# Role of the Cochaperone Tpr2 in Hsp90 Chaperoning[†]

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ABSTRACT: The molecular chaperones Hsp90 and Hsp70 are highly regulated by various cochaperones that participate in the activation of steroid receptors. Here we study Tpr2 (also called DjC7), a TPR domain-containing type III J protein implicated in steroid receptor chaperoning. We propose that Tpr2 plays a role in the Hsp90-dependent chaperoning of the progesterone receptor (PR). Tpr2 overexpression or knockdown resulted in slight reductions in PR transcriptional activity in HeLa cells. Immunoprecipitation and pulldown experiments indicated that Tpr2 associates with Hsp90 and Hsp70 complexes, some of which also contain the PR. Tpr2 can bind Hsp90 and Hsp70 simultaneously, which is also a property of the cochaperone Hop. However, unlike Hop, Tpr2 binding to Hsp70 in the presence of Hsp90 is ATP-dependent, and Tpr2 cannot replace Hop in Hsp90 chaperoning in vitro or in vivo. While Tpr2 was not detected as a component of PR heterocomplexes in cell lysates, purified Tpr2 bound the PR readily. Surprisingly, Tpr2 replaced type I and II J proteins in the Hsp90-dependent chaperoning of the PR and the protein kinase, Chk1. Unlike other J proteins, Tpr2 promoted the accumulation of Hsp70 in PR heterocomplexes in the presence of Hsp90. Thus, Tpr2 has the potential to regulate PR chaperoning.

Hsp90 is a highly abundant molecular chaperone that is ubiquitously expressed and associates with client proteins in near-native states (*1, 2*). Hsp90 oversees the ultimate activation step, which is mandatory for many vital regulatory proteins in eukaryotic cells. The growing family of Hsp90 clients includes important members of the signal transduction pathway as well as their mutant counterparts that act as oncogenes in cancer cells (*3, 4*). Steroid receptors, such as the progesterone receptor (PR)[1] and the glucocorticoid receptor (GR), are chaperoned by Hsp90 (*2*). Both of these receptors have been instrumental in understanding the mechanics and dynamics of Hsp90 complex assembly. Chaperone proteins interact with the steroid-binding domain (SBD), a well-conserved domain near the C-terminus of the receptors.

When the PR is isolated from cells in the absence of hormone, several chaperone and cochaperone proteins are associated with the apo receptor, including Hsp90, Hsp40, Hsp70, Hop, p23 [the five proteins required for in vitro chaperoning (*5*)], Hip (*6*), and one of several immunophilins, such as FKBP51, FKBP52, and Cyp40 (*6*). If Hsp90 is removed from the PR isolate, the hormone binding capacity of PR is greatly reduced in a time- and temperature-dependent manner (*5, 7*). Hormone binding activity can be restored using an in vitro system that includes the five above-mentioned purified recombinant chaperones and cochaperones with ATP (*5*). This in vitro system reconstitutes heterocomplexes closely resembling those originally found in vivo and provides a powerful tool for studying the sequence of events in the folding pathway of the PR.

PR chaperoning occurs in a series of steps, including association of the PR with Hsp40, which is believed to be the first step that will trigger Hsp90-dependent PR chaperoning (*7*). To become competent to bind hormone, an Hsp40 (J protein) recruits Hsp70 to the PR in an ATP-dependent manner (*8*). The interactions between Hsp40 and Hsp70 proteins are mediated by the J domain of Hsp40 and the ATP binding domain of Hsp70 (*9*). The Hsp70−Hsp40−PR early complex is followed by the formation of an intermediate state elicited by the incorporation of Hop (heat shock protein organizing protein) (*10, 11*). Hop transports Hsp90 into the multiprotein complex mediated by its tetratricopeptide repeat (TPR) domains (*6*). Hop contains three TPR domains, where TPR2a is required for Hsp90 binding (*6, 10*) and TPR1 is required for Hsp70 binding (*12*). When PR-associated Hsp90 binds ATP, p23 recognizes Hsp90 and release of the intermediate complex occurs (*1, 2*). This exchange allows the SBD of the PR to undergo a conformational rearrangement that permits hormone binding.

The first step in this pathway requires PR binding by Hsp40. Hsp40 is a member of the J protein family (*13*). The main shared characteristic among all family members is an HPD motif-containing J domain, capable of stimulating the ATPase activity of the Hsp70 partner (*9*). The J protein family comprises more than 40 members in humans (*14, 15*)

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[1] Abbreviations: TPR, tetratricopeptide repeat; Hsp, heat shock protein; PR, progesterone receptor; GR, glucocorticoid receptor; AR, androgen receptor; Hop, hsp organizing protein; Hip, Hsp70 interacting protein; FKBP, FK506 binding protein; Cyp, cyclophilin.

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and can be divided into three types based on sequence similarity and domain composition (*9*). Type I proteins have an amino terminal J domain followed by a zinc finger-like domain and a nonconserved carboxy-terminal domain which is believed to be involved in substrate recognition (*16, 17*) and can be farnesylated in vivo (*9*). Type II proteins share a J domain followed by a glycine-rich region (*18*), similar to type I, that is followed by a nonconserved C-terminal domain involved in substrate association (*16, 17, 19*). Type III proteins contain a J domain and lack any other sequence similarity to type I or II (*15*). Variations among these proteins raise the question of what specific roles each J protein plays in the cell, and little information is available on the expression, regulation, and function of most of these J proteins (*15*). In vitro, type I J proteins, such as yeast Ydj1 and human DjA1 (Hdj2), bind stably to one site on PR (*8*). Although a stable interaction is not readily detected between the type II J protein DjB1 and PR, it is able to promote PR chaperoning (*8*). On the other hand, the in vitro chaperoning of the Chk1 kinase was shown to be more effective with DjB1 than with DjA1 (*20*). Another Hsp90 client that has shown a preference for DjB1 is the reverse transcriptase of hepatitis B virus, for which DjA1 was apparently inactive (*21*).

It is becoming clear that while J proteins can overlap in some functions, each protein probably has an optimal role in the cell (*22*). Tpr2 (DnaJC7, DjC7, CCRP) (*23–25*) is a type III J protein that contains two TPR domains and a J domain. TPR domains are protein−protein interaction motifs that consist of a small group of tandem 34-amino acid sequences, where the amino acid sequence has very loose conservation yet the folding of this domain is conserved (*12*). Proteins containing TPR domains function in different cellular processes such as cell cycle control, transcription repression, protein kinase inhibition, and chaperoning (*26*).

Tpr2 is ubiquitously expressed (*23*), and although its role in the cell is not well understood, it has been hypothesized to play a role in the suppression of polyglutamine toxicity in the *Drosophila* eye (*27*), to bind Rad 9, which is an early cell-cycle checkpoint protein (*28*), and to bind neurofibromin, a promoter of the inactive form of p21 Ras (Ras-GDP) (*23*). Tpr2 has also been found to interact with the nuclear orphan receptors CAR and PXR (*25, 29*) and to play a role in the chaperoning of GR (*24*).

Our current study addresses some of the unanswered questions regarding the possible role of Tpr2 in the Hsp90-mediated chaperoning of a steroid receptor. Brychzy et al. (*24*) hypothesized that Tpr2 acts as a recycling cochaperone or a sensor of folding quality. Thus, in their hypothesis, Tpr2 works by facilitating the return of the client to early stages of chaperoning if further folding is required. Here we address the possibility that Tpr2 may play a role in PR chaperoning, similar to that found for GR.

Tpr2 is the first type III J protein that has been implicated in the chaperoning of steroid receptors. Here we show that, in vitro, Tpr2 is able to stimulate the formation of early PR complexes and to support PR chaperoning, as well as that of another Hsp90 client, the protein kinase, Chk1. Furthermore, Tpr2 can form complexes with Hsp90 and Hsp70 in the cell and form a three-way complex with purified Hsp90 and Hsp70. In the cell, Tpr2 appears to have only a modest influence on PR activity. The results from the previous publication by Brychzy et al. (*24*), and the evidence presented in this study, indicate that Tpr2 may be a recycling cochaperone in the Hsp90 chaperoning of steroid receptors.

