp70 (lane 3).

Elimination of hsp40 from the Purified hsp70—To purify hsp70, we normally adsorb rabbit brain cytosolic proteins to DE52 and pool the fractions eluting prior to hsp90 in the salt gradient. This pool, which we call DE52 fraction A (13), is then further purified by chromatography on an ATP-agarose column. Fig. 3A shows the further fractionation of DE52 fraction A by chromatography on a hydroxylapatite column. Two hsp40 bands were located in the column eluate by immunoblotting. The upper hsp40 band was arbitrarily designated hsp40-1 and the lower band hsp40-2. Fractions were pooled as indicated under the Western blot into three subfractions. Fraction A2 contains hsp70 that is separated from hsp40 and can be further purified by ATP-agarose chromatography to yield hsp40-free hsp70 (Fig. 2, lane 3).

The activity of each of the hydroxylapatite subfractions shown in Fig. 3A is tested in Fig. 3B. Stripped GR immune pellets (lane 1) were incubated with hsp40-free hsp90, bacterial lysate containing p60, and 20 mM molybdate (lane 2). As shown in lanes 3–5, addition of A1, A2, or A3 to this system yielded very little steroid binding activity. However, the combination of A2, which contains hsp70, with either A1 containing hsp40-2 (lane 6) or A3 containing hsp40-1 (lane 7) yielded a substantial number of steroid binding sites. This is consistent with the notion that contamination of hsp70 by endogenous rabbit hsp40 may play a role in GR-hsp90 heterocomplex assembly by our purified system.

The experiment of Fig. 4 shows the ability of YDJ-1 to potentiate GR-hsp90 heterocomplex assembly by a purified system containing hsp40-free hsp70 and hsp40-free hsp90. To restrict the assembly system to the minimum number of proteins (hsp90, p60, hsp70, and YDJ-1), the incubation contained molybdate rather than p23 to stabilize the newly assembled heterocomplexes. When stripped GR (lane 1) is incubated with hsp40-free hsp70 and hsp90 in the absence (lane 4) or presence

FIG. 2. The purified preparations of rabbit brain hsp70 and hsp90 contain low levels of hsp40. Samples of rabbit brain hsp70 and hsp90 purified by our routine methods or purified in a manner to eliminate contamination by hsp40 were resolved by SDS-polyacrylamide gel electrophoresis, and proteins were visualized by staining with Coomassie Blue or Western blotting with antibodies against hsp70, hsp90, and hsp40. Lanes 1 and 4, Coomassie Blue stain of hsp40-contaminated hsp70 and hsp90, respectively; lanes 2 and 5, Western blot of the samples run in lanes 1 and 4; lanes 3 and 6, Western blot of hsp70 and hsp90 purified free of hsp40.

