HSP40 Binding Is the First Step in the HSP90 Chaperoning Pathway for the Progesterone Receptor*

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The progesterone receptor (PR) can be isolated in its native conformationable to bind hormone, yet its ligand-binding domain rapidly loses its activity at elevated temperature. However, an in vitro chaperoning system consisting of five proteins (HSP40, HSP70, HOP, HSP90, and p23) with ATP is capable of restoring this function. The first step of this chaperoning mechanism is usually thought to be the binding of HSP70 to PR. Our findings here show that the binding of HSP40 to PR is, instead, the first step. HSP40 binding occurred rapidly and was not dependent on ATP or other proteins. The stoichiometry of HSP40 to native PR in these complexes was ∼1:1. HSP40 bound specifically and with a high affinity to native PR (Kd = 77 nM). The binding of HSP40 to PR was sustained and did not interact in the highly dynamic fashion that has been observed previously for HSP90 in this system. The HSP40-PR complex could be isolated as a functional unit that could, after the addition of the other chaperones, progress to a PR complex capable of hormone binding. These results indicate that HSP40 initiates the entry of PR into the HSP90 pathway.

A model system that has been central to the study of the HSP90 chaperoning pathway has been the use of the avian progesterone receptor (PR) (1–3). In the absence of hormone, PR is inactive, but able to bind and respond to progesterone. This inactive receptor is in a protein complex that contains the heat shock proteins HSP90 and HSP70, two proteins that bound the immunosuppressant drug FK506 (FKBP52 and FKBP51), and p23 (4). In some circumstances, an immunophilin protein that binds cyclosporin A (cyclophilin-40) and protein phosphatase-5 have also been shown to be part of inactive steroid receptor complexes (5, 6). This chaperone complex does not form through a simple association of the aforementioned proteins, but through an elaborate process that requires several additional proteins as well as ATP hydrolysis (7). Other proteins known to be involved in PR chaperone complex formation are three HSP70 co-chaperones: HSP40, HIP, and HOP (1–3). An in vitro chaperoning system has thus been used that consists of five proteins (HSP40, HSP70, HOP, HSP90, and p23) with ATP. Although this system lacks HIP and the immunophilins, it is capable of assembling a PR complex wherein the receptor has an active ligand-binding domain.

From studies with rabbit reticulocyte lysate (9) and recent studies with purified proteins (10), a multistep model pathway for PR complex assembly has emerged. There have been parallel studies using the glucocorticoid receptor (GR) instead of PR that further substantiate the validity of this model (11, 12). The binding of HSP70 to PR or GR is usually considered to be the first step in the chaperoning pathway (13, 14), followed by the recruitment of HOP and HSP90. This eventually leads to the formation of a mature complex containing mainly HSP90 and its co-chaperone p23, with only minor amounts of HSP70 and HOP. Genetic studies in yeast (15, 16) and biochemical evidence using purified proteins (10, 17) show that HSP40 is also involved in the formation of the early complex. Indeed, HSP40 is clearly involved in the HSP90 pathway in vivo, as mutants of the yeast HSP40 homolog Ydj1 affect the activities of both steroid receptors and another HSP90 client protein (pp60src) in yeast cells (15, 16, 18, 19). HSP40 binds to the ATPase domain of HSP70 and stimulates its ATP hydrolysis activity (20–23), which is needed for high affinity binding of HSP70 to substrates. However, in some other systems, HSP40 has been shown also to bind to substrate proteins, where it may act to target the chaperoning activity of HSP70 (24).

In PR complex assembly, the binding and functioning of HSP90, HOP, and p23 are dramatically reduced in the absence of HSP70 or HSP40, but these two proteins can bind to PR in the absence of HOP, HSP90, and p23 (10). Earlier studies with GR chaperoning did not indicate a requirement for HSP40 (25). However, HSP40 was more recently shown to be present as a contaminant in preparations of HSP70 and HSP90, and it is clearly needed for optimal chaperoning of GR (14, 17). These studies suggest that HSP70 and HSP40 are not only central to the chaperoning pathway, but may indeed be the first proteins to interact with PR. We sought to distinguish between the roles of HSP40 and HSP70 in the early complex so as to delineate the order of events concerning protein binding and release at the beginning of the PR chaperoning pathway. We now report the assembly of functional early PR complexes consisting of HSP40 and PR in the absence of HSP70. We describe the affinity, stoichiometry, and dynamics of these early PR complexes.

