Defining the Requirements for Hsp40 and Hsp70 in the Hsp90 Chaperone Pathway*

Nela S. Citron and David Toft1

From the Department of Biochemistry and Molecular Biology, Mayo Graduate School, Mayo Clinic and Foundation, Rochester, Minnesota 55905

The Hsp90 chaperoning pathway and its model client substrate, the progesterone receptor (PR), have been used extensively to study chaperone complex formation and maturation of a client substrate in a near native state. This chaperoning pathway can be reconstituted in vitro with the addition of five proteins plus ATP: Hsp40, Hsp70, Hop, Hsp90, and p23. The addition of these proteins is necessary to reconstitute hormone-binding capacity to the immuno-isolated PR. It was recently shown that the first step for the recognition of PR by this system is binding by Hsp40. We compared type I and type II Hsp40 proteins and created point mutations in Hsp40 and Hsp70 to understand the requirements for this first step. The type I proteins, Ydj1 and DjA1 (HDJ2), and a type II, DjB1 (HDJ1), act similarly in promoting hormone binding and Hsp70 association to PR, while having different binding characteristics to PR. Ydj1 and DjA1 bind tightly to PR whereas the binding of DjB1 apparently has rapid on and off rates and its binding cannot be observed by antibody pull-down methods using either purified proteins or cell lysates. Mutation studies indicate that client binding, interactions between Hsp40 and Hsp70, plus ATP hydrolysis by Hsp70 are all required to promote conformational maturation of PR via the Hsp90 pathway.

Molecular chaperones are known for several vital roles in the cell. These include the folding of newly synthesized polypeptides, translocation through membranes, maturation and assembly of client proteins, prevention of aggregation, promotion of degradation, and response to cell stress (1). While there are several chaperone networks, we are concerned with the Hsp90 chaperoning pathway and its model client substrate, the progesterone receptor (PR).2

More than 100 substrates or client proteins for Hsp90 have been identified (2–4). These proteins include a diverse family of kinases, transcription factors, and cell cycle regulators, many of which are involved in cancer (5–7). Steroid receptors are a family of transcription factors chaperoned by Hsp90. Of these, PR and glucocorticoid receptor (GR) have served as models to study this pathway in detail (2, 3). PR exists in the cell as two isoforms, PR-A and PR-B (8), which are products of a single gene and differ only in that PR-A lacks the first 164 amino acids from the N terminus. These isoforms are both ligand-activated and dimeric in their activated states. Previous studies have not shown any differences in the chaperone association of PR-A and PR-B (9, 10). Chaperones appear to associate with the C-terminal domain of PR, where the steroid binding domain (SBD) is located. The primary amino acid sequence of the SBD is highly conserved throughout the steroid receptor family and a crystal structure of this domain of human PR bound to hormone is available (11).

Steroid receptors isolated from cell cytosol are associated with several chaperone and co-chaperone proteins, including Hsp90. When PR is stripped of its association with Hsp90, it loses its hormone binding ability in a time- and temperature-dependent manner. However, this activity can be restored or maintained in vitro through the actions of a minimum of 5 proteins plus ATP: Hsp40, Hsp70, Hop, Hsp90, and p23 (12, 13). In vivo, other co-chaperones are involved, including HIP (14, 15) and one of several immunophilins (2, 3, 17). Incubation with the aforementioned five proteins regenerates the hormone binding ability of PR and reconstitutes a heterocomplex resembling that originally found in vivo. The hormone-binding cleft is believed to be collapsed through hydrophobic interactions in the absence of ligand, thus the SBD requires a change in conformation to bind hormone (18). These five proteins work together to confer this conformational change.

The Hsp90 chaperoning pathway occurs in a series of steps that include the formation of multichaperone complexes with the steroid receptor. It was recently shown that Hsp40 binding is the first step in the PR chaperoning pathway (19). This binding is followed by Hsp70 association, which is ATP dependent (19). Hsp70 binds ATP at its N-terminal domain, and the J-domain of Hsp40 binds to Hsp70 and stimulates its ATPase activity, thus causing the tight association of Hsp70 with the substrate (20). The intermediate complex that follows is formed with the assistance of Hop, which is an adaptor protein that can simultaneously associate with Hsp70 and Hsp90 and modulate their activities (21–23). It is capable of transporting Hsp90 into the complex. Hop senses conformational changes in Hsp70 and Hsp90, mainly brought about by ATP binding, hydrolysis, and release (24–26). After the intermediate complex is formed, ATP is bound to Hsp90. p23 recognizes ATP-bound Hsp90 and promotes the dissociation of the intermediate complex (27) and

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1 To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Mayo Clinic, 200 First St. Southwest, Rochester, MN 55905. Tel: 507-284-8401; Fax: 507-284-2053; E-mail: toft.david@mayo.edu.

2 The abbreviations used are: PR, progesterone receptor; SBD, steroid binding domain; GR, glucocorticoid receptor; WT, wild type; AR, androgen receptor.
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a transition to a receptor complex that is able to bind hormone. The Hsp90 chaperone pathway is thus highly regulated by ATP binding and hydrolysis as well as by a series of interactions with chaperones and cofactors.

We hypothesized that the initial recognition of PR by Hsp40 in one specific site is necessary for the reconstitution of hormone binding to PR. Here we characterized the assembly of the initial complex, PR-Hsp40-Hsp70, and compared type I and type II Hsp40 proteins in their association with PR. We also addressed the need for interaction between Hsp70 and Hsp40 in the context of Hsp90 chaperoning to gain insight on the requirements for client protein recognition by this chaperoning system.

