Differential interactions of p23 and the TPR-containing proteins Hop, Cyp40, FKBP52 and FKBP51 with Hsp90 mutants

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Abstract Hsp90 is required for the normal function of steroid receptors, but its binding to steroid receptors is mediated by Hsc70 and several *hsp*-associated accessory proteins. An assortment of Hsp90 mutants were tested for their abilities to interact with each of the following accessories: Hop, Cyp40, FKBP52, FKBP51, and p23. Of the 11 Hsp90 mutants tested, all were defective to some extent in associating with progesterin (PR) complexes. In every case, however, reduced PR binding correlated with a defect in binding of one or more accessories. Co-precipitation of mutant Hsp90 forms with individual accessories was used to map Hsp90 sequences required for accessory protein interactions. Mutation of Hsp90's highly conserved C-terminal EEVD to AAVD resulted in diminished interactions with several accessory proteins, most particularly with Hop. Deletion of amino acids 661–677 resulted in loss of Hsp90 dimerization and also caused diminished interactions with all accessory proteins. Binding of p23 mapped most strongly to the N-terminal ATP-binding domain of Hsp90 while binding of TPR proteins mapped to the C-terminal half of Hsp90. These results and others further suggest that the N- and C-terminal regions of Hsp90 maintain important conformational links through intramolecular interactions and/or intermolecular influences in homodimers.

INTRODUCTION

Steroid receptors are perhaps the best characterized targets for assembly with Hsp90 (reviewed in Pratt and Toft 1997). One major functional significance of Hsp90•receptor interactions is the establishment and maintenance of a high affinity hormone-binding conformation in the receptors for glucocorticoids (GR) and progestins (PR). In addition to Hsp90, several chaperone components, some directly associated with Hsp90, participate in the ordered assembly of receptor complexes. During cell-free assembly reactions, an early interaction occurs between Hsc70 and a receptor monomer followed by formation of intermediate and functionally mature multi-protein complexes. The intermediate complex contains Hsc70, an

Hsc70-interacting protein termed Hip (Hoehfeld et al 1995; Prapapanich et al 1996), Hsp90, and Hop, a protein that binds both Hsp90 and Hsc70 (Chen et al 1996). The mature complex contains Hsp90, and two Hsp90-associated proteins: p23 and any one of three large immunophilins – Cyp40, FKBP52, or FKBP51. Each of the receptor complexes is highly dynamic (Smith 1993; Smith et al 1995), and the details of transition from one complex to the next have not been worked out.

The ordering of protein interactions and the roles of *hsp* accessories are important, as suggested by the following observations. Functional Hsc70 is required for formation of intermediate and mature complexes (Smith et al 1992; Hutchison et al 1993; Dittmar et al 1996). Recent evidence with a Hip mutant suggests that normal Hip facilitates formation of intermediate complexes (Prapapanich et al 1998). Inhibiting the function of Hop inhibits formation of mature complexes while favoring the accumulation of the early receptor complex (Chen et al 1996). Finally, blocking p23 function prevents formation of mature complexes

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while enhancing the accumulation of both early and intermediate complexes (Johnson and Toft 1994, 1995; Smith et al 1995). Recent results from studies using a purified system for assembly of GR complexes suggest that Hsc70, Hsp90 and Hop are sufficient for establishing GR's hormone-binding conformation, but p23 is necessary to stabilize this conformation (Dittmar et al 1996, 1997; Dittmar and Pratt 1997). Immunophilins are not required for maturation of receptor's hormone-binding conformation *in vitro*, but the conservation and common appearance of immunophilins in mature receptor complexes hint at an important functional role for these proteins, perhaps a role that is most evident at the cellular level.

The peptide-binding domain of Hsc70 is well characterized. Surprisingly, though, there is no conclusive evidence for what region of Hsp90 comprises its target binding site or sites. Two recent reports (Prodromou et al 1997b; Stebbins et al 1997) on the crystallographic structure of Hsp90's N-terminal region have speculated that a target-binding site may reside in the N-terminus. The putative protein-binding site identified in one report (Stebbins et al 1997) corresponded to the binding site for geldanamycin (GA); however, this site was subsequently shown to be an ATP-binding site (Prodromou et al 1997a) and no longer seems the likely site for Hsp90 binding to targets. An alternate target-binding site has been proposed (Prodromou et al 1997b), a so-called molecular clamp that corresponds to a cleft observed in the dimeric crystal structure of a yeast Hsp90 N-terminal fragment, but no direct evidence was presented for a protein target binding to this site. The structure of Hsp90's C-terminal region is largely unknown, except for the presence of a strong dimerization function near the C-terminus.