## MATERIALS AND METHODS

*Plasmid Construction.* The cDNA for human Tpr2 with an N-terminal histidine tag was prepared as described previously (*24*). Tpr2 containing an N-terminal Flag tag was prepared using Topo technology (Invitrogen) and cloned into pcDNA3 for expression in mammalian cells through an *Eco*RI site.

*Protein Purification.* Human Hsp90, human Hsp70, Ydj1, DjA1, Hop, and p23 were all prepared as described previously (*5, 30*). Tpr2 was expressed in BL21 DE3 pLysS *Escherichia coli* by induction with 1 mM IPTG (isopropyl $\beta$-D-thiogalactopyranoside). This culture was incubated overnight at 15 °C. Cells were pelleted and later resuspended in buffer A [20 mM Hepes (pH 8), 150 mM KCl, 100 mM NaBr, and 5 mM imidazole] with Complete-Mini EDTA-free protease inhibitors from Roche. The cell suspension was sonicated three times for 30 s on ice, and the lysate was clarified by centrifugation. Tpr2 was purified using a nickel affinity column (Talon). The resin was washed using buffer A and 500 mM KCl and eluted with buffer A and 200 mM imidazole. The protein was stored at a concentration of 1−2 mg/mL in 20 mM Hepes (pH 8), 150 mM KCl, 100 mM NaBr, 10 mM NaSCN, and 1 mM DTT, at −70 °C.

*siRNA Knockdown of Tpr2.* We used HeLa cells stably expressing PR-B with a chloramphenicol acetyltransferase (CAT) reporter gene described previously (*31*). The HeLa cells were transfected with siRNA using DharmaFect 1 reagent according to the manufacturer's recommendations (Dharmacon) at 20−30% confluence in 24-well plates. We used 100 nM small interfering RNA (siRNA) duplexes to Tpr2 and FKBP51 or 50 nM small interfering RNA (siRNA) duplexes to Tpr2 and Hop (for a total of 100 nM RNAi). After 72 h, the HeLa cells were treated with 1 nM R5020 (Perkin-Elmer), a synthetic progestin, for 12 h. The cells were trypsin-treated, harvested by gentle centrifugation, and washed twice with PBS. Cells were lysed using the M-PER reagent (Pierce) following the manufacturer's recommendations with the addition of protease inhibitors. After lysate clarification by centrifugation, 10 $\mu$g protein samples were analyzed using SDS−PAGE. The gel was transferred to a PDVF membrane, and proteins were detected using Western blotting with the appropriate antibodies. The level of CAT protein was measured by an enzyme-linked immunosorbent assay (Roche) using 50 $\mu$g of protein.

The siRNAs used were purchased from Dharmacon: Tpr2, ON-TARGETplus SMARTpool L-019566-01; FKBP51, ON-TARGETplus SMARTpool L-004224-00; Hop, ON-TARGETplus SMARTpool L-019802-00.

*Overexpression of Tpr2.* HeLa PR-B cells (above) were transfected with cesium gradient centrifugation-purified pcDNA3-Flag-Tpr2 DNA using Lipofectamine LTX and plus reagent according to the manufacturer's specifications (Invitrogen). Forty-eight hours post-transfection, the cells were treated with 1 nM R5020 (Perkin-Elmer) overnight (12 h). Production of CAT (chloramphenicol acetyltransferase) was measured by an enzyme-linked immunosorbent assay (Roche) using 50 $\mu$g of protein according to the manufacturer's

recommendations. For the pull-down experiments, untreated cells (no R5020 added) were harvested and washed in lysate buffer [20 mM Tris (pH 7.5), 150 mM KCl, 20 mM $Na_2MoO_4$, 2 mM dithiothreitol, and 0.02% Nonidet P-40] and protease inhibitor mixture (Complete EDTA-free from Roche). The cells were sonicated for 30 s, and the cell debris was removed via centrifugation at 14000 rpm for 20 min. The lysate was added to protein A-Sepharose beads prebound with either PR-B antibody (PR6) or Hsp90 antibody (H9010). The lysate and beads were incubated on ice for 1.5 h and washed four times with 1 mL of lysate buffer without protease inhibitors. The beads were incubated with SDS sample buffer for 5 min at 90 °C, and proteins were resolved via 7.5% acrylamide SDS−PAGE, transferred to Immobilon-P transfer membranes (Millipore), and blotted with PR6 (against the PR) (*32*), H9010 (against Hsp90) (*20*), BB70 (against Hsp70) (*20*), anti-Flag (monoclonal anti-Flag M2, Sigma), or a polyclonal antibody against Tpr2 (*24*).

*PR Immuno-Isolation.* Chicken PR-A was expressed in a baculovirus system as described previously (*8*). A mouse monoclonal antibody, PR22 against chicken PR (*32*), was bound to protein A-Sepharose CL-4B (Amersham Biosciences) in PBS for 30 min at room temperature at a proportion of 5 $\mu$L of PR22 ascites per 20 $\mu$L of resin. The conjugated resin was washed in PBS and resuspended as a 1:1 slurry with ice-cold stripping buffer [20 mM Tris (pH 7.5), 500 mM KCl, 5 mM $MgCl_2$, 0.1% Nonidet P40, and 1 mM dithiothreitol]. To isolate the PR, 40 $\mu$L of a PR22/protein A resin slurry was added to 70 $\mu$L of salt-treated SF9 cell lysate, and this slurry was then incubated on ice for 1.5 h. This mixture was adjusted according to the number of reactions required. Receptor resin complexes were washed three times with cold stripping buffer to remove bound Hsp90, Hsp70, and cochaperones and once with reaction buffer. The term "stripped PR" in figures refers to receptor bound to resin previously washed in this manner.

*PR Chaperoning Assays.* PR resin pellets (20 $\mu$L) were suspended with 200 $\mu$L of cold reaction buffer [20 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, 0.01% Nonidet P-40, and 2 mM dithiothreitol (pH 7.5)] containing the specified amount of Hsp40 (either Ydj1, DjA1, or Tpr2), 20 $\mu$g of Hsp70, 20 $\mu$g of Hsp90, 5 $\mu$g of Hop, 5 $\mu$g of p23, and 2 mM ATP. The samples were incubated at 30 °C for 20 min and then chilled in ice. [$^3$H]Progesterone (100 nM) was added for further incubation on ice for 4 h (*7, 11*). The final samples were analyzed by adding 20 $\mu$L of SDS sample buffer, followed by a 5 min incubation at 90 °C. A small portion, 10 $\mu$L, was removed for measurement of radioactivity, and the proteins were separated by SDS−PAGE.

*Chk1 Chaperoning Assay.* In addition to the chaperones described above, GST-Chk1-(1−265) and GST-Cdc25C (residues 200−256) were provided by the Karnitz laboratory (*33*). The chaperoning assays for GST-Chk1-(1−265) were performed as described previously (*33*) using Hsp90, Hsp70, Hop, an Hsp40 protein together with casein kinase 2 (Sigma), and [$\gamma$-$^{32}$P]ATP (4500 Ci/mmol) (MP Biomedicals). However, in this study, we used either 2 $\mu$g of DjB1 or various amounts of Tpr2 as indicated in the figures. The recovered kinase activity of GST-Chk1-(1−265) was measured by calculating the phosphorylation levels of substrate GST-Cdc25C, which was radiolabeled and measured after membrane transfer, using a Storm 840 PhosphorImager (Amersham Biosciences). The procedures were performed as described previously (*20, 33*).