EXPERIMENTAL PROCEDURES

HeLa Cell Lysate Pull-downs—HeLa cells stably expressing PR-B (28) were grown to 70% confluency in MEM medium enriched with 5% fetal bovine serum (HyClone Laboratories, Logan, UT), 6 ng/µl insulin (Invitrogen), nonessential amino acids, penicillin, and streptomycin at 37 °C. The cells were trypsinized and washed in lysate buffer that included 20 mM Tris 7.5, 50 mM KCl, 1 mM EDTA, 2 mM dithiothreitol, 0.02% Nonidet P-40 and protease inhibitor mixture (Complete® EDTA-free from Roche). The cells were sonicated five times using the following cycle: 1 s pulse with 30 s rest on ice. The lysates were centrifuged in a microcentrifuge at maximum speed for 20 min. The lysate was added to protein A-Sepharose beads that were cross-linked using dimethylpimelimidate (DMP; Sigma-Aldrich) (29) with either PR-B antibody (PR6 (30)) or DjA1 antibody (Neomarkers® HDJ-2/DNAJ Ab-1). The lysate and beads were incubated on ice for 1.5 h, and washed four times with 1 ml of lysate buffer minus protease inhibitors. The beads were incubated with SDS sample buffer, boiled for 5 min at 90 °C, and proteins were resolved by 7.5% acrylamide SDS-PAGE.

Construction of Mutants—Human DjA1 D36N, Hsp70 K71M and Hsp70 R171H were prepared using the QuikChange® site-directed mutagenesis kit from Stratagene. These constructs were prepared in a pET23C vector (Novagen). The Ydj1 G315D mutant was a gift from Dr. Douglas Cyr. All mutant proteins were overexpressed in the BL21 DE3 PLysS E. coli strain with the addition of 1 mM isopropyl-β-D-thiogalactopyranoside at an A 600 nm between 0.6 and 0.8 for 3 h at room temperature.

Protein Purification—Human Hsp90, human Hsp70, Ydj1, DjA1, DjB1, Hop, and p23 were all expressed and purified as described previously (12, 19). The mutant DjA1 D36N and the mutant Ydj1 G315D were purified as described previously (31) with the following modifications. Bacterial lysates were fractionated by FPLC using first a Q-Sepharose, followed by UnoQ and Superdex 200. Both proteins were eluted early in the salt gradient of the ionic exchange columns. The Hsp70 mutations Hsp70 K71M and Hsp70 R171H were purified using the same procedure as the wild-type Hsp70 (19).

PR-A Expression and Cytosol Preparation—The procedure followed for the baculovirus-mediated expression of PR-A in SF9 cells included co-expression of p23, which enhances the expression of PR in a native state. This method has been described previously for GR expression (32). SF9 cells were co-infected with viral vectors coding for chicken PR-A and human p23 in the Recombinant Protein Expression Proteomics Core, The Cancer Center, Baylor College of Medicine. A final concentration of 10 mM glucose was added to the cultures at 24-h postinfection. The pellets were washed in phosphate-buffered saline prior to freezing at −80 °C. The cytosol was prepared from these pellets in the following manner. A pellet containing ~756 × 10^6 cells was thawed in 20 ml of lysis buffer (20 mM Tris, pH 7.5, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and protease inhibitors: Complete® EDTA-free). Cells were lysed by sonication, and the lysate was then centrifuged at 40,000 rpm for 1 h and stored at −80 °C. Prior to use, the cytosol was thawed on ice and adjusted to 500 mM KCl, 5 mM MgCl₂, 5 mM ATP. The lysate was salt-treated for 30 min on ice to dissociate chaperone proteins from PR.

PR Immuno-isolation—We used a mouse monoclonal antibody PR22 (IgG) against chicken PR described previously (30). Antibody resin was prepared by incubating PR22 with a slurry of protein A-Sepharose CL-4B (Amersham Biosciences) in PBS for 30 min at room temperature prior to use. Proportions were 7 µl of PR22 ascites for every 20 µl of resin volume. The conjugated resin was washed three times in PBS and then resuspended as a 1:1 slurry with ice-cold stripping buffer, and the immunoprecipitate was incubated for 30 min on ice with gentle resin suspension.

PR Binding Assays—PR resin pellets (20 µl) were suspended with 200 µl of cold reaction buffer (20 mM Tris-HCl, 50 mM KCl, 5 mM MgCl₂, 0.01% Nonidet P-40, and 2 mM dithiothreitol, pH 7.5) containing the specified amount of wild type or mutant Ydj1, DjA1, or DjB1. Reactions that assess the stimulation of Hsp70 binding to PR also include wild type or mutant Hsp70 plus 2 mM ATP. These reactions proceeded at 30 °C for 20 min; the samples were chilled on ice for 2 min, then washed four times with 1 ml of reaction buffer. The final samples were suspended in 20 µl of SDS sample buffer (2% SDS plus 5% mercaptoethanol), heated for 5 min at 95 °C, and analyzed by SDS-PAGE. The determination of association constants was performed as published previously (19).

Progesterone Receptor Reconstitution—PR resin (20 µl) was suspended with 200 µl of cold reaction buffer containing 20 µg of Hsp70, 5 µg of Ydj1, DjA1, or DjB1, 5 µg of Hop, 20 µg of Hsp90, 5 µg of p23, and 5 mM ATP unless otherwise noted. Incubation proceeded at 30 °C for 20 min. The samples were chilled on ice for 2 min and supplemented with 100 nM [³H]progesterone (American Radiolabeled Chemicals, Inc, St. Louis, MO, 50 Ci/mmol) plus 100 nM of unlabeled progesterone. The samples were incubated for 3 h on ice with gentle resin suspen-
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As seen in Fig. 1A, type I and type II Hsp40 proteins contain a J-domain at the N terminus of the protein (20, 33). In some cases the J domains of type I and II Hsp40 proteins are nearly identical and interchangeable (34, 35). This domain interacts with Hsp70 and is responsible for stimulating the ATPase activity of Hsp70. The zinc finger-like domain (ZFLD), unique to the type I Hsp40, is essential for proper recognition and delivery of substrates to Hsp70 (36, 37). The glycine-phenylalanine (G/F)-rich region is required for the proper function of these proteins (35), while the significance of the glycine-methionine (G/M) region, unique to the type II Hsp40 proteins, is unclear (38).