A number of Hsp90 mutagenic analyses have been performed in which various Hsp90 mutants were found to be defective in associating with steroid receptors and/or defective in supporting steroid receptor function *in vivo*. However, little emphasis was given in these analyses to the roles played by Hsp90-associated accessories and the potential disruption of accessory protein interactions that might indirectly prevent productive binding of Hsp90 to receptor.

Several Hsp90 accessories contain tetratricopeptide repeats (TPRs) that are required for binding to Hsp90. TPRs are repeats of a 34-amino acid, loose consensus motif (Lamb et al 1995), and the number of repeats in Hsp90 accessories vary from 2 to 5. Individual repeat units within a protein or between different proteins are highly degenerate. Hop binds Hsp90 through a central set of TPRs, but Hop also binds Hsc70 through an N-terminal TPR region (Chen et al 1996). Cyp40 (Ratajczak and Carrello 1996), FKBP52 (Radanyi et al 1994), and FKBP51 (Barent et al 1998) bind Hsp90 through their respective TPR regions, each a set of three repeats in the

C-terminal half of the protein. Likely, then, there is a TPR-recognition site in Hsp90, but this presumed site has not been mapped nor have the interactions of different TPR proteins with Hsp90 been carefully compared.

The fifth Hsp90-associated accessory, p23, does not contain a TPR, and unlike the TPR-containing accessories, its binding to Hsp90 is ATP-dependent (Johnson and Toft 1994, 1995; Grenert et al 1997) and blocked by the nucleotide-competitor GA (Johnson and Toft 1995; Smith et al 1995).

In this report, we examined 11 Hsp90 mutants *in vitro* for their abilities to associate with PR complexes and to bind Hsp90 accessories. The patterns of accessory protein interactions with Hsp90 provide novel insight into the structural/functional relationships between Hsp90 domains and between dimeric partners.

MATERIALS AND METHODS

Antibodies

Mouse monoclonal IgGs used were anti-PR PR22 (Sullivan et al 1986), anti-Hop F5 (Smith et al 1993a), anti-p23 JJ3 (Johnson et al 1994), anti-FKBP52 Hi52C (Nair et al 1997) and anti-FKBP51 Hi51 (Nair et al 1997). Anti-Cyp40 rabbit antiserum PA3-022 was purchased from Affinity Bioreagents.

Plasmids expressing Hsp90 forms

Wild-type chicken Hsp90 α cDNA in pGEM-4Z and altered forms of this plasmid construct encoding mutants 381-C, Δ 381-441, Δ 521-540, Δ 548-567, Δ 601-620, Δ 661-677, and N-708 were described previously (Sullivan and Toft 1993). Point mutations were introduced into wild-type Hsp90 α DNA by site-directed mutagenesis using the QuikChange kit (Stratagene). Oligonucleotide primers containing the desired base changes were used according to the supplier's directions. To produce mutant AAVD, codons for glutamic acid in the C-terminal EEVD sequence were altered to alanine codons. For the point mutant expressing G329N the codon encoding glycine at position 329 of chicken Hsp90 α was mutated to a codon for asparagine; likewise, to generate T541I, a codon for threonine at position 541 was altered to code for isoleucine. Altered sequences in the point mutants were verified by automated sequencing.

To produce a protein lacking the first 112 amino acids of Hsp90 α (mutant 113-C), two subcloning steps were used. First, wild-type Hsp90 α /pGEM4Z was digested with SalI and SphI to cut at pGEM 4Z polylinker sites flanking the Hsp90 cDNA insertion site. The digested insert was subcloned into similarly digested pSPUTK (Stratagene) and subsequently digested with KpnI, targeting a unique

site that overlaps codon 113 in the Hsp90 α cDNA, and NcoI, targeting a site in pSPUTK downstream from the insertion site. This product was subcloned into pET30A (Novagen) that had been digested with KpnI and NcoI. The resulting plasmid encodes a 652-amino acid fusion protein whose first 36 amino acids are encoded by pET30a sequence and whose remainder corresponds to amino acids 113–728 of Hsp90 α . For *in vitro* expressions, the plasmids for 113-C and Δ 661–677 contain a T7 promoter, and all other plasmids contain an SP6 promoter.