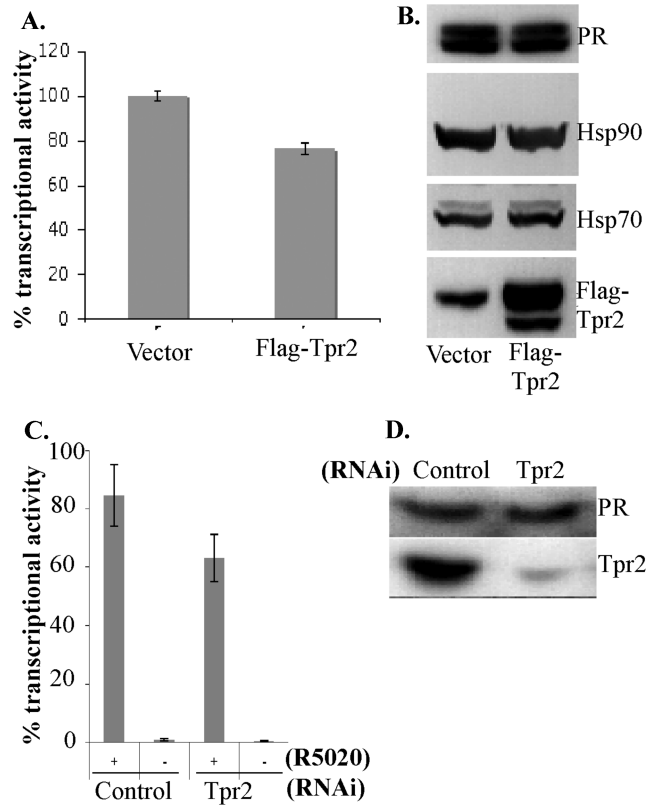


FIGURE 1: PR activity is slightly affected by variations in Tpr2 levels. (A) HeLa cells were transfected with control vector or vector containing Flag-Tpr2 using Lipofectamine LTX from Invitrogen. CAT enzyme produced was measured from cell lysates after overnight treatment with 1 nM R5020. (B) The protein levels in cells from panel A were assessed by Western blot analysis. (C) HeLa cells were transfected using the DharmaFECT1 reagent with 100 nM scrambled siRNA control (#3 Dharmacon) or siRNA specific for Tpr2. Three days after transfection, the cells were treated overnight with 1 nM R5020 or the equivalent volume of ethanol. The amount of CAT enzyme produced was measured from cell lysates. (D) The protein levels in cells from panel C were assessed by Western blot analysis.

## RESULTS

*Changes in the Cellular Levels of Tpr2 Mildly Affect the Transcriptional Activity of PR.* Tpr2 has the ability to bind Hsp90 and Hsp70 (*24, 34*), and altering the cellular protein levels of Tpr2 has been shown to negatively affect GR transcriptional activity (*24*). In view of the fact that PR interacts with both Hsp70 and Hsp90 and it is thought to be chaperoned in a manner similar to that of GR (*2*), we sought to determine if altering protein levels of Tpr2 in cells would have an effect on PR transcriptional activity. Flag-tagged Tpr2 was overexpressed in HeLa cells stably expressing human PR-B and a PRE (PR response element)-CAT reporter gene described previously (*31*). The effect of overexpression observed in multiple experiments was that the transcriptional activity of PR decreased by approximately 20% (Figure 1A), compared to the activity observed in cells transfected with an empty vector. Western blot analysis showed that protein levels of PR, Hsp90, and Hsp70 were unaffected by the overexpression of Flag-Tpr2 (Figure 1B).

The data published by Brychzy et al. indicated that the transcriptional activity of GR was negatively affected when

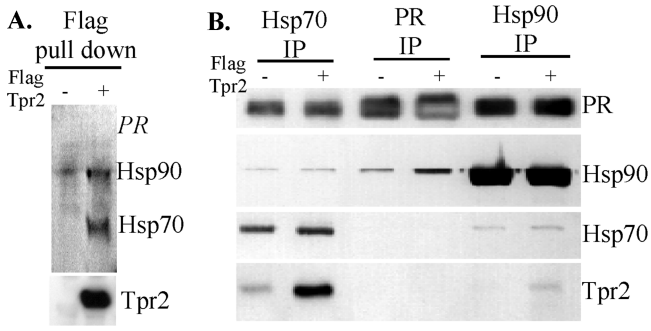


FIGURE 2: Tpr2 interacts with Hsp70 and Hsp90 complexes. HeLa cells transfected with control vector or vector containing Flag-Tpr2 were harvested as described in Materials and Methods. (A) The cell lysates were used to perform a pull-down using Flag antibody beads. (B) The cell lysates were used to perform immunoprecipitation of Hsp70, PR, and Hsp90. Associated proteins were identified by Western blot analysis using the appropriate antibodies.

Tpr2 was either overexpressed or suppressed using siRNA knockdown (*24*). To test PR activity when the levels of Tpr2 were reduced, HeLa cells stably expressing PR-B were used, and Tpr2 was knocked down using RNAi against endogenous Tpr2. Transfected cells were exposed to 1 nM R5020, and the transcriptional activity of PR was measured (Figure 1C). The transcriptional activity of PR was only mildly affected by the reduction of Tpr2 protein levels. In Figure 1D, Western blot analysis shows that reducing the levels of Tpr2 does not affect PR protein levels. Together, the overexpression of Flag-tagged Tpr2 and the knockdown of endogenous Tpr2 both result in modest reductions in PR activity. A reduction in activity under both situations was also observed previously for GR; however, PR was apparently less sensitive than GR, since in the GR system more marked changes were observed with a change in Tpr2 level (*24*). Thus, Tpr2 may play some role in chaperoning PR, but it appears not to be an essential component of this process.

*Tpr2 Exists in Complexes with Hsp90 and Hsp70.* To investigate whether Tpr2 exists in complex with PR in the cell, pull-down experiments were performed using lysates of HeLa-PR-B cells transfected either with empty vector or with Flag-Tpr2. Figure 2A shows that a pull-down of Flag-Tpr2 contains Hsp70 and some Hsp90, but no PR was detected. These results indicate that, while this protein may play a role related to both Hsp70 and Hsp90, it does not appear to associate with PR with enough strength to be detected using pull-down techniques. However, when Hsp90 was immuno-isolated from cell lysates, Flag-Tpr2 was found present in Hsp90 complexes (Figure 2B), as well as Hsp70 and PR. An immuno-isolation of Hsp70 from HeLa-PR-B cells overexpressing Flag-Tpr2 contained PR, Hsp90, and Tpr2. Tpr2 was not found associated with PR when PR immunoprecipitations (Figure 2B) in which Hsp90 was the predominant chaperone were performed.

The results described above indicate that Tpr2 is not in complex with cellular PR or its association is such that it is readily disrupted by our pull-down conditions. However, due to association of Tpr2 with both Hsp90 and Hsp70, the possibility that Tpr2 might play a role in some aspect of PR chaperoning was pursued further, by testing the activity of Tpr2 using in vitro chaperoning assays.