FIG. 3. Separation of rabbit hsp40 from hsp70. A, chromatography of DE52 fraction A from rabbit brain on hydroxylapatite. DE52 fraction A was chromatographed on a column of hydroxylapatite as described under "Experimental Procedures" (solid line, absorbance at 280 nm; dotted line, KH₂PO₄ gradient). Aliquots of every other fraction were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted for hsp70 and hsp40. Fractions were pooled (A1–A3) as indicated by the brackets under the immunoblots. Fraction pools A1, A2, and A3 were dialyzed against HKD buffer, concentrated to 0.5 ml, and stored at –70 °C. B, steroid binding activity for samples of stripped GR incubated with the various combinations of subfractions A1, A2, and A3. Stripped GR immune pellets were incubated for 20 min at 30 °C with 3 μl of bacterial lysate containing p60, 12 μg of purified hsp40-free hsp90, and 20 mM molybdate with the indicated additions in the presence of an energy generating system. The immunopellet was incubated with [3H]TA to determine steroid binding activity. Conditions are as follows: lane 1, stripped GR; lane 2, stripped GR plus p60 and hsp90; lane 3, GR plus p60, hsp90, and A1; lane 4, GR plus p60, hsp90, and A2; lane 5, GR plus p60, hsp90, and A3; lane 6, GR plus p60, hsp90, A1, and A2; lane 7, GR plus p60, hsp90, A2, and A3; lane 8, GR plus p60, hsp90, A1, and A3.
hsp40 Is a Component of the Foldosome—We have previously shown that immunoadsorption of p60 from reticulocyte lysate results in co-adsorption of a protein folding complex (foldosome) that converts the GR HBD to a steroid binding state (18, 19). Both hsp90 and hsp70 have been identified in this p60 immune pellet (17, 43), and in Fig. 5A, we show that hsp40 is also present. In this experiment, rabbit reticulocyte lysate was immunoadsorbed with the F5 antibody against p60, and as shown in lane 2 (Fig. 5A), hsp90, hsp70, and hsp40 were co-adsorbed but p23 was not. It is possible to make a complex in reticulocyte lysate that contains all five proteins. Sullivan et al. (21) have shown that binding of purified p23 to purified hsp90 requires elevated temperature, and ATP/Mg^2+ and is strongly promoted by the non-ionic detergent Nonidet P-40 and molybdate. Thus, we incubated reticulocyte lysate at 30 °C with an ATP-generating system, Nonidet P-40, and molybdate and then immunoadsorbed p60. As shown in lane 4 of Fig. 5A, the five proteins are now immunoadsorbed in a common complex.

The experiment of Fig. 5B shows that the p60 immune pellets prepared in Fig. 5A convert the GR HBD to the steroid binding conformation. Stripped GR (lane 1) was incubated with the p60 immune pellet of lane 2 in Fig. 5A, which contains hsp90, hsp70, p60, and hsp40. This four-protein immune pellet has little ability to produce a stable, steroid binding GR-hsp90 heterocomplex by itself (Fig. 5B, lane 3), but if p23 is added to the incubation, stable heterocomplexes are produced (lane 4). We have emphasized in previous work that p23 interacts in a highly dynamic fashion with the GR-hsp90 heterocomplex, and it must be continuously present to stabilize the heterocomplex (19). This is highlighted by the fact that the p60 immune pellet containing all five proteins, including p23, also generates little steroid binding activity (Fig. 5B, lane 5) unless purified p23 is added (lane 6) to permit dynamic stabilization during GR-hsp90 heterocomplex assembly.

In the experiment of Fig. 5C, we asked whether the hsp40 homolog YDJ-1 can directly enter the foldosome complex. p60 was immunoadsorbed from reticulocyte lysate to yield the hsp40-containing four-protein complex shown in lane 2. When this pellet was incubated in buffer containing YDJ-1, some of the rabbit hsp40 dissociated and YDJ-1 associated with the foldosome (lane 4). Thus, the Dnaj-like proteins appear to