According to a recently published tertiary structure for both type I and type II Hsp40 (39), the dimer of the type I forms a horseshoe with the J domains close together in space, while the dimer of the type II forms a more elongated structure where the J domains are at a distance. These peculiarities, together with the non-conserved C terminus of the Hsp40 proteins (Fig. 1A), suggest that they would behave differently and recognize different client proteins (34). In the present study we compared the activities of two type I Hsp40 proteins, yeast Ydj1 (40) and human DjA1 (HDJ2, HSDJ, HSJ2, HSPF4, Dj-2) (41), and one type II protein, human DjB1 (HDJ1, dj1, HSJ2, HSPF1) (42), in the context of Hsp90 chaperoning (see Ref. 43 for classification and nomenclature). These were compared for their interaction with PR and ability to promote hormone-binding activity.

To study the cellular interaction of PR with Hsp40 proteins, we used a HeLa cell line that had been modified to express human PR-B (28). DjA1 and DjB1 are readily detected in the soluble cytosolic fraction of HeLa cells, as seen in Fig. 1B (lane 5), and both are potential PR-interacting proteins. Because the Hsp40 type that interacts with PR in the cell has not been identified, we tested for the association of DjA1 and/or DjB1 with PR in cell lysates. Using PR pull-down experiments (Fig. 1B) DjA1 showed a clear association with PR, whereas DjB1 was not detected (lane 5). When the pull-down was performed using antibody to DjA1, the co-isolation of PR was not observed (lane 6). This lack of detection may be explained by the much greater abundance of DjA1 over PR in the lysate. It is also possible that the DjA1 antibody interferes with the interaction. A pull-down of DjB1 was not able to detect PR association (data not shown).

Using the PR reconstitution system, we have previously shown that the binding of Ydj1 to PR is rapid, has a high affinity, can be assembled in vitro, and is independent of nucleotide and Hsp70 (19). Because these conditions have been well established for the yeast Hsp40, Ydj1, we tested the human Hsp40s, DjA1, and DjB1. As seen in Fig. 1C, both type I proteins Ydj1 and DjA1 interact with PR in the absence of other proteins (lanes 1 and 2) and stimulate Hsp70 association in the presence of ATP (lanes 4 and 5). DjB1 does not visibly interact with PR (lane 3); yet, it is able to promote the association of Hsp70 with PR (lane 5). This also results in some binding of DjB1 in the complex which may represent its binding to Hsp70, PR, or both. These in vitro results agree with the in vivo analysis above in that DjA1 binds readily to PR in a pull-down experiment but DjB1 does not. However, the in vitro results suggest that DjB1 may bind transiently to PR to promote Hsp70 binding.

Both Type I and II Hsp40 Proteins Are Able to Promote Hsp90 Chaperoning—In Fig. 2A we depict the hormone binding profile of PR when increasing amounts of either Ydj1 or DjA1 are used in the reconstitution reaction. In addition to Hsp40, this reaction includes purified Hsp90, Hsp70, Hop, and p23. These proteins work together to form complexes with PR to promote hormone binding through the opening of the hormone-binding site. Both Hsp40 proteins are able to promote PR chaperoning to the hormone binding state, although there are some differences in the response profiles. Ydj1 is somewhat more potent than DjA1, but it is slightly inhibitory when in excess. Fig. 2B represents the complex formation of the reaction described above. Protein association occurs in a timely and organized manner (44–46). Generally, the presence of proportional quantities of Hsp90 and p23 is indicative of a mature complex that is able to bind hormone. Both Hsp40 proteins are able to promote Hsp90 binding to PR. The main difference between a
reconstitution that is triggered by DjA1 or Ydj1 is the presence of Hsp70 in the isolated complexes, which is more prominent throughout when Ydj1 is used. This difference is subtle and does not appear to affect substantially the hormone binding of PR although this may account for the inhibitory effect seen when excess Ydj1 is used. The association of Ydj1 can be seen in this gel while association of DjA1 is not, because it is not resolved from the antibody heavy chain in this gel system (10% acrylamide gels). In experiments performed with beads containing cross-linked antibody or 7.5% acrylamide gels, the amounts of DjA1 and Ydj1 seen associated to the PR complexes are comparable (data not shown).

Surprisingly, whereas DjB1 binding to PR is not observed using our methods, it is able to promote PR chaperoning in a similar manner to Ydj1 and DjA1. Fig. 2, C and D show that, when adding increasing amounts of DjB1 to a reconstitution reaction, we are able to achieve hormone binding by PR. As seen in Fig. 2C, the maximum potency of DjB1 is less than that of Ydj1, but very similar to that of DjA1 (Fig. 2A). In Fig. 2D, both the association of Hsp90 and Hsp70 are similar whether Ydj1 or DjB1 is used although the association of Hsp70 in the presence of Ydj1 is higher, as seen previously in Fig. 2B.

Hsp40 Binding to PR Is Specific and Limited—DjA1 and Ydj1 are able to bind similarly to PR, promote Hsp70 binding, and promote hormone binding of PR when used in a reconstitution assay. The PR binding affinities for these two proteins were compared as shown in Fig. 3A. The \( K_d \) for Ydj1 is 225 nM, while the \( K_d \) for DjA1 is 278 nM. Thus, these two proteins have similar affinities for PR. In an earlier study, a higher affinity of Ydj1 for PR was reported with a \( K_d \) of 77 nM (19). The presence of detergent (0.01% Nonidet P-40) in the present study apparently lowers the affinity for Ydj1 binding somewhat, but enhances the efficiency of PR chaperoning overall. The Scatchard analysis in Fig. 3A depicts a linear relationship between the binding of PR and each Hsp40, Ydj1, and DjA1. Therefore, a single type of binding site is expected from type I Hsp40. A binding affinity constant was not calculated for DjB1 because no measurable binding of DjB1 to PR was detected.