*Purification of Recombinant His$_6$-Tpr2.* To test the activity of purified His$_6$-Tpr2, we had to overcome the tendency of

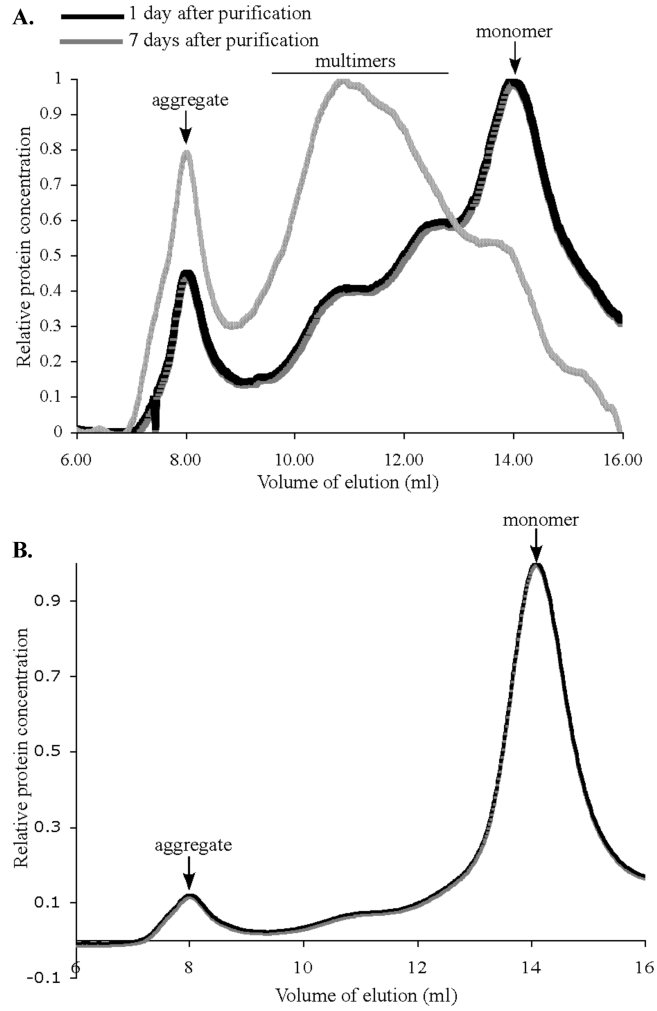


FIGURE 3: Prevention of the time-dependent precipitation of purified Tpr2. His$_6$-Tpr2 was purified as described in Materials and Methods. (A) Purified Tpr2 was stored on ice in 20 mM Tris (pH 7.5), 150 mM KCl, and 2 mM DTT. Samples were analyzed after being stored for 1 or 7 days. (B) Tpr2 was stored in 20 mM Hepes (pH 8), 150 mM KCl, 100 mM NaBr, 10 mM NaSCN, and 1 mM DTT for 2 months and analyzed by gel filtration.

this protein to precipitate from solution after purification. When in solution and in a buffer containing 20 mM Tris (pH 7.5), 150 mM KCl, 0.01% Triton X-100, and 2 mM DTT, a tendency of Tpr2 to precipitate over time was observed. This was enhanced by large changes in temperature such as freezing−thawing cycles. Aliquots of Tpr2 were analyzed by analytical gel filtration during the first week after purification. Figure 3A shows the size distribution of Tpr2 on day 1 and day 7 after purification. The monomer of Tpr2 was the major molecular form initially; however, it aggregated within days, and the concentration of aggregated protein predominated in the span of 1 week when it was kept on ice.

Storing Tpr2 at −20 °C with 30% glycerol, in addition to the buffer described above, helped slow the precipitation process, yet after 2 months, the protein was visibly precipitated in the form of a white pellet. Along with precipitation, the activity of the protein deteriorated. Additional detergent accelerated the precipitation of Tpr2 (unpublished observations). Brychzy et al. used high salt (500 mM NaCl) to maintain the solubility of Tpr2 for their studies (*24*). Since salt can alter the in vitro chaperoning assay, we sought to

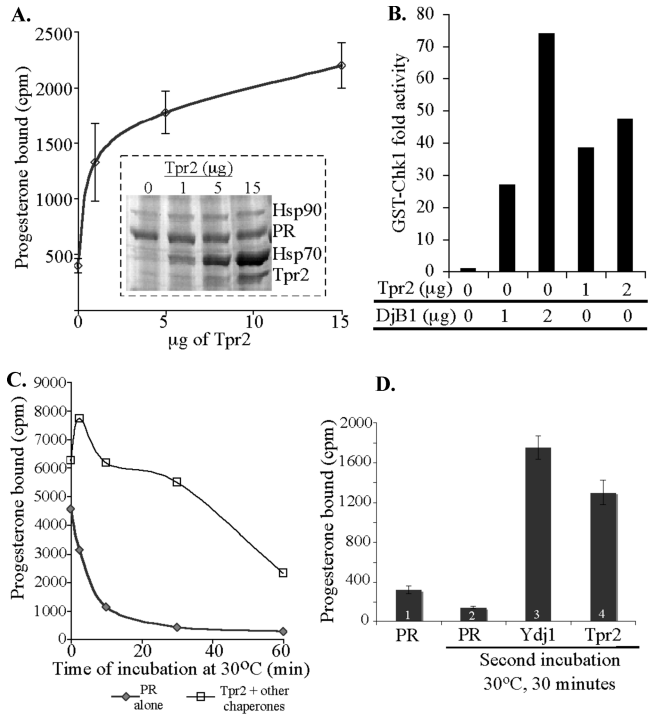


FIGURE 4: Tpr2 is able to stimulate chaperoning of PR and Chk1. (A) Stripped chicken PR-A immobilized on antibody resin was incubated at 30 °C for 30 min. The samples (200 $\mu$L) contained 5 mM ATP, 20 $\mu$g of Hsp90, 20 $\mu$g of Hsp70, 5 $\mu$g of Hop, and 5 $\mu$g of p23. Tpr2 was added as indicated. Chaperoning was monitored by measuring hormone binding activity as described in Materials and Methods. The inset is a Coomassie-stained SDS−PAGE gel showing complex formation after addition of increasing amounts of Tpr2 as indicated. (B) Chaperoning of GST-Chk1 using indicated amounts of Tpr2 or DjB1. The reaction mixtures contained Hsp90, Hsp70, CK2, Hop, and Cdc37, and chaperoning was assessed by measuring kinase activity as described in Materials and Methods. (C) Stripped chicken PR-A immobilized on antibody resin was incubated without (◆) or with 5 mM ATP, 20 $\mu$g of Hsp90, 20 $\mu$g of Hsp70, 5 $\mu$g of Hop, 5 $\mu$g of p23, and 5 $\mu$g of Tpr2 (□) at 30 °C for the indicated times. The samples were analyzed for hormone binding activity. (D) PR-A was inactivated by preincubation for 10 min at 30 °C (bar 1). Samples were then incubated for an additional 30 min at 30 °C in buffer alone (bar 2) or the five-protein system in the presence of 5 $\mu$g of Ydj1 (bar 3) or 5 $\mu$g of Tpr2 (bar 4).

identify a salt condition that did not affect, in great measure, the chaperoning activity of the in vitro PR chaperoning system. After testing several salts, the addition of 100 mM NaBr and 10 mM NaSCN was chosen. Figure 3B shows a Tpr2 protein preparation that contained a buffer of 20 mM Hepes (pH 8), 150 mM KCl, 100 mM NaBr, 10 mM NaSCN, and 2 mM DTT. This sample contained mostly a monomeric molecular form, as assessed by gel filtration after being stored for 2 months on ice. In this case, no precipitation was observed, although a small amount of aggregate was detected upon gel filtration (Figure 3B).

*Tpr2 Is Able To Support PR Chaperoning in Vitro.* Purified $His_6$-Tpr2 was tested in an in vitro five-protein chaperoning system for avian PR-A. This system requires Hsp40 (DjA1, Ydj1, or DjB1), Hsp70, Hsp90, Hop, and p23 (*5, 7, 8*). Hsp40 was replaced with Tpr2 to determine if this type III J protein was able to promote PR chaperoning. We found that Tpr2 was able to replace Hsp40 in the reaction (Figure 4A). It promoted the chaperoning of PR in a dose-dependent manner as measured by hormone binding activity. The resulting chaperoning activity was quite similar to that obtained when using DjA1 or DjB1, and all ingredients of the in vitro system were required for optimal activity (results not shown) (*8*). The inset of Figure 4A shows the analysis of complexes by SDS−PAGE. Increasing doses of Tpr2 resulted in enhanced binding of Hsp70. However, Hsp90 binding was fairly constant and generally weaker than that observed when using the standard conditions with DjA1 or Ydj1, as we will discuss later.