EXPERIMENTAL PROCEDURES

Materials—Mouse monoclonal (IgG) antibody PR22 made against avian PR has been described previously (26). PR is expressed from a single gene as two distinct molecular forms: PR-A (72 kDa) and PR-B (86 kDa). This antibody recognizes both the PR-A and PR-B isoforms. Approximately equal amounts of these receptor isoforms exist in chick oviduct cytosol extracts.

Protein Purification—A bacterial expression system for Ydj1 (GenBankTM/EBI accession number X56560) was generously supplied by Dr.
Avrom Caplan and has been described previously (27). Bacterial lysates were fractionated by DEAE-cellulose column chromatography, followed by hydroxylapatite column chromatography. Additional purification was achieved by fractating the pool from hydroxylapatite on a MonoQ 10/10 FPLC column (Amersham Biosciences) that was eluted with a linear gradient of 0 to 0.5 M KCl. The preparation was 94% pure as assessed by densitometry of SDS-polyacrylamide gels.

Human HOP (GenBank™/EBI accession number M86752) was expressed in bacteria was prepared essentially as described previously (30). Bacterial lysates were fractionated by DEAE-cellulose column chromatography, followed by hydroxylapatite column chromatography. Additional purification was achieved by fractating the pool from hydroxylapatite on a MonoQ 10/10 FPLC column (Amersham Biosciences) that was eluted with a linear gradient of 0 to 0.5 M KCl. The preparation was 94% pure as assessed by densitometry of SDS-polyacrylamide gels.

Radiolabeled HSP40 Protein—[35S]Methionine-labeled Ydj1 was translated in vitro using the T7/T system (Promega) with T7 RNA polymerase. For Ydj1 binding analysis, each 200-μl sample contained 1 μl of translation solution with radiolabeled Ydj1 and 5 μl of unlabeled Ydj1.

Isolation of PR (Cytosol Preparation)—Chicken oviduct cytosol was prepared from chicks that had been stimulated with estradiol benzoate for 5 weeks as described previously (33). Oviduct tissue was stored at −70°C and homogenates were prepared in 4 volumes of TTg buffer (10 mM Tris-HCl (pH 7.5) and 10 mM thiglycoloyl) containing a protease inhibitor mixture of 0.1 mM leupeptin, 0.1 mg/ml bacitracin, 77 μg/ml aprotinin, 1.5 μg pepstatin, and 1 mg/ml benzene-sulfonyl fluoride. To remove receptor-associated proteins, the cytosol was adjusted to 0.5 mM KC1 and 10 mM ATP for 30 min on ice prior to the isolation of PR. In the case of hormone-activated PR, 50 μM progesterone was added at this step.

Immune Isolation of PR—Antibody resin was prepared by incubating the PR22 antibody with a slurry of protein A-Sepharose CL-4B (Amersham Biosciences) in 0.1 M potassium phosphate (pH 8.0) for 30 min at room temperature. The PR22-conjugated resin was washed three times with TTg buffer. For the purification of receptor, 60 μl of PR22/protein A slurry (1:1) was added per 1.5 ml of salt-treated cytosol. This mixture was maintained on ice for 1.5 h with gentle resin suspension. Receptor resin complexes were rapidly washed two times with 1 ml of TTg buffer supplemented with 0.5 M KC1 and 5 mM ATP and then two times with 1 ml of TTg buffer alone. Resin pellets were then used under various experimental conditions.

Experimental Conditions for Incubations—Packets of receptor-bound resin were suspended in 200 μl of incubation buffer (10 mM Tris-HCl, 50 mM KC1, 5 mM MgCl2, and 2 mM dithiothreitol (pH 7.5)), ATP, ADP, or AMP-PNP was added where indicated at 5 mM. Where noted, an ATP regeneration system was used that consisted of 10 mM phosphocreatine (di-Tris salt, Sigma) and creatine phosphokinase (3.5 units/100 μl of lystate; type I from rabbit muscle, Sigma). Untreated rabbit reticulocyte lysate (20 μl purchased from Green Hectares (Oregon, WI). Incubations were at 30 or 48°C with agitation at 100 rpm, in a water bath, with resuspension of the samples every 5 min. To isolate complexes for a subsequent incubation, samples were washed two times with 1 ml of incubation buffer by centrifugation at 4°C and resuspension before proceeding to the next step.