Our laboratory has previously calculated the stoichiometry of Ydj1 binding to PR in the early complex as \( \sim 1:1 \) (19). Since the PR exists as a monomer in chaperone complexes, Ydj1 may also bind PR in a monomeric state or the Ydj1 dimer may be able to bind two PR molecules. This stoichiometry argues for one binding site on PR for Hsp40. Therefore, a single type of binding site on PR for each Hsp40, Ydj1, and DjA1.

FIGURE 2. Ydj1, DjA1, and DjB1 are able to reconstitute the hormone binding activity of PR. Reaction samples (200 \( \mu l \)) contained a mix of 5 mM ATP and four purified proteins as indicated under "Experimental Procedures" and increasing amounts, as specified, of Hsp40. A shows progesterone bound to the receptor for reactions containing increasing amounts of Ydj1 or DjA1. B, complex formation when performing a reconstitution reaction using increasing amounts of WT Ydj1 (lanes 1–7) or WT DjA1 (lanes 8–15) shown here in a Coomassie-stained 10% SDS-PAGE. C, bound progesterone is shown when using Ydj1 or DjB1. D shows complex formation when performing a reconstitution reaction using increasing amounts of Ydj1 (lanes 1–7) or DjB1 (lanes 8–15). Legend: H-chain, heavy chain of PR22 antibody, and L-chain, light chain of PR22 antibody.
Hsp40. The same displacement or competition pattern occurs in the reverse experiment when DjA1 is used to occupy the site for Ydj1 binding on PR (data not shown).

DjB1 does not stably bind PR when using our methods and it is not an effective competitor for Ydj1 binding. Thus, we considered a functional approach. We used a dominant negative Hsp40, DjA1 D36N, which binds to PR (see Table 1 and Fig. 5), is unable to bind Hsp70 (47) and is also unable to reconstitute the hormone binding ability of PR (Fig. 5). Fig. 4A shows a competition experiment where Hsp90, Hsp70, Hop, DjA1, and p23 were used in a reconstitution assay. We then added increasing amounts of DjA1 D36N to this reaction. We show here that increasing amounts of DjA1 D36N inhibit the maturation of PR in the presence of DjA1 WT, which is able to bind to PR with high affinity in a single site. We then performed a similar experiment using DjB1. Fig. 4B shows a very similar inhibition of DjB1 by DjA1 D36N. These data argue that Hsp40 proteins, both type I and II, bind PR in a single specific site and that this site must be available for the activity of DjB1 and DjA1.

**Conditions for the Association of Hsp40 to PR**—We have proposed that the chaperoning of PR is initiated by the binding of Hsp40, which can also bind and recruit Hsp70 (19). This interaction leads to ATP hydrolysis by Hsp70 and its tight binding to PR. This sequence of events is based on previous models derived from studies on a limited number of peptides and proteins (20, 48, 49), and whether all of these events are needed for chaperoning PR or other Hsp90 clients has not been established. To investigate the requirements for Hsp40 in the formation of the initial complex of the Hsp90 chaperoning pathway, we constructed point mutations in the type I Hsp40 proteins, Ydj1 and DjA1 (Table 1). First, we chose to eradicate Hsp70 binding and the stimulation of Hsp70 ATPase activity by the J-domain using DjA1 D36N. This mutation is located on the histidine-proline-aspartic acid (HPD) motif, which is required for Hsp70 binding and the stimulation of ATPase activity (20, 47). The second property we focused on was the proper recognition of substrate by Hsp40. For this purpose we utilized Ydj1 G315D (50). This mutation is located in the C-terminal domain of Ydj1, the least conserved region of the Hsp40 family of proteins. It has an intact J domain, and is capable of fully activating the ATPase activity of Hsp70 (data not shown). This protein has been characterized for its lack of substrate recognition, because studies have shown that it renders Ydj1 unable to suppress the aggregation of rhodanese and to complex with unfolded luciferase (36), and it depresses GR transcriptional activity in yeast (50).

To characterize the requirement for Hsp40-Hsp70 interaction, and proper identification of substrate in the formation of the initial complex, we tested these two mutations in a PR binding assay. First, we looked at the binding of DjA1 D36N and Ydj1 G315D to PR. Fig. 5A shows that DjA1 D36N was able to recognize and bind PR at a similar level as the wild-type DjA1. However, unlike the wild-type DjA1, it was not able to stimulate

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**TABLE 1**

| Chaperone | Mutation | Net result | Ref. |
|-----------|----------|------------|-----|
| DjA1      | D36N     | Cannot stimulate Hsp70 ATPase activity (47) |    |
| Ydj1      | G315D    | Unable to effectively recognize substrate (50) |    |
| Hsp70     | K71M     | ATPase mutant (51) |    |
|           | R171H    | Unable to interact with Hsp40 (52) |    |

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**FIGURE 3.** Ydj1 and DjA1 have similar affinities for PR and are able to compete for a binding site. A shows a Scatchard plot of Hsp40 binding to PR. The representative set of data shown in the graph are as follows: for DjA1, the equation of the line was $y = -3.6035x + 0.9586$, $R^2 = 0.9546$ and for Ydj1 the equation was $y = -4.407x + 0.739$, $R^2 = 0.8636$. The average $K_d$ for DjA1 was 279.3 nM ($n = 3$) and for Ydj1 was 221.0 nM ($n = 3$). B, each 200-μl sample of 5 μg of DjA1 and increasing amounts of Ydj1 were incubated with PR to show competition. C, densitometry measurements of B are presented in graphic form.
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Fig. 4. DjB1 and DjA1 require a common site on PR. For each experiment (n = 3) the dotted line represents the radioactive counts in the absence of any Hsp40, and the solid line after incubation with 5 μg of DjA1 D36N. In A, each sample contained 5 μg of DjA1 and increasing amounts of DjA1 D36N (closed circles). In B, each sample contained 5 μg of DjB1 and increasing amounts of DjA1 D36N (open circles).