To establish if Tpr2 is able to promote chaperoning of other Hsp90 clients, Tpr2 was tested in the Hsp90-dependent activation reaction that has been developed in vitro to study the chaperoning of the checkpoint kinase, Chk1 (*33*). As a protein kinase, Chk1 requires different cochaperones. In particular, while it does not need p23, Cdc37 is essential. Cdc37 is a cochaperone of Hsp90 which targets kinases for Hsp90 chaperoning, and it is activated by phosphorylation using CK2 (*35*). Chk1 also shows a strong preference for DjB1 as the Hsp40 protein. The proteins required for the chaperoning assay of GST-Chk1 are as follows: DjB1, Hsp90, Hsp70, Hop, Cdc37, and CK2 (*33*). As shown in Figure 4B, Tpr2 was sufficiently active as a J protein to promote the chaperoning of GST-Chk1, in vitro, in a dose-dependent manner. Its activity was substantial, but generally somewhat lower than that of DjB1. Thus, Tpr2 has been validated as a J protein able to promote maturation of a protein kinase and a steroid receptor in vitro together with the chaperones and cochaperones mentioned above.

The PR chaperoning system was used to study the activity of Tpr2 in greater detail. A time course for PR chaperoning is shown in Figure 4C. When isolated from cell lysate, the PR maintains some hormone binding activity if kept on ice; however, this is rapidly lost upon incubation at 30 °C as shown (*5, 7*). When in the presence of the chaperoning system with Tpr2, hormone binding activity was maintained over 30 min at 30 °C. However, a marked loss of binding activity occurred between 30 and 60 min. Such a loss was not generally observed when the system contained DjA1 or Ydj1 (data not shown).

The experiment shown in Figure 4D was designed to test whether Tpr2 has the ability to chaperone the PR starting from an inactive state. As shown in Figure 4C, the stripped PR retains some ability to bind hormone when kept on ice. This ability is enhanced by the addition of chaperones and further incubation at 30 °C (*5, 7, 8*). However, when stripped PR is preincubated without chaperones for 10 min at 30 °C, the PR is inactivated as shown in Figure 4D (bar 1). These inactivated samples were further incubated at 30 °C for 30 min in the following manner: stripped PR in reaction buffer with no additional chaperones (bar 2) or PR incubated with Hsp90, Hsp70, Hop, p23, and either Ydj1 (bar 3) or Tpr2 (bar 4). While Ydj1 was more efficient at supporting PR chaperoning (bar 3), both Tpr2 and Ydj1 were quite effective as Hsp40 proteins at promoting the activation of PR together with the other chaperones mentioned above.

*Tpr2 Interactions during PR Chaperoning.* To further compare Tpr2 and Ydj1, a time course for the chaperoning reaction with PR was performed at 30 °C. Figure 5A shows a SDS−PAGE gel with receptor complexes formed over a period of 60 min. These reaction mixtures contained Hsp90, Hsp70, Hop, and p23. Lanes 1−5 contained reaction mixtures with 5 $\mu$g of Tpr2, while lanes 6−10 included 5 $\mu$g of Ydj1.

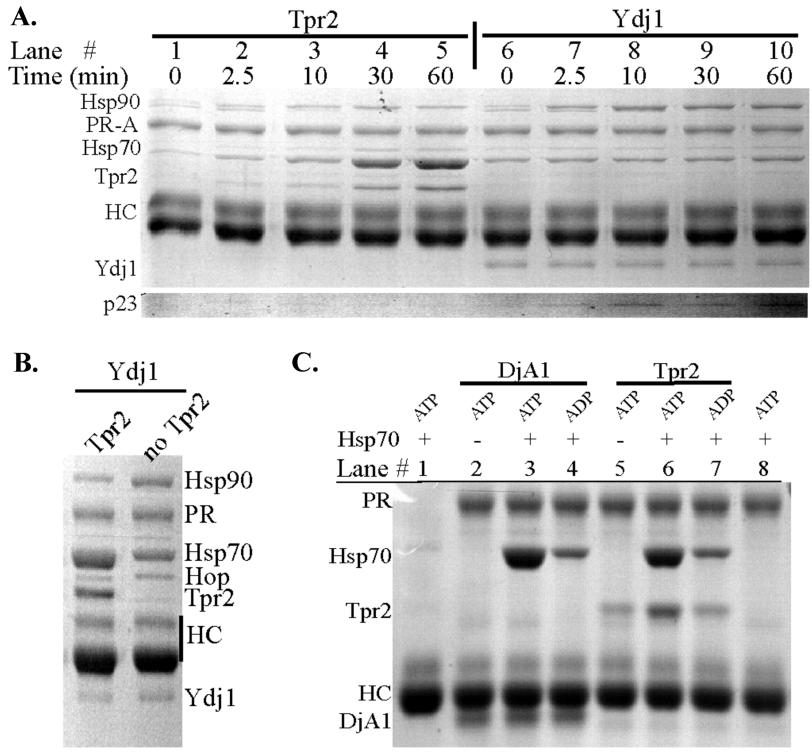


FIGURE 5: Tpr2 promotes extensive binding of Hsp70. (A) Stripped PR-A immobilized on antibody resin was incubated at 30 °C with 5 mM ATP, 20 $\mu$g of Hsp90, 20 $\mu$g of Hsp70, 5 $\mu$g of Hop, and 5 $\mu$g of p23 for the times indicated. Samples contained 5 $\mu$g of Tpr2 (lanes 1−5) or 5 $\mu$g of Ydj1 (lanes 6−10). The Coomassie-stained SDS−PAGE gel shows PR heterocomplexes. (B) PR chaperoning reaction mixtures were incubated at 30 °C for 30 min. The samples contained 5 mM ATP, 20 $\mu$g of Hsp90, 20 $\mu$g of Hsp70, 5 $\mu$g of Hop, 5 $\mu$g of p23, and 5 $\mu$g of Ydj1 with (lane 1) or without (lane 2) 30 $\mu$g of Tpr2. (C) A binding reaction was performed using stripped PR-A. In this case, 20 $\mu$g of Hsp70 was incubated with either 10 $\mu$g of Tpr2 or 10 $\mu$g of DjA1, in the presence of either 1 mM ATP or 1 mM ADP as indicated. Complexes are shown from a Coomassie-stained SDS−PAGE gel. HC indicates antibody heavy chain.

The level of incorporation of Hsp90 was generally lower in chaperoning reaction mixtures containing Tpr2 (lanes 1−5) than in those containing Ydj1 (lanes 6−10). Concomitant with the level of Hsp90 association, when Ydj1 was used, p23 association was observed (lanes 6−10), but this was much less evident when Tpr2 was used. The most obvious difference in this comparison was that the level of incorporation of Hsp70 into PR complexes increased greatly over time when Tpr2 was present (lanes 1−5), but this did not occur when Ydj1 was used (lanes 6−10) (*7*).

Figure 5B shows a PR chaperoning experiment in which both reaction mixtures contained Hsp90, Hsp70, Hop, Ydj1, and p23 and were incubated for 30 min. However, the sample in lane 1 also contained 30 $\mu$g of Tpr2. This caused an accumulation of Hsp70 in the PR complex comparable to the previous example in Figure 5A. These results suggest that Ydj1 and Tpr2 differ in either their abilities to recruit Hsp70 to PR complexes or the ability of PR complexes to progress beyond that stage.

In previous publications, direct comparisons between Ydj1, DjA1, and DjB1 established that these proteins stimulate Hsp70 binding to PR in an ATP-dependent manner very similarly (*7, 8*). Therefore, we tested whether Tpr2 was able to promote the ATP-dependent formation of early complexes more efficiently than, in this particular comparison, DjA1. In the absence of an Hsp40 (Figure 5C, lane 8) or ATP (lanes 2 and 5), no Hsp70 was bound to the PR. Some binding occurred in the presence of ADP, but optimal Hsp70 binding required ATP and a J protein. Tpr2 was very comparable to DjA1 in promoting the recruitment of Hsp70.