SDS-PAGE and Hormone Binding—After incubations, samples were chilled, and 5 ml of 12.5% [3H]progesterone (1.9 TBq/mmol; PerkinElmer Life Sciences) and 50 nm unlabeled progesterone were added to each 200-μl sample. Samples were left on ice for 2 h with gentle resin suspension. They were subsequently washed four times with 1 ml of incubation buffer by centrifugation at 4°C and resuspension. 0.1 ml was removed for the measurement of [3H]progesterone using a Beckman LS 1800 liquid scintillation counter, and the remainder was analyzed by SDS-PAGE on 10% gels, except for the experiments with only HSP40 and/or HSP70, in which the samples were run on 7% gels. The SDS-polyacrylamide gels were stained with Coomassie Blue. Images shown here were scanned using a digital camera.

Quantification of Protein Levels—Arbitrary densitometric values for the indicated bands were obtained using IP Lab Gel 1.5e software (Signal Analytics Corp., Vienna, VA), which assigns a number corresponding to the pixel count in the area indicated. To quantify the amount of radiolabeled protein present, the proteins on the gel were transferred to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA), and the level of radioactivity was measured using a Storm 840 PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). The membrane was also Coomassie Blue-stained to assess the total amount of protein bound, and this was compared with the amount of radiolabeled protein bound.

Calculation of Stoichiometry—Protein bands stained with Coomassie Blue were quantified by densitometry to obtain an arbitrary number termed the “volume” after correcting for background binding. The amount of chaperone both bound and unbound was compared with total amount of PR (PR-A and PR-B) in those samples. The molar ratios were calculated using the following three formulas: (a) HSP40/PR molar ratio = (volume Ydj1/44.6 kDa)/(volume PR-B/86 kDa) + (volume PR-A/72 kDa); (b) HSP70/PR molar ratio = (volume HSP70/70 kDa)/(volume PR-B/86 kDa) + (volume PR-A/72 kDa); and (c) HSP70/HSP40 molar ratio = (volume HSP70/70 kDa)/(volume Ydj1/44.6 kDa). It should be noted that there is some degree of error in these measurements because all proteins do not stain equally.

RESULTS

PR Complex Assembly in Vitro—Avian PR, if kept cold, can be isolated on an antibody resin in its native conformation capable of binding hormone. The PR hormone-binding domain is very unstable and rapidly loses its binding activity at elevated temperatures (13). However, the hormone binding ability can be restored after a transient loss using an in vitro five-protein system containing HSP40, HSP70, HOP, HSP90, and p23. An example of this is shown in Fig. 1, which illustrates the time course of PR complex assembly. When PR was incubated at 30°C without any additional proteins, it lost most of its hormone binding ability by 5 min and could not bind any hormone above background levels after 30 min (Fig. 1A). However, when PR was incubated with the five-protein system and ATP, about half of its hormone binding ability was transiently lost, yet recovered by 5 min. Furthermore, the five-protein system was able to restore some of the PR that no longer had an active hormone-binding domain after it had been stripped of its endogenous chaperone proteins, as shown by the higher levels of hormone binding at 10 min with the five-protein system compared with PR right on ice.

Fig. 1B shows the protein content of PR complexes after incubation, including the heavy chain of the PR22 antibody used for the immunoprecipitation. The two receptor forms (PR-A and PR-B) were present and have been shown to be very similar in their binding of chaperone proteins (4). In the absence of added chaperone proteins, the levels of full-length PR remained constant throughout the 30-min time period (lanes 2 and 11–13). This shows that the loss of hormone-binding activity by PR is not due to proteolysis from contaminants or from large conformational changes leading to loss of binding to the receptor, but could be due to inhibition of PR activity from the rapid effects of mild heat on the highly unstable hormone-binding domain. The pattern of associated chaperone proteins show that as PR complexes initially formed, the most significant binding was that of HSP40 to PR, whereas HSP70 and HSP90 remained at relatively minor levels on ice (lane 3). HSP40 was also the first protein in the system to reach its
maximally bound state, at ~1–2 min (lanes 4 and 5). HSP70, HSP90, and p23 appeared to reach their maximal binding by 10 min (lane 7). In earlier studies on PR complex formation in rabbit reticulocyte lysate, the binding of HSP70 was found to precede that of HSP90 and p23 (13); however, that was not evident in the present experiment with purified proteins. HOP, which only participated as an intermediate, was not apparent under the conditions used. As shown previously (13), HSP90 and p23 binding correlate with the gain of hormone-binding activity. In this study, Ydj1 was used as the HSP40 homolog. This yeast protein contains all of the functional domains of bacterial DnaJ and is thus a type I homolog of DnaJ (34). It is highly homologous to HDJ2, a human HSP40 protein that functioned interchangeably with Ydj1 in our in vitro PR system (Fig. 2). Studies in yeast have shown that only HDJ2 among three human HSP40 homologs assayed suppresses the hormone binding defect of the Ydj1 deletion mutant (19). In our hands, we were able to more reproducibly purify Ydj1 than HDJ2, so it was used in the experiments for this study. Because the other chaperone proteins in our system are of human origin, it is possible that HDJ2 may function more effectively than Ydj1 in this system. However, limited comparisons have not indicated any differences.