hsp40 and Receptor Heterocomplex Assembly

(lane 5) of YDJ-1, there is essentially no heterocomplex assembly. Incubation of the receptors with hsp40-free hsp70 and hsp90 in the presence of p60 (lane 6) yields some steroid binding activity, and the presence of YDJ-1 (lane 7) increases binding activity essentially to the level that is achieved with the purified chaperones that are contaminated with rabbit hsp40 (lane 3). The Western blot above the bar graph in Fig. 4 shows the GR-hsp90 heterocomplexes formed under each condition.
Formation of a foldosome from individual components. A, creation of foldosomes. Purified hsp40-free hsp90, hsp40-free hsp70 and p23, and 3 μl of bacterial lysate containing p60 were incubated for 20 min at 30 °C with an ATP-generating system, 20 mM molybdate, and 0.02% Nonidet P-40 in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of YDJ-1. Samples were immunoadsorbed with nonimmune IgG (lanes 1 and 3) or with F5 antibody against p60 (lanes 2 and 4); the immune pellets were washed, and pellet-associated proteins were assayed by immunoblotting. B, hsp90 and hsp70 are required for the presence of p23 and YDJ-1, respectively. The indicated proteins were mixed with 3 μl of bacterial lysate containing p60, and the mixtures were incubated for 20 min at 30 °C with an ATP-generating system, molybdate, and Nonidet P-40. At the end of the incubation, samples were immunoadsorbed with nonimmune IgG (lanes 1, 3, 5, and 7) or F5 antibody against p60 (lanes 2, 4, 6, and 8). The immune pellets were washed, and pellet-associated proteins were assayed by immunoblotting. Protein mixtures were as follows: lanes 1 and 2, p60, hsp70, and p23; lanes 3 and 4, p60, hsp90, and p23; lanes 5 and 6, p60, hsp90, and YDJ-1; lanes 7 and 8, p60, hsp90, hsp70, and YDJ-1. C, reconstitution of GR steroid binding activity with the immunoadsorbed foldosome prepared from the individual proteins. Stripped GR immune pellets were incubated with the indicated additions for 20 min at 30 °C in the presence of an ATP-generating system. The immune pellets were then washed and incubated with [3H]TA to assay steroid binding activity. Conditions are as follows: lane 1, stripped GR; lane 2, GR incubated with reticulocyte lysate; lanes 3 and 4, GR incubated with bacterial lysate containing p60, purified p23, hsp40-free hsp90, and hsp40-free hsp70 in the absence (lane 3) or presence (lane 4) of 1 μg of YDJ-1; lane 5, GR was incubated with p23 and a p60 immune pellet prepared from a mixture of p60, hsp40-free hsp70, and hsp40-free hsp90; lane 6, GR incubated with p23 and a p60 immune pellet prepared from a mixture containing p60, hsp40-free hsp70, hsp40-free hsp90, and YDJ-1; lane 7, same as in lane 5 except that YDJ-1 was added to the incubation along with p23 and the p60 immune pellet. D, concentration dependence of YDJ-1 effect on GR steroid binding activity. Stripped GR immune pellets were incubated with p60, purified p23, hsp40-free hsp90, hsp40-free hsp70, and the indicated amounts of YDJ-1 for 20 min at 30 °C, and steroid binding activity was assayed. The dashed line represents the amount of steroid bound by the stripped GR.

interact dynamically with the preformed foldosome complex.

Formation of a Foldosome from the Individual Proteins—In the experiment of Fig. 6A, a mixture of proteins was incubated under conditions that promote the presence of p23 (i.e. elevated temperature, ATP-generating system, Nonidet P-40, and molybdate), and p60 was then immunoadsorbed. Lane 2 shows the formation of a four-protein heterocomplex without YDJ-1 and lane 4 shows the formation of a five-protein heterocomplex.

It is established that p60 binds to both hsp90 and hsp70 to form an hsp90-p60-hsp70 heterocomplex (14). In that p23 binds to hsp90 and hsp40 binds to hsp70, then hsp90 and hsp70 should be required for the presence of p23 and hsp40, respectively, in a foldosome complex. That this is the case is shown in Fig. 6B where p23 is not co-immunoadsorbed with p60 from a mixture of p60, hsp70, and p23 (lane 2) but is co-immunoadsorbed if hsp90 is also present (lane 4). Similarly, YDJ-1 is not co-immunoadsorbed with p60 from a mixture of p60, hsp90, and YDJ-1 (lane 6) but is co-immunoadsorbed if hsp70 is also present (lane 8). Thus, the five-protein foldosome can be written as a p23-hsp90-p60-hsp70-hsp40 unit, with the dots indicating the major protein-protein interactions required for complex formation.

Fig. 6C shows that a foldosome prepared from purified proteins can reconstitute GR steroid binding activity. In this experiment, p60 immune pellets were prepared from a mixture of p60, hsp40-free hsp70, and hsp40-free hsp90 or from a mixture of these proteins plus YDJ-1. When stripped GR (lane 1) was incubated with the hsp90-p60-hsp70 pellet (lane 5) or the hsp90-p60-hsp70-YDJ-1 pellet (lane 6) in the presence of p23 a similar modest number of stable steroid binding sites was produced. If purified YDJ-1 was added to the mixture of GR and synthetic (hsp90-p60-hsp70) foldosome, then twice as many steroid binding sites were generated (lane 7). This stimulation by the presence of free YDJ-1 is consistent with the notion that foldosome complexes are dynamic and that the DnaJ-like proteins may cycle in and out during GR-hsp90 heterocomplex assembly. This is also consistent with the fact that we are providing only 1 μg of YDJ-1 per 20 μg of hsp70 in the soluble assembly system.