*Tpr2 Can Bind Directly to the PR, Hsp70, and Hsp90.* To compare Tpr2 and DjA1 in more detail, we tested the ability of Tpr2 to bind the PR on its own, which is a characteristic of the type I J proteins Ydj1 and DjA1 (*8*). Figure 6A shows that, in vitro, Tpr2 binds PR directly in a dose-dependent manner when incubated at 30 °C. In this case, immuno-isolated PR was incubated with different amounts of purified Tpr2, as indicated, and the isolated complexes were analyzed by SDS−PAGE. Each pair of lanes shows specific association of a different amount of Tpr2 with the PR, as indicated, with no background binding to antibody beads. We then tested whether Tpr2 binds to the same site on PR as Ydj1 and DjA1 (Figure 6B). A competition experiment in which a constant amount of DjA1 was bound to the PR and increasing amounts of either Ydj1 or Tpr2 were added as a competitor was performed. As shown previously, competition was observed using Ydj1 (*8*), but Tpr2 did not compete with DjA1 for PR binding. It appears, therefore, that the binding of Tpr2 to the PR occurs either at a different binding site or at multiple sites on the PR. While the specific site of binding of Hsp40 proteins to the PR is not known, chaperones in general are believed to bind steroid receptors in the SBD (*2*).

*Requirements for the Association of Tpr2 with Hsp70 and Hsp90.* While a difference in PR binding may contribute to the excessive recruitment of Hsp70 observed when using Tpr2 in the five-protein system, this may also reflect a deficiency in Hsp90 recruitment. The TPR domains of Tpr2 have been shown to bind C-terminal peptides of Hsp90 and Hsp70 (*24*). The binding of Hsp70 and Hsp90 to Tpr2 was

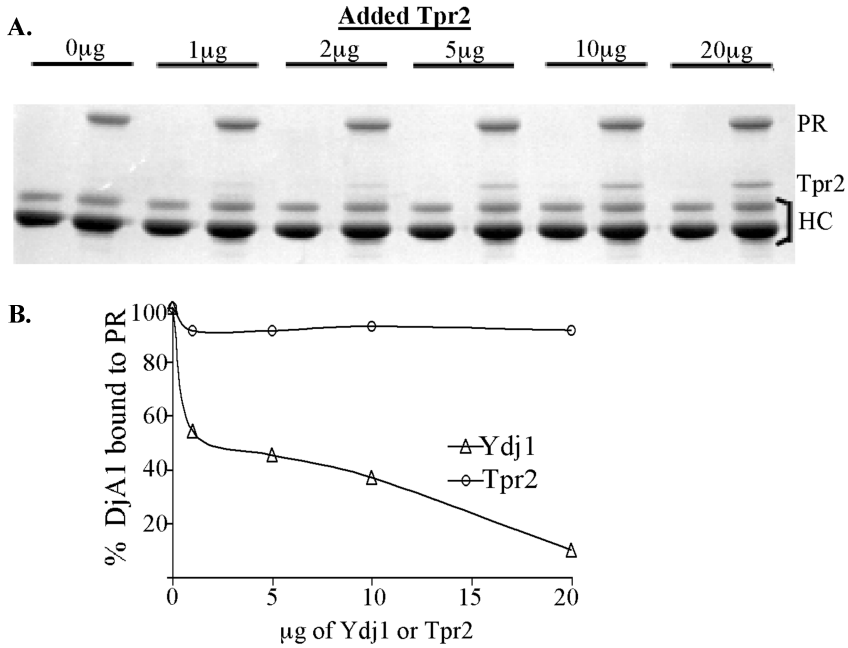


FIGURE 6: Tpr2 binds directly to the PR. (A) Samples of stripped PR-A immobilized on antibody resin, or antibody resin alone, were incubated with the indicated amounts of DjA1 at 30 °C for 10 min. PR complexes were isolated and analyzed by SDS−PAGE. (B) Immobilized PR was incubated with 5 $\mu$g of DjA1 and various amounts of either Ydj1 or Tpr2 at 30 °C for 10 min. DjA1 bound to PR was assessed using SDS−PAGE, and the bands were quantified by densitometry. The graph shows the percentage of DjA1 that remained associated with the PR.

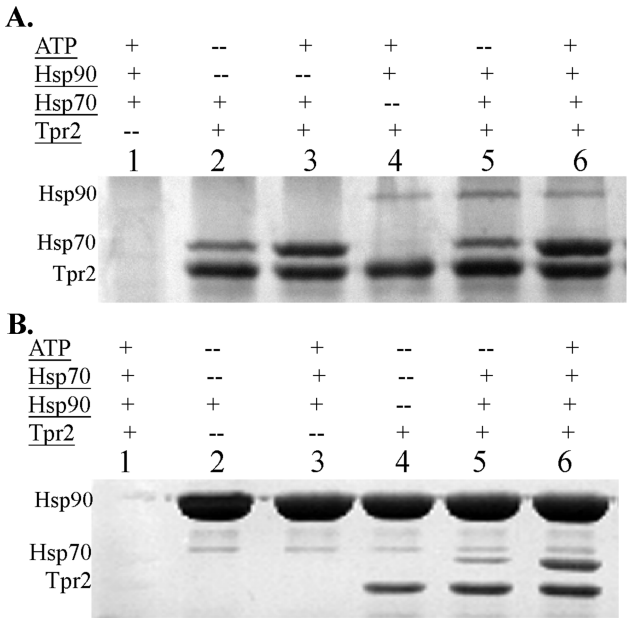


FIGURE 7: Tpr2 binds Hsp90 and Hsp70 simultaneously. (A) Samples with $His_6$-Tpr2 (5 $\mu$g) immobilized on nickel beads (lanes 2−6) or beads alone (lane 1) were incubated with 20 $\mu$g of Hsp90 and/or 20 $\mu$g of Hsp70. Where indicated, 1 mM ATP was added. Reaction mixtures were incubated for 20 min at 30 °C. (B) Samples contained Hsp90 immobilized on antibody beads (lanes 2−6) or beads alone (lane 1). The reaction mixtures contained 10 $\mu$g of Tpr2, 20 $\mu$g of Hsp70, and 1 mM ATP where indicated and were incubated for 20 min at 30 °C.

confirmed in our system. Figure 7A shows the interactions of immobilized $His_6$-Tpr2 with purified Hsp90 and/or Hsp70. Hsp70 associated with Tpr2 in the absence of ATP (lane 2), while the addition of ATP enhanced this association (lane 3). Tpr2 was previously shown to stimulate Hsp70 ATPase activity through its J domain and to bind Hsp70 using its TPR domains (*24*). Thus, it is likely that the presence of ATP enhances Tpr2−Hsp70 interaction by engaging both the TPR domains and the J domain of Tpr2. Hsp90 associated with Tpr2 with an affinity apparently lower than that of Hsp70 (lane 4), and this association did not change significantly in the presence of ATP or ADP (data not shown). Lanes 5 and 6 show that the association of Hsp90 with Tpr2 is not affected by the addition of Hsp70 or Hsp70 and ATP, respectively. Tpr2 did not bind to either Hop or p23, and their presence did not enhance or weaken the association of Tpr2 with either Hsp90 or Hsp70 in the presence or absence of nucleotide (data not shown).