**HSP40 Binds PR Independently and Is Required for HSP70 Binding**—To gain more insight into the mechanism of PR complex assembly, we looked in detail at the early steps of PR complex assembly. Specifically, we wanted to determine whether the role of HSP40 in the early complex is indeed a critical one, particularly in introducing PR to the HSP90 chaperoning pathway. We first examined the binding of both the yeast HSP40 protein Ydj1 and HSP70 to PR under various conditions as summarized in Fig. 2. As shown in Fig. 2A, Ydj1 clearly bound to PR in the absence of other proteins of the chaperoning pathway (lanes 1 and 5). The binding of HSP40 was specific to the conformation of PR without bound hormone. Identical incubations were set up in the presence (lane 4) or absence (lane 5) of 50 nM progesterone. Previous studies have shown that the binding of progesterone to PR suppresses the interaction of PR with chaperones (7), and this is consistent with the specificity of HSP40 observed here. Ydj1 binding to PR did not depend on nucleotide, as a high level of binding could be obtained both without nucleotide and with ATP (lanes 5 and 6). Comparable binding to PR was observed with the human HSP40 protein HDJ2 (lane 7). The binding of Ydj1 to PR was very tight considering that with a high salt concentration (1 M KCl), there was still residual HSP40 binding (lane 3). Thus, HSP40 bound tightly to PR, even in the absence of HSP70, in a nucleotide-independent manner. This shows that an HSP70-HSP40 complex does not need to be formed prior to the binding of HSP40 to PR.

In contrast, the binding of HSP70 to PR has different requirements compared with HSP40, as is shown in Fig. 2B. HSP70 in the presence of ATP did not have much observable binding in the absence of HSP40 (lane 1). Studies have shown that HSP70 binds substrate loosely when there is ATP bound and tightly after hydrolysis of ATP to ADP (35, 36). It has been observed that HSP40 binds to the ATPase domain of HSP70 and stimulates ATP hydrolysis activity (21, 22, 37, 38), which is needed for high affinity binding of HSP70 to substrates. HSP70 binding increased substantially when either Ydj1 or HDJ2 was present, even in the absence of nucleotide (lanes 2 and 6), indicating that HSP40 recruits HSP70 to PR. However, this binding was much less than that observed in the presence of
ATP (lane 5). The addition of ADP had little effect on HSP70 binding (lane 3). This underscores that although HSP40 can bind to PR independently of HSP70 and nucleotide, both HSP40 and ATP are essential for optimal binding of HSP70 to PR. As observed with HSP40 alone, HSP70 did not bind well to the conformation of PR activated with bound progesterone, although not as much binding was lost as with HSP40 (compare lanes 3 and 4). HDJ2 functioned interchangeably with Ydj1 in our in vitro PR system. This is apparent, for example, in its stimulation of HSP70 binding to PR (lanes 6–8) where it functioned in the same manner as Ydj1 (lanes 2, 4, and 5).

HSP40 Binds to Native PR with High Affinity—The requirements for targeting the HSP90 chaperoning system to particular substrates such as PR are not well understood. It has been observed that both HSP70 and HSP40 interact with peptides and denatured protein substrates (22, 24, 42–44). It has also been suggested in other systems that HSP40 might serve to target HSP70 to its substrate (22, 24, 42–44), but this has not been established for PR complex assembly. Unlike many HSP70 substrates, PR is a native protein. We sought to test the importance of native structure for PR chaperoning by subjecting PR to mild heat denaturation. Fig. 3A shows the results of an experiment that investigated the effect of a 10-min incubation at various elevated temperatures on the ability of the five-protein chaperone system to restore the hormone-binding activity of PR. The initial incubations were done at the temperatures indicated without any chaperone proteins added at this step. It was only in the subsequent 30-min incubation at 30 °C that the chaperones were included. It is clear that PR is highly susceptible to heat treatment. In the absence of chaperones, hormone-binding activity would be lost at all temperatures tested, as shown in Fig. 1A. However, if the preincubation occurred at 30 °C, most of the hormone-binding activity could be restored by the subsequent incubation with chaperones. Also, significant stabilization by progesterone was observed in the absence of chaperones when the hormone was included during the course of the experiment (Fig. 3A, open circles); but at higher temperatures, the capacity to restore hormone binding was lost irreversibly both in the presence and absence of progesterone. These results indicate that the hormone-binding domain of the receptor, where chaperone interaction occurs, can be easily denatured to a state that is resistant to chaperoning by the HSP90 pathway.