The formation of a complex containing Hsp70, Hsp40, and PR (lanes 8 and 11). Therefore, interactions between Hsp40 and Hsp70 are required for the formation of an initial tripartite complex.

Fig. 5A also illustrates wild-type Ydj1 and Ydj1 G315D binding to PR. Lanes 3 and 6 show the nonspecific binding of the proteins to the immuno-resin. Note the appearance of significant background binding with Ydj1 G315D, consistent with a protein that is lacking substrate binding specificity. Ydj1 G315D stimulated some Hsp70 binding (lane 5), but not as efficiently as the wild-type Ydj1 (lane 2). We hypothesize that this instability is because of poor recognition of a binding site on PR by Ydj1 G315D. In support of this, we have shown that Ydj1 G315D is not able to compete with DjA1 for PR binding (data not shown).

To functionally test the importance of the initial complex in the Hsp90 chaperoning of PR, we used DjA1 D36N and Ydj1 G315D in the in vitro reconstitution system and measured the resulting progesterone binding activity. These reactions were performed by adding standard amounts of Hsp90, Hsp70, Hop, p23, 5 mM ATP, reaction buffer, and either 10 μg of wild-type Ydj1, DjA1 D36N, or Ydj1 G315D. Neither mutant has any activity for supporting PR chaperoning and hormone binding (Fig. 5B). Fig. 5C illustrates the complex formation during the reconstitution reactions. Generally, the amount of Hsp90 associated with the receptor correlates with the amount of hormone binding activity of PR. In the case of the mutant Hsp40s, only traces of Hsp90 can be seen associated with PR. When using Ydj1 G315D, some of the mutant and Hsp70 are present in the complex, but these do not lead to the recruitment of Hsp90. This was not improved by using higher amounts of the mutant Ydj1 (results not shown). DjA1 D36N was present in the PR complex as expected, but it was unable to recruit Hsp90 into the complex. Taken together, these findings indicate that proper recognition of substrate by Hsp40, and its interaction with Hsp70 are necessary for the subsequent recognition of PR by Hsp90.

Conditions for the Formation of an Hsp40-Hsp70-PR Complex—Table 1 enumerates the mutations we used to investigate the role of Hsp70. Previously published data on the structural characteristics of the ATPase domain of Hsp70 have identified residue lysine 71 as essential for ATP hydrolysis (51). This positively charged residue interacts with the negative γ-phosphate of ATP and helps drive its release. The McKay group changed this residue from lysine to methionine, glutamic acid, or alanine. They found that all three amino acid changes removed all ATPase activity from Hsp70 (51). We investigated K71M in this report, which is capable of binding ATP in a similar manner as Hsp70 WT. This ability was assessed as part of the purification protocol where the protein was affinity-purified using ATP-agarose (data not shown).

The second mutation used was Hsp70 R171H (52, 53). Residue Arg171 in human Hsp70 is homologous to residue Arg167 in the bacterial Hsp70, DnaK. This arginine was found to be required for binding of the corresponding Hsp40, DnaJ, through a screen performed by Gross and co-workers (52) to restore binding to DnaJ D35N. They found that DnaK R167H was unable to interact with DnaJ WT, and that Asp35 of DnaJ requires interaction with Arg167 for the proper ATP hydrolysis and change into a polypeptide binding conformation of DnaK.

Fig. 6, A and B show that, unlike Hsp90 WT, the mutants Hsp70 K71M and Hsp70 R171H exhibit some background binding to PR in the absence of Hsp40. Hsp70 K71M binds poorly to PR in the presence of Hsp40 since it lacks ATPase activity (51) (Fig. 6A, lanes 4 and 8). Hsp70 R171H cannot be stimulated to bind PR when Hsp40 is present, because it is not able to interact with Hsp40 (52) (Fig. 6B, lanes 4 and 8), and it is apparently unable to interact with PR productively on its own. Hsp70 R171H has intact ATPase activity, which cannot be stimulated by Hsp40 (data not shown). Hence, the formation of an initial complex requires Hsp40 interaction with Hsp70 plus the ATPase activity of Hsp70.

These two Hsp70 mutants were tested in progesterone binding assays in vitro with immuno-isolated PR (Fig. 6, C and D). Neither of the Hsp70 mutants was able to support the chaperoning of PR to a hormone binding state (Fig. 6C). Hsp70 K71M was present in the PR complex at a level comparable to Hsp70 WT, unlike its low level of interaction shown in Fig. 6A. We have found the binding of this mutant to be somewhat variable, but it may be influenced by the additional chaperone proteins used in the experiment in Fig. 6D or by the ratio of ATP/ADP that might be generated under these conditions. However, the binding of Hsp70 K71M did not lead to the binding of Hop, Hsp90, and p23. This indicates a need for ATP hydrolysis which...
is probably required both for productive binding to PR and for interaction with the co-chaperone Hop (24). Similar results were observed using either Hsp70 K71A or Hsp70 K71E (not shown). Only a low level of Hsp70 R171H was present in the PR complex and this did not promote any binding of Hop, Hsp90, or p23. We can thereby conclude that both the ATPase activity of Hsp70 and the interaction of Hsp70 with Hsp40 are essential for the maturation of PR.