To determine if Tpr2 can bind to Hsp90 and Hsp70 at the same time, we tested complex formation using immobilized Hsp90 and purified Hsp70 and Tpr2 (Figure 7B). Hsp70 was not able to bind to Hsp90 on its own (lane 3), and as expected, Tpr2 could bind to Hsp90 by itself (lane 4) and promote a slight amount of Hsp70 binding in the absence of ATP (lane 5). The presence of ATP greatly enhanced the incorporation of Hsp70 into the Tpr2−Hsp90 complex (lane 6). We tested Ydj1 and DjB1 in this manner. However, these proteins did not bind Hsp90 or stimulate Hsp70 binding to Hsp90 (data not shown). Thus, Tpr2 can bind to Hsp70 and Hsp90 in a three-way complex, but Hsp70 binding is ATP-dependent and probably occurs mainly through its J domain.

*Tpr2 Does Not Appear To Influence Hop Activity.* Our study shows that Tpr2 has the potential to bind simultaneously to Hsp70 and Hsp90, which is also a key property of the cochaperone Hop (*11, 36*). Therefore, we asked whether Tpr2 could replace the cochaperone Hop in the chaperoning process. To test this possibility, we compared PR chaperoning by Hsp90, Hsp70, p23, and ATP and added either DjA1 or Tpr2 in the presence or absence of Hop (Figure 8A). The data showed that both DjA1 and Tpr2 can similarly support PR chaperoning for 30 min at 30 °C. While

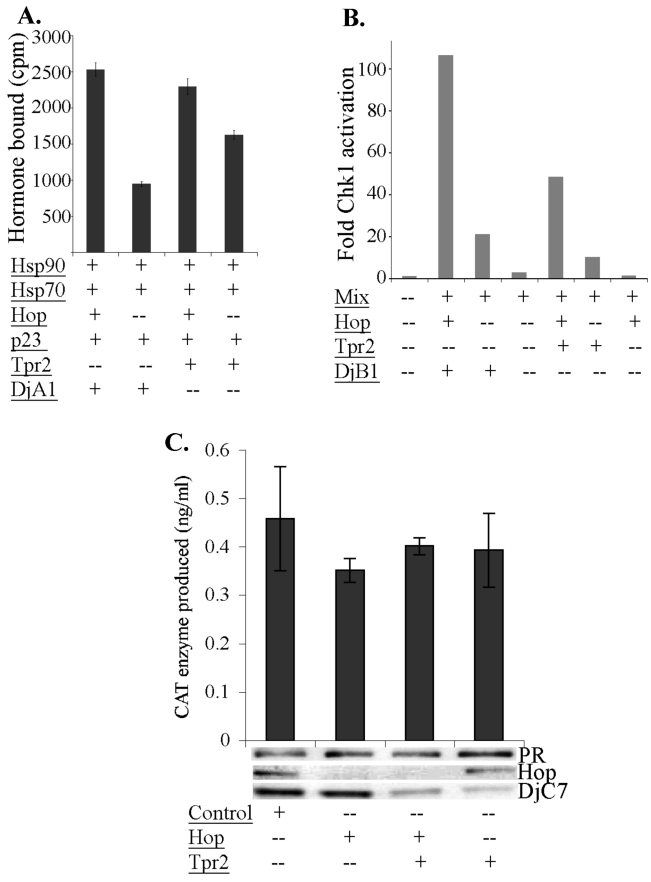


FIGURE 8: Tpr2 does not appear to substitute for Hop. (A) Stripped PR-A immobilized on antibody resin was incubated at 30 °C for 30 min with 5 mM ATP, 20 $\mu$g of Hsp90, 20 $\mu$g of Hsp70, and 5 $\mu$g of p23. Reaction mixtures contained either 5 $\mu$g of Tpr2 or 5 $\mu$g of DjA1. Additionally, 5 $\mu$g of Hop was included where indicated. (B) Chaperoning GST-Chk1 was performed with reaction mixtures containing Hsp90, Hsp70, CK2, Cdc37 (labeled as "Mix"), and Hop, DjB1, or Tpr2 as indicated. The graph presents the fold activation of the Chk1 kinase activity. (C) HeLa cells were transfected using 100 nM scrambled siRNA control (control #3 Dharmacon) or 50 nM siRNA specific for Tpr2 and/or Hop. Three days after transfection, the cells were treated overnight with 0.5 nM R5020. The amount of CAT enzyme produced was measured from cell lysates. Protein levels were assessed by Western blot analysis.

both systems show some dependency on Hop, the Tpr2 system consistently showed a weaker dependency.

Since PR chaperoning involves a combination of maintenance and restoration of activity (Figure 4), we tested the Hop requirement in the more rigorous system for chaperoning Chk1 (Figure 8B). Here, the client is initially inactive and the signal to background ratio is much greater than for PR chaperoning. As shown in Figure 8B, the chaperoning of Chk1 using DjB1 as the optimal Hsp40 resulted in >100-fold activation. Approximately 50-fold activation was observed using Tpr2. In both cases, omitting Hop resulted in an 80% loss of chaperoning activity. Thus, the systems with Tpr2 and DjB1 are equally dependent on Hop in properly promoting GST-Chk1 chaperoning.

To further test for any relationship between Hop and Tpr2 activities in the cell, we performed a simultaneous knockdown of Hop and Tpr2 in HeLa PR-B cells (Figure 8C). Like that of Tpr2, the knockdown of Hop resulted in only a slight marginal loss of PR activity. This was somewhat surprising since Hop is thought to be quite central in the Hsp90 chaperoning machine. While there may be another cellular protein that can replace Hop, that is apparently not a property of Tpr2 since the knockdown of both proteins does not result in further loss of PR activity.

## DISCUSSION

In this report, we show that Tpr2, a type III J protein, is able to support the Hsp90 chaperone machine using two different Hsp90 clients: a steroid receptor (PR) and a protein kinase (Chk1). Changes in the cellular levels of Tpr2 resulted in only slight effects on PR activity. While a complex between Tpr2 and PR in the cell could not be documented, Tpr2 was able to bind PR in a cell-free system and promote the recruitment of Hsp70. Tpr2 binds both Hsp90 and Hsp70. However, this protein could not replace the cochaperone Hop in in vitro assays and did not influence Hop activity in cellular assays.

When purified from bacterial cell lysates, Tpr2 was initially soluble, but it showed a strong tendency to aggregate while being stored. We found that modest concentrations of NaBr and NaSCN were very effective in preventing this behavior. This tendency to aggregate suggests that Tpr2 may normally form complexes with other proteins in the cell. Also, in a eukaryotic cell, it might be modified to a more soluble form by posttranslational modifications.

In earlier studies using the GR as a client, reductions or increases in the cellular level of Tpr2 were shown to lower GR-dependent transcriptional activity by approximately 50% (*24*). Here, we show that only a slight loss of PR transcriptional activity was observed when Tpr2 was overexpressed. Because the ubiquitous type I and II Hsp40 proteins, DjA1 and DjB1, can support PR chaperoning (*8*), it is not surprising that the Tpr2 knockdown experiments had only a small effect on PR transcriptional activity. These results show that Tpr2 is not essential for the chaperoning or activity of PR, although it could play a role under some circumstances. No degradation of PR was apparent in lysates from treated cells when inspected by Western blotting; thus, an excess of Tpr2 or reduction of Tpr2 levels does not appear to play a role in PR turnover. The GR and PR have been shown to be somewhat different in the way they bind to Hsp40 proteins in vitro (*37*), offering a possible explanation for the difference between the GR and PR in responding to varying levels of Tpr2.

Pull-down and immunoprecipitation experiments from HeLa cells demonstrated that, while Tpr2 interacts with Hsp90 and Hsp70, no association was evident between PR and Tpr2. However, Tpr2 associates readily with PR in a purified system as do DjA1 and Ydj1. Thus, it appears that Tpr2 and PR have the potential to transiently bind each other in vivo. Previous studies showed that DjA1 and Ydj1 compete for one binding site on PR (*8*); however, Tpr2 did not act as a competitor. These results are an indication that the Tpr2−PR association occurs at a different site, perhaps with a difference in binding dynamics.