The effects of heat denaturation on chaperone binding to PR are shown in Fig. 3B. We found that once PR had been heated for 10 min at 48 °C, HSP40 binding was reduced extensively (lanes 3 and 4), suggesting that HSP40 needs a discrete site for binding that has been damaged. HSP70 binding was dependent on the presence of HSP40, regardless of whether PR was denatured or not (compare lanes 6 and 7 with lanes 9 and 10). Yet in the presence of HSP40, HSP70 binding levels were constant under the two conditions, whereas HSP40 binding decreased substantially despite the addition of HSP70 (lanes 9 and 10). In the five-protein system, both HSP90 and HSP40 binding decreased, whereas HSP70 binding increased at 48 °C, presumably because of its enhanced affinity for denatured substrates. Although the binding of HSP70 was maintained or enhanced by partial denaturation of PR, this binding did not lead to the assembly of complexes with HSP90 and did not generate native PR.

Because HSP40 is able to bind PR in the absence of other proteins, its binding was measured throughout a range of concentrations (0.06–3.4 μM) and expressed in a Scatchard plot (Fig. 4). Native PR that had been left on ice (closed circles) and PR that had been preincubated at 48 °C for 10 min prior to its...
Fig. 5. Quantitation of HSP40 and HSP70 binding to PR. A, saturation conditions for the binding of Ydj1 to PR. Each 200-μl sample of resin blank without PR (odd-numbered lanes) or with ~1 μg of PR adsorbed to antibody resin (even-numbered lanes) was incubated for 10 min at 30 °C with 1 (lanes 1 and 2), 2 (lanes 3 and 4), 5 (lanes 5 and 6), 10 (lanes 7 and 8), or 20 (lanes 9 and 10) μg of Ydj1. Samples were isolated and analyzed for protein content by SDS-PAGE. Stained gels were scanned, and arbitrary densitometric values (Volume) were obtained for Ydj1 (HSP40), PR-A, and PR-B (lanes 6, 8, and 10). The HSP40/PR molar ratio was calculated as described under “Experimental Procedures.” B, saturation conditions for the binding of HSP70 to PR. The resin blank (lane 1) contained 5 μg of Ydj1, 20 μg of HSP70, 5 mM ATP, and antibody resin without PR. The next sample (lane 2) contained PR with 30 μg of HSP70 and 5 mM ATP in the absence of Ydj1. The remaining samples contained PR bound to resin plus 5 μg of Ydj1, 5 mM ATP, and increasing amounts of HSP70 as follows: 0 (lane 3), 5 (lane 4), 10 (lane 5), 20 (lane 6), and 30 (lane 7) μg of HSP70. After 20 min at 30 °C, samples were isolated and analyzed for protein content by SDS-PAGE. Stained gels were scanned, and arbitrary densitometric values (Volume) were obtained for the indicated bands (lanes 5–7). The HSP70/PR and HSP70/HSP40 molar ratios were calculated as described under “Experimental Procedures.” H-chain, heavy chain.

binding with HSP40 (open circles) were compared in their binding to HSP40. As we had suspected given the level of HSP40 binding to native PR under high salt conditions (Fig. 2A, lane 3), HSP40 bound tightly to native PR, with a Kd of 77 nM (Fig. 4, closed circles). This affinity agrees with at least one other HSP40 substrate complex that has been observed. The DnaJ–GroES complex in Escherichia coli has a Kd of 20 nM as measured using a BIACore system (45). In that system, the E. coli homolog for HSP70 (DnaK) can bind to the substrate independently of HSP40 and in the absence of nucleotide, and it does so with a Kd of 5 μM. Clearly, as is shown here in Fig. 4, there is a high affinity interaction between HSP40 and native PR. In contrast, when PR had been first incubated at 48 °C for 10 min prior to its binding with HSP40 (open circles), the affinity of HSP40 dropped considerably, as reflected in a Kd of 550 nM. In both cases, the Scatchard analysis showed a linear relationship consistent with a single type of binding interaction. These results show that HSP40 binds much more readily to PR in its native form than in its denatured form, further emphasizing the contrast between this HSP90 client protein and a more general chaperoning target such as a denatured protein.