**DISCUSSION**

The results shown here demonstrate crucial roles for the full activities of human Hsp40 and Hsp70 in the early stages of PR chaperoning. In previous studies involving Hsp40, Hsp70, and PR, the complete human chaperone system had not been used, and the requirement of their full activities had not been addressed (12, 19). This is therefore, the first report addressing this problem together with a comparison of how type I and type II Hsp40 proteins affect PR maturation in the context of the Hsp90 chaperoning pathway. Both type I and type II Hsp40 proteins are effective in this chaperoning system, but they must be able to interact with Hsp70 and PR. Hsp70 must be able to interact with Hsp40 and PR, and to hydrolyze ATP. Proteins that lack any of these activities are unable to support the chaperoning process and, in some cases, they can block progression of PR chaperoning.

The differences between the functions of type I and type II Hsp40 proteins are fairly well established in yeast (54), where the type II (Sis1) is essential for survival (55), and the type I (Ydj1) is not (40). Overexpression of Sis1 can compensate for the slow growth defects in YDJ1-null yeast while the absence of Sis1 causes lethality in yeast and cannot be rescued by Ydj1 (40, 55). Using a yeast system, Caplan and co-workers (56) have studied the Hsp40 requirement for in vivo chaperoning of the androgen receptor (AR). They found that DjA1, but not DjB1, was able to suppress the hormone binding deficiency of AR in a YDJ1-null strain of yeast. While not directly comparable to the present study, their results indicate a specific need for type I Hsp40 in chaperoning AR. Conversely, our in vitro studies have found that PR is able to bind hormone using either DjA1/Ydj1 or DjB1 in our reconstitution system. The cellular actions of these proteins are not well understood (20, 34, 37, 57). However, a DjA1−/− mouse has been generated (58). Female mice grow normally, are fertile and appear to have normal mammary gland development. The male mice, on the other hand, have severe spermatogenesis defects, are infertile, and on average are smaller than their normal male counterparts, having an average size similar to that of normal females. The main defect of these mice is aberrant AR signaling due to accumulated AR. The reason for this accumulation and apparent AR hyperactivity is unknown. It may be that DjA1 is more important in the turnover of AR than in its activation. This phenotype correlates somewhat with our data, because the normal phenotype of the DjA1−/− female mice indicate that PR is functioning normally in these mice, and we have shown that PR is able to function well with either DjA1 or DjB1, thus suggesting that DjB1 may be able to compensate for the lack of DjA1. However, there is another ubiquitous type I protein, DjA2 (DNAJA2, dj3, rjd2, crp3, DNNJ3, HIRIP4) (59, 60), which may also compensate for the lack of DjA1 (61). The AR

**FIGURE 5.** Hsp40 type I activity requires interaction with PR and Hsp70. In A, each reaction sample (200 μl) contained 10 μg of Hsp40 protein as indicated and was incubated with or without PR at 30 °C for 10 min in reaction buffer supplemented with 2 mM ATP and/or 10 μg of Hsp70 as depicted. In all cases the proteins were isolated and are shown here resolved in a Coomassie-stained 7.5% SDS-PAGE. B and C, Ydj1 WT (lane 1), Ydj1 G315D (lane 2), DjA1 WT (lane 3), or DjA1 D36N (lane 4) or no Hsp40 (lane 5), or no PR + Ydj1 WT (lane 6) were used in a reconstitution assay containing Hsp90, Hsp70, Hop, p23, and 2 mM ATP as described under “Experimental Procedures.” In C, the graph shows bound progesterone in each case as noted (n = 3).
Roles of Hsp40 and Hsp70 in Hsp90 Chaperoning

One of the main roles of Hsp40 proteins is the regulation of the ATPase activity of Hsp70. In vitro, both, Ydj1 and Sis1 can stimulate the ATPase activity of Hsp70, bind to denatured proteins and cooperate with Hsp70 in the folding of firefly luciferase (54). However, it was somewhat surprising that the two human proteins DjB1 and DjA1, have very comparable activities for facilitating the binding of Hsp70 to PR and supporting the chaperoning process. Not only do they differ in domain composition and tertiary structure (39), but also in their interaction with PR. While DjA1 binds tightly to PR in the absence of other proteins and in vivo, the binding of DjB1 to PR was not evident and this interaction could only be indicated indirectly.

Whether PR ever associates with DjB1 in the cell, remains unknown. However, our in vitro results show that DjB1 is capable of stimulating Hsp70 binding to PR and progression through the Hsp90 chaperoning pathway. Evidently, a sustained, high affinity binding of Hsp40 to PR as seen with Ydj1 (19) and DjA1, is not necessary, and one could argue that Hsp40 interaction with Hsp70, rather than with PR, is of primary importance to PR chaperoning.

To assess this question we utilized the Hsp70 mutant R171H, which is unable to interact with Hsp40. We found that it is an inactive protein, and thus prevents the progression of the Hsp90 pathway, indicating that Hsp70 must interact with Hsp40. Additionally, Hsp40 interaction with PR remains an important priming step in the pathway because the Ydj1 mutant, G315D, is inactive in PR chaperoning even though it has an intact J domain, and it is comparable to WT Ydj1 in stimulating the ATPase activity of Hsp70. Also, the J domain alone is not sufficient for this process. We have found that the J domain fragment of DjB1 (residues 1–76) is totally inactive (results not shown) and it has very little ability to stimulate Hsp70 ATPase activity, as previously reported (62), because additional domains of Hsp40 proteins are essential to the process. These results argue that interaction of Hsp40 with PR is essential. A requirement for the Hsp40 priming step in a single site is supported by use of the inactive DjA1 mutant, D36N, which was able to block DjB1 activity, presumably by occupying a site on the receptor that was required for DjB1 interaction.