In addition to the difference in PR binding by Tpr2 and Ydj1, we observed marked differences in the protein composition of PR heterocomplexes. When using Tpr2, the binding of Hsp90 to PR complexes was weaker while the amount of Hsp70 association was abnormally high, in comparison to the use of Ydj1 or DjA1. When the J proteins

were compared for their ability to simply recruit Hsp70 to the PR, they were very similar. However, the presence of Hop, Hsp90, and p23 normally promotes the transition to late or mature complexes showing a modest amount of Hsp70 and extensive binding of Hsp90 and p23 (*5, 7, 8*). On the other hand, in the presence of Tpr2, PR complexes appeared to accumulate in the early and intermediate stages with an abundance of Hsp70, less Hsp90, and negligible amounts of p23. These observations are consistent with the role for Tpr2 proposed by Brychzy et al. (*24*). These investigators identified Tpr2 as an Hsp90 cochaperone involved in GR chaperoning. Rather than functioning to initiate chaperoning, Tpr2 was proposed to facilitate the retrograde transfer of poorly folded clients from later complexes containing Hsp90 to early complexes containing mostly Hsp70. In fact, they were able to show that Tpr2 can displace Hsp90 from GR complexes in the absence of ATP in a manner different from that of cochaperones Hop and p23. Although we show that Tpr2 can initiate PR chaperoning in the absence of any other J protein, Tpr2-supported chaperoning is not stable over time. Tpr2 efficiently promoted early complex formation and enriched association of Hsp70 with PR even in the presence of Hsp90. This study confirms that Tpr2 facilitates and enhances client binding to Hsp70. Our results are consistent with the hypothesis in which Tpr2 promotes a retrolocation of the client to an earlier stage of chaperoning. It is surprising that such complexes can lead to a PR with hormone binding activity, although this chaperoning appears unstable and much loss of activity occurred with longer incubation (30−60 min). Because the in vitro reaction mixtures contained an excess of Hsp90, the resulting hormone binding may be an indication that transient interactions with Hsp90 take place in the purified system to promote the appearance of the active PR.

The transition between early and late PR complexes requires the action of the adaptor protein Hop. Hop is a well-characterized cochaperone known to simultaneously bind Hsp90 and Hsp70 using its TPR domains (*11*). Like Hop, Tpr2 contains TPR domains and has been shown to use these domains to bind Hsp90 and Hsp70. However, previous studies indicate that the TPR domains of Tpr2 can bind to only one protein at a time (Hsp70 or Hsp90, in the absence of ATP) (*24*). Here we show that Tpr2 can bind Hsp70 and Hsp90 simultaneously, but this requires the presence of ATP to promote Hsp70 binding, most likely through association with the J domain of Tpr2. Thus, the mechanisms of interaction with Hsp70 and Hsp90 differ between Tpr2 and Hop. Because it could bind both Hsp70 and Hsp90, we tested the ability of Tpr2 to replace Hop in the chaperoning process. The chaperoning PR in the presence of Tpr2 did not remove the need for Hop, although the process seemed somewhat less dependent on Hop than that using Ydj1. The Chk1 chaperoning system provided clearer results, with equal dependencies on Hop when Tpr2 or DjB1 was used as the J protein.

The possibility of overlap in cochaperone activities was further tested in a cellular context. As with Tpr2, the knockdown of Hop had only a small impact on PR activity in the cell. Thus, while Hop is thought to play a major role in the Hsp90 chaperoning machine, the cell can clearly accommodate lower levels of this cochaperone. The deletion or inactivation of yeast Hop (Sti1) has been shown to cause a marked reduction in the activities of two Hsp90 clients, $pp60^{v\text{-}src}$ and GR (*38, 39*). Such studies have not been conducted in higher eukaryotes. We found that the knockdown of both Hop and Tpr2 did not result in further loss of chaperone activity, indicating that the two cochaperones do not overlap in activity. This is not surprising since Hop not only brings Hsp70 and Hsp90 together in one complex but also modulates their activities (*36, 40, 41*). Also, the cellular level of Tpr2 is approximately 10-fold lower of than that of Hop (*24*) which is less abundant than Hsp90 or Hsp70. Thus, only a small percentage of cellular Hsp90 or Hsp70 would be in complex with Tpr2.

Tpr2 had not been tested previously using a protein kinase client, and the protein kinase Chk1 provides a useful comparison with PR. In vitro, Chk1 chaperoning shows similarities to that of PR in requiring input by an Hsp40 protein with Hsp70, Hop, and Hsp90. It also needs activated Cdc37, but it does not require p23 (*33*). Unlike the PR which can be chaperoned equally using Ydj1, DjA1, or DjB1, Chk1 has a clear preference for DjB1 (*20*). Thus, it was interesting that Tpr2 was able to activate Chk1, although it was less active than the preferred Hsp40, DjB1. These results offer evidence for a potential role for Tpr2 in steroid receptor chaperoning, as well as a novel role in the chaperoning of protein kinases.

While some human J proteins are well-known, most members are not yet fully characterized, and more evidence is required to assign appropriate substrate specificity and function to J proteins (*15*). Both redundancy and specific binding to clients appear to be important characteristics among J proteins (*42*). For example, J protein redundancy is evident in mitochondrial import, where different J proteins show a degree of partial specialization (*22*). In the case of PR, which requires a J protein for proper chaperoning (*5, 8*), some J proteins appear to effectively replace one another. Type I (Ydj1 and DjA1) and II (DjB1) J proteins have been shown to play a role in initiating the Hsp90 chaperoning pathway by binding the client and stimulating Hsp70 association (*7, 8, 43*). However, in yeast, the activity of steroid receptors is dependent upon Ydj1 (*43, 44*), and the closely related J protein DjA1 is associated with PR complexes isolated from human cell lysates (*8*). On the other hand, all steroid receptors may not have the same requirements. Recently, a $DjA1^{-/-}$ mouse was generated that resulted in aberrant androgen receptor (AR) signaling. No obvious defects relating to PR were observed (*45*), suggesting that other J proteins could substitute for the DjA1-primed chaperoning of PR, but not of AR, which appears to have more specific J protein requirements (*43*). In addition to Chk1, other Hsp90 clients that have shown a preference for DjB1 include the reverse transcriptase of hepatitis B virus (*21*) and HSF1 (heat shock factor 1) (*46*).

To the best of our knowledge, only one other type III J protein, DjC3, better known as $p58^{IPK}$ (*15*), has a domain composition similar to that of Tpr2. $p58^{IPK}$ has nine TPR motifs predicted to form either two or three TPR domains that are followed by a C-terminal J domain (*47*). Like Tpr2, $p58^{IPK}$ is able to bind Hsp70 and stimulate ATP hydrolysis by Hsp70 (*47*). $p58^{IPK}$ has been shown to be necessary for cotranslational degradation of some ER proteins (*48*) and has been characterized as an inhibitor of the interferon-induced double-stranded RNA-activated protein kinase (PKR)

(*49*). This latter inhibitory function is apparently relieved by association of DjB1 with p58$^{IPK}$ (*47*). A role for p58$^{IPK}$ in Hsp90 chaperoning is still unclear, although PKR has been shown to be an Hsp90 client (*50*). Further investigation is needed to assess whether Tpr2 and p58$^{IPK}$ play similar roles in the cell.

The role of Tpr2 in the sequence of steroid receptor chaperoning is not fully established, and the mechanisms underlying the role of this new cochaperone are not well understood. Future structural information describing interaction surfaces between protein complexes formed by Tpr2, Hsp70, and Hsp90 and clearer information about the role of Tpr2−Hsp90 binding will improve our understanding of Tpr2 in the context of Hsp90 chaperoning. In addition, more information about the relevance of Tpr2−nuclear receptor binding (*25, 29*), and a better understanding of Tpr2 in the context of the Hsp40 family (*15*), will aid in elucidating the molecular mechanisms exhibited by this protein.

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