To assess the protein composition of this early step, we measured the stoichiometry of the proteins in the HSP40–PR and HSP70–HSP40–PR complexes. Titrations of HSP40 binding to PR (Fig. 5A) and of HSP70 binding to PR (Fig. 5B) were used to determine the saturating amounts of chaperone proteins. The protein bands stained with Coomassie Blue were quantified by densitometry to obtain an arbitrary number termed the volume after correcting for background binding. The amounts of HSP40 and HSP70 bound were compared with the total amount of PR (PR-A and PR-B) in those samples. From these values, the molar ratios were calculated assuming equivalent staining/mass for all proteins (see “Experimental Procedures”).

The HSP40/PR molar ratio is ~1:1, and so the two proteins can exist in equimolar concentrations in the early complex. Yet the HSP70/PR molar ratio at saturation of binding is ~2:1, as is the HSP70/HSP40 molar ratio. It is evident that HSP70 binding did not affect binding of HSP40 to PR and that HSP70 binding could exceed that of HSP40, suggesting that HSP40 can promote either the binding of multiple HSP70 monomers or the formation of HSP70 dimers. Because the efficiency of Coomassie Blue staining can vary among proteins, these measurements should be considered only to be reasonable estimates of stoichiometry.

HSP40 Binding to PR Is Rapid and Persistent—The results of Fig. 1B indicate that HSP40 binding to PR precedes HSP70 binding. In this case, with all five chaperones present, HSP70 binding was never very extensive. Thus, we wished to analyze the early time course for HSP40 and HSP70 binding to PR in the absence of HOP, HSP90, and p23. As shown in Fig. 6, HSP40 bound quickly to PR. Within 2.5 min, HSP40 had reached the maximal level of binding. This occurred both in the presence and absence of HSP70 (data not shown). There was no apparent effect of the other chaperone proteins on the relative speed with which HSP40 bound to PR. HSP70 also bound to PR quickly, reaching its maximally bound state by 5 min of incubation at 30 °C. However, it is important to note that not only did HSP40 reach its maximally bound state sooner than HSP70, a higher percentage of it bound early on, even on ice. HSP40 reached ~55% of its maximally bound state at time 0, the time required to mix the ingredients on ice immediately followed by centrifugation and washing. This binding is unique to HSP40 as compared with the other four proteins in the system. This gives further support that HSP40 binding is the first step of the pathway. The binding of HSP70 shown in Fig. 6 was more rapid and much more extensive than observed in the five-protein system (Fig. 1B). Evidently, the interplay
among these chaperones alters the dynamics of HSP70 binding quite significantly.

Next, we examined the stability of the interaction between Hsp40 and PR. The dynamics of the Hsp40-PR complex were assessed under different conditions by measuring the dissociation of bound Hsp40 from the complex after removal of free Hsp40 with PR. The early PR complex was assembled with [35S] Met-labeled Ydj1 during the first incubation. These samples were washed to remove free radiolabeled Ydj1 not bound to the complex. In the dissociation experiments, no Ydj1 was added in the second incubation; in the exchange experiments, unlabeled Ydj1 (in excess) was added in the second incubation. In the protein complexes isolated from the exchange experiments, the total bound Hsp40 levels (radiolabeled plus unlabeled) were constant throughout the time course, as shown by Coomassie Blue staining (data not shown). This indicates that the PR complex remained saturated with Hsp40 and that Hsp40 dissociation and exchange were constant throughout the time course, as shown by Coomassie Blue staining (data not shown). This indicates that the PR complex remained saturated with Hsp40 and that any loss of radiolabeled Hsp40 occurred by exchange with unlabeled Hsp40. The extent to which this occurred was measured by observing how much radiolabeled Hsp40 remained after a given time.

A representative graph of the time course of Hsp40 dissociation and exchange is shown in Fig. 7, with the open symbols corresponding to the progress of dissociation after removal of free Hsp40 and the closed symbols representing the extent of exchangeability. The open symbols illustrate the dissociation of Hsp40 from a PR complex when it was subsequently incubated at 30 °C in buffer alone (open circles) or with only Hsp70 and ATP (open squares). The amount of radiolabeled Hsp40 bound to PR under these conditions stayed relatively constant, with 90% remaining after a 20-min incubation at 30 °C. These dissociation data, especially when considered along with the data in Table I, show that under the conditions assayed, there was no marked loss of the complex. In fact, without added Hsp40 present to replace Hsp40 already bound to PR, the protein remained stably bound to PR. The stability of Hsp40 binding to PR occurred independently of Hsp70. This is shown in Table I, where the Hsp40 dissociation was minimal in both the presence of Hsp70 binding conditions (with ATP, with ATP and ATP regeneration system, or with ATP and HOP) and conditions not conducive to Hsp70 binding (with no ATP or ADP). Taken together, the dissociation data are in line with the fact that this is a high affinity interaction with a very low rate of dissociation.