That YDJ-1 is acting at levels that are substantially substoichiometric with respect to hsp70 is shown in the concentration curve of the YDJ-1 effect on the generation of steroid binding activity by the hsp40-free, purified system in Fig. 6D. Peak activity is achieved at ~0.5 μg, which represents a ratio of 1 molecule of YDJ-1 to ~20 molecules of hsp70.

Recruitment of hsp40 with the GR-hsp90-p60-hsp70 Intermediate Assembly Heterocomplex—Fig. 7A presents the composition of native and reconstituted GR heterocomplexes. As shown in lane 2, when the GR is immunoadsorbed from cytosol of hor-
mone-free L cells, the receptor is bound to hsp90 and p23 (several immunophilins that are present in GR-hsp90 heterocomplexes (43) have not been assayed here). As we have reported previously (44), native L cell GR-hsp90 heterocomplexes do not contain hsp70, and as shown in lane 2 of Fig. 7A, they do not contain hsp40 either. When GR-hsp90 heterocomplexes are assayed in reticulocyte lysate, the complexes contain hsp70, p23, and hsp40 (lane 4). In this experiment, we detect a trace of p60 in this complex as well (lane 4). In kinetic experiments, Smith (8) has shown that p60 is present at an intermediate stage of receptor heterocomplex assembly. The presence of a small amount of p60 in lane 4 indicates that a small fraction of the GR-hsp90 heterocomplexes are at this intermediate stage in this incubation. Geldanamycin, an antibiotic that binds specifically to hsp90 (45), blocks receptor-hsp90 heterocomplex assembly at this intermediate complex (46). As shown in lane 6 of Fig. 7A, GR heterocomplexes that were reconstituted in the presence of geldanamycin contain less hsp90 and an increased amount of p60, but hsp70 and hsp40 are unchanged. As reported by Johnson and Toft (47), geldanamycin-bound hsp90 does not bind p23, and p23 is not in the intermediate stage receptor-hsp90 heterocomplexes formed in the presence of geldanamycin (Ref. 46 and Fig. 7A, lane 6). The presence of hsp40 in the GR-hsp90 intermediate complex assembled in reticulocyte lysate but not in the final GR heterocomplex isolated from L cell cytosol is consistent with the presence of hsp70 in the former complex but not in the latter.

In the experiment of Fig. 7B, GR-hsp90 heterocomplexes that were assembled in reticulocyte lysate were washed and incubated for a further 20 min at 30 °C in the presence of YDJ-1. Molybdate was also present during the second incubation to stabilize the receptor heterocomplex. During the second incubation, some of the rabbit hsp40 dissociated (cf. lane 4 with lane 2), but either no or only a trace amount of YDJ-1 is recovered with the GR-hsp90 heterocomplex (lane 4). Thus, in contrast to their dynamic interaction with the foldosome (Fig. 5C), the DnaJ-like proteins do not seem to associate with the hsp70-containing GR-hsp90 heterocomplex assembled in reticulocyte lysate. Another possibility is that YDJ-1 associates with hsp70 in the preassembled GR-hsp90 heterocomplex much more weakly than with hsp70 in the foldosome, and we don’t detect this weak binding in our assay.