The role of Hsp40 in the chaperoning of various Hsp90 clients may differ significantly. For example, while evidence supports Hsp40 binding as the first step in the recognition of PR, Hsp70 is believed to be the first protein to recognize GR (63). When Hsp90 is stripped from isolated GR, the receptor rapidly converts to an alternate conformational state that lacks hormone binding activity (63). Conversely, PR free of Hsp90 maintains its hormone binding ability if kept on ice. The hormone binding function will be lost over time at 30 °C if no chaperones are present (19, 44). Thus, the Hsp90-free conformations of GR

D. Toft, unpublished observation.
and PR differ in stability and, perhaps, in conformation of the hormone binding domain.

On the other hand, Hu and co-workers (16) found that DjB1 was the favored Hsp40 protein for facilitating the in vitro chaperoning of the reverse transcriptase of hepatitis B virus to its functional state. Ydj1 showed some activity in this system, but DjA1 was inactive. Also, as mentioned earlier, AR requires DjA1 and not DjB1 for full chaperoning through the Hsp90 pathway (56, 58). Thus, it appears that various Hsp90 client proteins can differ in their Hsp40 requirements.

The Hsp90 client proteins are diverse and do not appear to be identifiable through any common sequence or structural properties, but there is little information on the initial site(s) of interaction on Hsp90 clients. Many clients contain a region of structural instability that may provide the ability to assume multiple conformational states needed for their function or regulation. Thus, specialized chaperoning through Hsp90 may be a necessary cost for this structural flexibility. Much more information is needed on the sites and components involved in the initial chaperone interaction of Hsp90 clients before a generalized view of this process can be obtained.

The present results support a model for PR chaperoning that begins with the binding of Hsp40 to a specific site on the PR that rapidly leads to recruitment of Hsp70-ATP. Hsp70-ATP may bind very transiently to the PR yet, ATP hydrolysis, triggered by interaction with Hsp40 and the client (19), alters Hsp70 to a state that binds tightly to PR. Hsp70 ATP hydrolysis must be stimulated by Hsp40 or Hsp70 will not associate tightly with PR and chaperoning will not take place, as supported by our experiments using the Hsp70 mutants R171H and K71M. These early interactions lead directly to recruitment of Hop and Hsp90 which, as a complex, can bind to Hsp70-ADP, but not to Hsp70-ATP (24). However, Hsp40 and Hsp70 can function as a cycle or an iterative process (20) and it is possible that more than one cycle of interaction is needed before the recruitment of Hop and Hsp90 (19, 27). Studies with GR chaperoning indicate a sustained need for Hsp70 throughout the Hsp90 chaperoning process (63) suggesting multiple cycles of Hsp70 interactions. However, it should be noted that the mature PR complex is dynamic and is thought to dissociate and re-assemble continuously to maintain a high proportion of active PR. Once the intermediate complex including Hop and Hsp90 is formed, this can proceed to the binding of ATP to Hsp90 and conversion to a mature PR complex that also contains p23 and other co-chaperones. Further studies are needed to clarify these latter interactions.