However, there is some exchange of Hsp40 on PR, and this occurs rather slowly in the context of the time course of PR chaperoning (Fig. 1). This is clearly in contrast to the highly dynamic nature of Hsp70, which appears to function through the rapid cycles of binding and release of unfolded protein substrates that have been observed in other model systems (46) and with PR (9). After 20 min, 60–70% of Hsp40 remained bound, as shown in Fig. 7 and Table II. This implies that 30–40% of radiolabeled Hsp40 was exchanged with unlabeled Hsp40 under these conditions, given that the levels of total Hsp40 (radiolabeled plus unlabeled) in the PR complexes were constant. Clearly, the majority of Hsp40 did not exchange in a 20-min period at 30 °C. However, there was some variability, albeit fairly limited, in the extent of exchangeability under different conditions. Generally, conditions that do not allow much Hsp70 binding (with no ATP, ADP, AMP-PNP (a non-hydrolyzable ATP analog), or ATP regeneration system) with AMP-PNP) led to 20–30% exchange of Hsp40 in 20 min. These time courses were similar to the closed squares in Fig. 7 which represent exchange in the presence of unlabeled Hsp40, without any additional proteins. In contrast, those conditions that foster Hsp70 binding (with ATP; with ATP and ATP regeneration system; and with ATP and HOP) appeared to enhance exchange by ~10%. These time courses were similar to the closed circles in Fig. 7, which represent exchange in the
presence of unlabeled HSP40, HSP70, and ATP. Yet even in the complete five-protein system with HSP40, HSP70, HOP, HSP90, p23 and ATP or with rabbit reticulocyte lysate, the level of exchangeability did not reach background levels, theoretically ~10%. Instead, the percent of radiolabeled HSP40 remaining was 58%, roughly equal to the extent of exchangeability seen under HSP70 binding conditions. Given that only 30–40% of HSP40 exchanged under these conditions, it is possible that there may be some heterogeneity in HSP40 that binds to PR. Nonetheless, in the presence of both rabbit reticulocyte lysate and progesterone (50 nM), the loss of radiolabeled HSP40 from PR was increased, as shown by the closed triangles in Fig. 7. This was due, however, to the disassembly of the PR complex that occurred upon activation with hormone and not because of exchangeability. This is apparent when looking at the protein content per PR complexes under such conditions with hormone where a lower level of the various proteins in the mature complex is evident (data not shown), as is typical upon hormone activation (2, 7).

The Early Complex Can Progress to a Mature PR Complex Capable of Hormone Binding—To establish that the early complex with HSP40-PR was indeed a functional complex in the context of the PR chaperoning pathway, we sought to isolate this complex and demonstrate that it could support PR chaperoning after the addition of the necessary factors. It was crucial to see if HSP40 that remained bound to PR, as described for Fig. 7, was indeed still capable of performing its activities when combined with the other chaperones: HSP70, HOP, HSP90, and p23. As such, we assembled the five-protein complex in two steps. This was done by first binding HSP40 to PR and washing away any free HSP40 so that only HSP40 bound to the PR complex remained. Then either the remaining four proteins (HSP70, HOP, HSP90, and p23) or the complete five-protein system including unbound HSP40 was added for a further incubation at 30 °C for 30 min. As shown in Fig. 8, these conditions led to a viable complex of bound HSP90 with PR capable of binding hormone, similar to the assembly with the five-protein system in one step. When PR was left on ice without any other proteins added (lane 2), a high level of hormone-binding activity was retained. HSP40 binding alone was not sufficient to chaperone PR (lane 3), as when it was incubated at 30 °C for 5 min, low levels of hormone binding were achieved, despite the formation of the early complex. Nonetheless, HSP40 is necessary for chaperoning, as seen when HSP40 was excluded from a 30-min incubation at 30 °C with the other four chaperone proteins (lane 4). Under these conditions, there were also low levels of hormone binding as well as HSP90 binding (Fig. S8B). As seen before, when PR was incubated for 30 min at 30 °C with HSP40, HSP70, HOP, HSP90, and p23 along with ATP (lane 5), high levels of hormone binding and HSP90 binding were obtained. The chaperoning pathway was also separated into two steps. This was done by first assembling a PR complex with HSP40, followed by incubation with the remaining four proteins (lane 6) or with all five proteins including unbound HSP40 (lane 7). In both cases, high levels of HSP90 binding were obtained, corresponding to high levels of hormone binding. These were similar to those in lane 5, in which the five proteins were all added at once. It is apparent that the early complex can be isolated as a functional unit. Pre-bound HSP40 was sufficient to assist in the chaperoning pathway without additional free HSP40 (lane 6), and having additional free HSP40 made only a slight difference to hormone binding (lane 7). These results are thus further evidence that HSP40 binding is the first step in the HSP90 chaperoning pathway for PR.