**DISCUSSION**

It is clear that both the hsp70 and hsp90 preparations that we have been using are contaminated with small amounts of hsp40 (Fig. 2). Separation of hsp70 and hsp90 from hsp40 lowers the activity of the purified GR-hsp90 heterocomplex assembly system, and activity is returned by addition of purified YDJ-1 (Fig. 4). Addition of YDJ-1 to the hsp40-contaminated assembly system yields only a small increase in steroid binding activity (Fig. 1A), suggesting that hsp40 is not limiting under these conditions. At this time, we can say that hsp40 (in this case the yeast homolog YDJ-1) potentiates GR-hsp90 heterocomplex assembly in a purified system that is hsp40-free by immunoblotting, but we do not know whether or not it is obligatory for assembly. We always see a basal level of assembly activity in the hsp40-free system (e.g. Fig. 4 and Fig. 6C).

In the experiments of this paper, the p60 is added in a bacterial lysate that contains a small quantity of E. coli DnaJ, and it has been shown that bacterial DnaJ can stimulate polypeptide translocation across membranes (48) and luciferase refolding (40) mediated by yeast hsp70s. However, we find that a commercial preparation of DnaJ has no stimulating activity in our hsp40-free assembly system (data not shown), and we still obtain the basal GR-hsp90 heterocomplex assembly activity when the stripped GR is incubated with purified human p60 (kindly provided by David Toft) and hsp40-free hsp90 and hsp70 (data not shown). Thus, at this time, we favor a mechanism in which GR-hsp90 assembly proceeds at a basal level without hsp40, and addition of hsp40 in the form of its homolog YDJ-1 increases activity over that of the basal assembly system.

In the purified GR-hsp90 heterocomplex assembly system, YDJ-1 is active at levels that are markedly substoichiometric with respect to hsp70 (Fig. 6D). This was also the case in a luciferase refolding system consisting of purified hsp70, hsp90, and YDJ-1, where Schumacher et al. (37) found that the amount of luciferase renatured using 1.4 μM hsp70 and 10 nM luciferase was maximal when the YDJ-1 concentration was between 40 and 100 nM or roughly a ratio of 1 molecule of YDJ-1 to 20 molecules of hsp70. This low stoichiometry implies a dynamic system in which the DnaJ homolog interacts with hsp70 only briefly during receptor-hsp90 heterocomplex assembly.

The intermediate in heterocomplex assembly is shown as the product of step 2 in Fig. 8, which presents an evolving model of GR-hsp90 heterocomplex assembly derived from reconstitution studies with the purified assembly system. The model has been modified to include the hsp40 data of this work. In the model of Fig. 8, we show hsp40 entering the preformed foldosome directly, as was shown with YDJ-1 in Fig. 5C. However, hsp40 probably also enters as a bimolecular hsp70-hsp40 complex when the foldosome assembles. When hsp90-p60-hsp70-hsp40

![FIG. 8. The heterocomplex assembly pathway with sites of hsp40 interaction.](http://www.jbc.org/)
complexes are immunoadsorbed from reticulocyte lysate with anti-p60 and incubated in buffer with YDJ-1, we see dissociation of hsp40 as well as entry of YDJ-1 into the foldosome (Fig. 5C), suggesting a reversible interaction of the DnaJ homologs with hsp70 in the foldosome complex. In Fig. 8, the interaction of hsp40 with the foldosome is defined by double arrows to indicate that this is a dynamic process. In contrast, YDJ-1 does not seem to enter the preassembled GR-hsp90 heterocomplex, despite the presence of hsp70 in the complex (Figs. 7B). Thus, in Fig. 8, hsp40 is presented in a cycle of entry via the foldosome. In this work, we have shown that incubation of either reticulocyte lysate (Fig. 5A) or purified proteins (Fig. 6A) under conditions that favor binding of purified p23 to hsp90 (21) results in formation of a p23-hsp90-p60-hsp70-hsp40 complex. It is important to note that all of the five proteins proven to be involved in GR-hsp90 heterocomplex assembly can under appropriate conditions be brought together in a complex independent of the presence of any protein that is being chaperoned (Figs. 5A and 6A). Several years ago, we made the observation that all of the factors required for assembly of the GR into a functional heterocomplex with hsp90 are preassociated with hsp90, and we suggested that they form a self-sufficient protein folding structure that we called a foldosome (49). An activity required for stable heterocomplex assembly was subsequently shown not to be an integral component of the foldosome, and this activity was identified as p23 (16). We have not shown p23 in the foldosome complex in Fig. 8, and it should be emphasized that we do not obtain a foldosome containing p23 in the absence of ATP, Nonidet P-40, and molybdate (Ref. 19 and Fig. 5A, lane 2). These conditions apparently convert hsp90 to the ATP-bound state that is required for p23 binding (21). This same conversion of hsp90 to the ATP-bound conformation occurs during formation of the GR-hsp90 heterocomplex, and p23 then binds to GR-hsp90 in an ATP-independent manner to stabilize the complex (19).