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REFERENCES
1. Young, J. C., Agashe, V. R., Siegers, K., and Hartl, F. U. (2004) Nat Rev Mol. Cell. Biol. 5, 781–791
2. Pratt, W., and Toft, D. (2003) Exp. Biol. Med. (Maywood) 228, 111–133
3. Pratt, W. B., Galigniana, M. D., Morishima, Y., and Murphy, P. J. (2004) Essays Biochem. 40, 41–58
4. Zhao, R., Davey, M., Hsu, Y., Kaplanek, P., Tong, A., Parsons, A., Krogan, N., Cagney, G., Mai, D., Greenblatt, J., Boone, C., Emili, A., and Houry, W. (2005) Cell 120, 715–727
5. Whitesell, L., and Lindquist, S. (2005) Nat. Rev. Cancer 5, 761–772
6. Workman, P. (2004) Cancer Lett. 206, 149–157
7. Chiosis, G., Vilenchik, M., Kim, J., and Solit, D. (2004) Drug Discov. Today 9, 881–888
8. Conneely, O., Maxwell, B., Toft, D., Schrader, W., and O’Malley, B. (1987) Biochem. Biophys. Res. Commun. 149, 493–501
9. Schowalter, D., Sullivan, W., Mahle, N., Dobson, A., Conneely, O., O’Malley, B., and Toft, D. (1991) J. Biol. Chem. 266, 21165–21173
10. Smith, D., Schowalter, D., Kost, S., and Toft, D. (1990) Mol. Endocrinol. 4, 1704–1711
11. Williams, S., and Sigler, P. (1998) Nature 393, 392–396
12. Kosano, H., Stensgaard, B., Charlesworth, M., McMahon, N., and Toft, D. (1998) J. Biol. Chem. 273, 32973–32979
13. Dittmar, K. D., Banach, M., Galigniana, M. D., and Pratt, W. B. (1998) J. Biol. Chem. 273, 7358–7366
14. Prapanich, V., Chen, S., and Smith, D. F. (1998) Mol. Cell. Biol. 18, 944–952
15. Prapanich, V., Chen, S., Nair, S. C., Rimerman, R. A., and Smith, D. F. (1996) Mol. Endocrinol. 10, 420–431
16. Hu, J., Flores, D., Toft, D., Wang, X., and Nguyen, D. (2004) J. Virol. 78, 13122–13131
17. Smith, D. F. (2004) Cell Stress Chaperones 9, 109–121
18. Gee, A., and Katzenellenbogen, J. (2001) Mol. Endocrinol. 15, 421–428
19. Hernandez, M., Chadli, A., and Toft, D. (2002) J. Biol. Chem. 277, 11873–11881
20. Fan, C., Lee, S., and Cyr, D. (2003) Cell Stress Chaperones 8, 309–316
21. Chen, S., and Smith, D. (1998) J. Biol. Chem. 273, 35194–35200
22. Lassle, M., Blatch, G., Kundra, V., Takatori, T., and Zetter, B. (1997) J. Biol. Chem. 272, 1876–1884
23. Smith, D., Sullivan, W., Marion, T., Zaitsev, K., Madden, B., McCormick, D., and Toft, D. (1993) Mol. Cell. Biol. 13, 869–876
24. Hernandez, M., Sullivan, W., and Toft, D. (2002) J. Biol. Chem. 277, 38294–38304
25. Johnson, B., Schumacher, R., Ross, E., and Toft, D. (1998) J. Biol. Chem. 273, 3679–3686
26. Odunuga, O., Longshaw, V., and Blatch, G. (2004) BioEssays 26, 1058–1068
27. Sullivan, W., Owen, B., and Toft, D. (2002) J. Biol. Chem. 277, 45942–45948
28. Chadli, A., Graham, J., Abel, M., Jackson, T., Gordon, D., Wood, W., Felts, S., Horwitz, K., and Toft, D. (2006) Mol. Cell. Biol. 26, 1722–1730
29. Sisson, T., and Castor, C. (1990) J. Immunol. Methods 127, 215–220
30. Sullivan, W., Beito, T., Proper, J., Krco, C., and Toft, D. (1986) Endocrinology 119, 1549–1557
31. Caplan, A., Tsai, J., Casey, P., and Douglas, M. (1992) J. Biol. Chem. 267, 18890–18895
32. Morishima, Y., Kanelakis, K. C., Murphy, P. J., Lowe, E. R., Jenkins, G. J., Osawa, Y., Sunahara, R. K., and Pratt, W. B. (2003) J. Biol. Chem. 278, 48754–48763
33. Hennessy, F., Nicoll, W., Zimmermann, R., Cecheta, M., and Blatch, G. (2005) Protein Sci. 14, 1697–1709
34. Fan, C., Lee, S., Ren, H., and Cyr, D. (2004) Mol. Cell. Biol. 15, 761–773
35. Yan, W., and Craig, E. (1999) Mol. Cell. Biol. 19, 7751–7758
36. Lu, Z., and Cyr, D. (1998) J. Biol. Chem. 273, 5970–5978
37. Fan, C., Ren, H., Lee, P., Caplan, A., and Cyr, D. (2005) J. Biol. Chem. 280, 695–702
38. Lee, S., Fan, C., Younger, J., Ren, H., and Cyr, D. (2002) J. Biol. Chem. 277, 21675–21682
39. Borges, J. C., Fischer, H., Craievich, A. F., and Ramos, C. H. (2005) J. Biol. Chem. 280, 13671–13681
40. Caplan, A., and Douglas, M. (1991) J. Biol. Chem. 164, 609–621
41. Davis, A., Alevy, Y., Chelliah, A., Quinn, M., and Mohanakumar, T. (1998) Int. J. Biochem. Cell Physiol. 30, 1203–1211

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42. Raabe, T., and Manley, J. L. (1991) Nucleic Acids Res. 19, 6645
43. Ohtsuka, K., and Hata, M. (2000) Cell Stress Chaperones 5, 98–112
44. Smith, D. (1993) Mol. Endocrinol. 7, 1418–1429
45. Smith, D., Whitesell, L., Nair, S., Chen, S., Prapapanich, V., and Rimerman, R. (1995) Mol. Cell. Biol. 15, 6804–6812
46. Chen, S., Prapapanich, V., Rimerman, R. A., Honore, B., and Smith, D. F. (1996) Mol. Endocrinol. 10, 682–693
47. Suh, W., Burkholder, W., Lu, C., Zhao, X., Gottesman, M., and Gross, C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15223–15228
48. Kim, S., Sharma, S., Hoskins, J., and Wickner, S. (2002) J. Biol. Chem. 277, 44778–44783
49. Han, W., and Christen, P. (2003) J. Biol. Chem. 278, 19038–19043
50. Kimura, Y., Yahara, I., and Lindquist, S. (1995) Science 268, 1362–1365
51. O’Brien, M., Flaherty, K., and McKay, D. (1996) J. Biol. Chem. 271, 15874–15878
52. Suh, W., Lu, C., and Gross, C. (1999) J. Biol. Chem. 274, 30534–30539
53. Landry, S. (2003) Biochemistry 42, 4926–4936
54. Lu, Z., and Cyr, D. (1998) J. Biol. Chem. 273, 27824–27830
55. Luke, M., Sutton, A., and Arndt, K. (1991) J. Cell Biol. 114, 623–638
56. Fliss, A., Rao, J., Melville, M., Cheetham, M., and Caplan, A. (1999) J. Biol. Chem. 274, 34045–34052
57. Cyr, D., and Douglas, M. (1994) J. Biol. Chem. 269, 9798–9804
58. Terada, K., Yomogida, K., Imai, T., Kiyonari, H., Takeda, N., Kadomatsu, T., Yano, M., Aizawa, S., and Mori, M. (2005) EMBO J. 24, 611–622
59. Andres, D. A., Shao, H., Crick, D. C., and Finlin, B. S. (1997) Arch. Biochem. Biophys. 346, 113–124
60. Edwards, M. C., Liegeois, N., Horecka, J., DePinho, R. A., Sprague, G. F., Jr., Tyers, M., and Elledge, S. J. (1997) Genetics 147, 1063–1076
61. Terada, K., and Mori, M. (2000) J. Biol. Chem. 275, 24728–24734
62. Karzai, A., and McMacken, R. (1996) J. Biol. Chem. 271, 11236–11246
63. Murphy, P. J., Morishima, Y., Chen, H., Galigniana, M. D., Mansfield, J. F., Simons, S. S., Jr., and Pratt, W. B. (2003) J. Biol. Chem. 278, 34764–34773