**DISCUSSION**

The HSP90 pathway for PR is very complex, yet it is presumed to be an ordered pathway of assembly that involves multiple proteins in the molecular chaperone machinery. Ear-
A proposed model for the assembly of PR complexes in the five-protein chaperoning pathway is illustrated in Fig. 9. This model depicts three distinct complexes that are formed prior to arriving at the mature PR complex with a functional hormone-binding domain. The initiation of the pathway (step 1) involves the binding of HSP40 to PR. In previous studies on PR complex formation in rabbit reticulocyte lysate, the first protein interaction observed was the ATP-dependent binding of HSP70 (13). We propose here that, in fact, HSP40 binds to PR prior to the binding of HSP70 and that this interaction recruits HSP70 to the complex.

To proceed from the early complex to step 2, HSP70 binds PR through an ATP-dependent mechanism. This step is likely to involve an intermediate where HSP70-ATP binds to HSP40 and loosely to PR. These interactions stimulate the ATPase activity of HSP70 to achieve tight PR binding by HSP70-ADP.

This would be consistent with previous reports on the modulation of HSP70 binding by ATP binding and hydrolysis (20, 22, 23, 25). The complex of PR with HSP40 and HSP70 then assembles with HOP and HSP90 (step 3) to form what has generally been referred to as the intermediate complex (13, 51). Through two separate domains, HOP can bind to both HSP70 and HSP90 simultaneously and is believed to act as an adaptor to promote the binding of HSP90 to pre-existing early complexes (52–54). Indeed, binding of HOP to HSP70 and HSP90 occurs directly with pure proteins and without the need for nucleotide (55). However, HOP has been shown to favor HSP70 in its ADP-bound conformation over that bound to ATP (55). Even though HSP90 is a part of this complex, the receptor is still unable to bind hormone, as this occurs when the intermediate complex is converted to a mature complex.

The intermediate complex containing HSP70, HOP, and HSP90 is then converted to a mature complex containing HSP90 and p23 (step 4). The mechanism for this transition is unknown, but involves the binding of ATP to HSP90, perhaps facilitated by interaction of HSP90 with PR. p23 binds selectively to HSP90 in an ATP-bound state through interactions with the N-terminal and middle regions of dimeric HSP90 (56). This interaction stabilizes the HSP90-receptor complex so as to maintain the ligand-binding domain of the receptor in a conformation capable of binding hormone (25, 57, 58).
pathway, suggesting a unique role for Ydj1 in repressing steroid receptor activity. It was thus proposed that entry of substrates into the HSP90 pathway involves a critical interaction of the substrate with Ydj1. The apparent “leakiness” caused by Ydj1 mutations might be explained by the results shown in this study. We found that Ydj1 was bound to PR throughout the entire assembly process and did not readily dissociate like the other chaperone proteins. This sustained binding may act to constantly promote the assembled complex with HSP90, but defective Ydj1 may allow receptors to frequently escape from the chaperone complex.

Finally, this study shows that HSP40 binding requires a native PR structure, a specific recognition site that identifies PR as an HSP40 target and consequently as an HSP90 target. The high affinity binding of HSP40 to PR could be abolished either by the binding of progesterone to the receptor or by mild heat denaturation. Thus, this binding seems to require a specific conformation of the native receptor, in contrast to the more general interactions of HSP40 and HSP70 in chaperoning the folding of denatured proteins. It has been suggested that HSP40 serves to target HSP70 to its substrates through a direct interaction between the two chaperones. An example is the J domain of auxilin, which targets HSP70 to the basket-triskelion-auxilin complex for the uncoating of clathrin-coated vesicles (49, 50). As such, HSP40 may serve to select a particular substrate for chaperoning. The known HSP90 client proteins are numerous and diverse, yet it has not been clear how the chaperone machinery is targeted to them. An attractive possibility is that HSP40 may serve to identify this kind of substrate as opposed to a severely misfolded protein that has arisen because of cell stress. In future studies, it will be important to identify and characterize the HSP40-binding site on PR and to investigate whether analogous sites exist on other HSP90 client proteins.

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