Previously, we reported that incubation of purified hsp90, p60, hsp70, and p23 with ATP, Nonidet P-40, and molybdate did not produce a four-membered complex that immunoadsorbed with an antibody against p23 (19). However, as we show here that we can immunoadsorb a five-membered complex with antibody against p60, our failure to do so with antibody against p23 may reflect inaccessibility of the epitope when p23 is in this four-membered complex.

Although this and other studies (13, 18, 19) of GR-hsp90 heterocomplex assembly by the purified system have led us to the assembly model of Fig. 8, it should be emphasized that there are differences between GR heterocomplexes formed by the purified system and those formed in reticulocyte lysate or complexes formed in reticulocyte lysate except when ATP is limiting (10), at early times in the assembly process (8), or when geldanamycin is present (18, 46). Yet, substantial amounts of p60 are present in GR heterocomplexes formed by the purified system system and those formed in reticulocyte lysate or complexes formed in reticulocyte lysate except when ATP is limiting (10), at early times in the assembly process (8), or when geldanamycin is present (18, 46). Yet, substantial amounts of p60 are present in GR heterocomplexes formed by the purified system.
43. Owens-Grillo, J. K., Czar, M. J., Hutchison, K. A., Hoffmann, K., Perdew, G. H., and Pratt, W. B. (1996) *J. Biol. Chem.* 271, 13468–13475
44. Sanchez, E. R., Hirst, M., Scherrer, L. C., Tang, H.-Y., Welsh, M. J., Harmon, J. M., Simons, S. S., Jr., Ringold, G. M., and Pratt, W. B. (1990) *J. Biol. Chem.* 265, 20123–20130
45. Whitesell, L., Mimnaugh, E. G., De Costa, B., Myers, C. E., and Neckers, L. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 8324–8328
46. Smith, D. F., Whitesell, L., Nair, S. C., Chen, S., Prapapanich, V., and Eimerman, R. A. (1995) *Mol. Cell. Biol.* 5, 6804–6812
47. Johnson, J. L., and Tuft, D. O. (1995) *Mol. Endocrinol.* 9, 670–678
48. Caplan, A. J., Cyr, D. M., and Douglas, M. G. (1992) *Cell* 71, 1143–1155
49. Hutchison, K. A., Dittmar, K. D., and Pratt, W. B. (1994) *J. Biol. Chem.* 269, 27894–27899
50. Deleted in proof
51. Rexin, M., Busch, W., and Gehring, U. (1991) *J. Biol. Chem.* 266, 24601–24605
52. Alexis, M. N., Mavridou, I., and Mitsiou, D. J. (1992) *Eur. J. Biochem.* 204, 75–84
53. Czar, M. J., Galigniana, M. D., Silverstein, A. M., and Pratt, W. B. (1997) *Biochemistry* 36, 7776–7785
54. 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
The Role of DnaJ-like Proteins in Glucocorticoid Receptor·hsp90 Heterocomplex Assembly by the Reconstituted hsp90·p60·hsp70 Foldosome Complex
Kurt D. Dittmar, Maria Banach, Mario D. Galigniana and William B. Pratt

J. Biol. Chem. 1998, 273:7358-7366.
doi: 10.1074/jbc.273.13.7358

Access the most updated version of this article at http://www.jbc.org/content/273/13/7358

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 52 references, 32 of which can be accessed free at http://www.jbc.org/content/273/13/7358.full.html#ref-list-1