Crosslinking of Hsp90 dimers

Wild-type and mutant cDNAs were expressed *in vitro* (TnT kit, Promega) in the presence of [35 S]methionine. A 5 μ l aliquot was removed from each synthesis reaction and quantitated for incorporated radioactivity by SDS-PAGE, autoradiography and laser densitometry (Personal Densitometer, Molecular Dynamics). Aliquots (2.5–12 μ l) of each synthesis mixture containing equal amounts of incorporated radioactivity were added to 100 μ l reaction buffer containing 50 mM potassium phosphate, pH 7.0, and 500 mM NaCl. Either the amine-reactive cross-linker Bis(sulfosuccinimidyl)suberate (Pierce) or the non-crosslinking compound sulfo-N-hydroxysuccinimide (Pierce) were added at 5 mM final concentration to a duplicate sample for each Hsp90 product. After 30 min at room temperature, the reactions were quenched by adjusting each to 25 mM Tris and incubating an additional 15 min. Ten microliters of each reaction mixture were resolved by SDS-PAGE and autoradiographed to visualize radiolabeled Hsp90 products. Samples of purified Hsp90 (5 μ g each) were treated with crosslinker or control compound and stained with Coomassie blue following SDS-PAGE.

Co-precipitations of Hsp90 forms with Hsp90-associated proteins

WT and mutant Hsp90 cDNAs were expressed *in vitro* and labeled with [35 S]methionine. A 2 μ l aliquot was removed from each synthesis reaction and quantitated for incorporated radioactivity by SDS-PAGE, autoradiography and laser densitometry. To determine equimolar amounts, O.D. values were adjusted for the number of methionines in each Hsp90 form.

Each set of immunoaffinity resins (PR22 control, F5, Hi51, Hi52C, anti-Cyp40, or JJ3) was prepared by pre-adsorbing antibody to protein A-Sepharose (Pharmacia). The immunoprecipitation media were prepared by combining 150 μ l rabbit reticulocyte lysate (RL) supplemented with an ATP regenerating system (ATPRS; 4.5 mg phosphocreatine and 7 units of creatine phosphokinase per ml RL) and 3–15 μ l of one of the radiolabeled Hsp90

synthesis mixtures (11 different Hsp90 forms tested). Since FKBP51 and FKBP52 are present at low concentrations in RL, the immunoprecipitation media for these proteins were supplemented with purified recombinant human protein (20 μ g/ml final concentration) (Nair et al 1997). A 4 μ l aliquot of each final mixture was removed for gel analysis to monitor the actual levels of incorporated radioactivity present. Individual precipitation reactions were performed in triplicate with each tube containing 50 μ l of the appropriate immunoprecipitation medium and a 10 μ l resin pellet adsorbed with 10 μ g monoclonal antibody or, in the Cyp40 samples, antibody from 3 μ l antiserum.

Precipitation mixtures were all incubated at 30°C for 45 min with a brief vortex every 3 min. to keep resin suspended. At the end of the incubation period, samples were immediately diluted with 1 ml ice-cold wash buffer (WB; 20 mM Tris, pH 8.0, 50 mM NaCl, 10 mM monothioglycerol, 0.5% Tween 20). Resin pellets were collected by briefly centrifuging the reaction tubes, and each pellet was washed three additional times with 1 ml ice-cold WB. Proteins were extracted from resin pellets by boiling with SDS sample buffer and separated by SDS-PAGE. Gels were Coomassie stained to visualize total proteins in each sample, and dried gels were autoradiographed to visualize radiolabeled Hsp90 forms. Due to the different levels of coprecipitating Hsp90 in the different sets, exposure times for autoradiographs ranged from 7 h (Hop and p23) to 4 days (FKBP51, FKBP52, Cyp40 and the PR22 control).

Association of Hsp90 forms with progesterone complexes

PR for assembly studies was generated through a baculoviral expression plasmid in Sf9 cells. Briefly, the cDNA for chicken PR form A was subcloned into the pAclIT transfer vector and co-transfected with baculoviral DNA into Sf9 cells following the manufacturer's recommendations (BaculoGold kit; PharMingen). Soluble cell extracts were prepared from Sf9 cells expressing recombinant PR by freeze-thawing in 20 mM Hepes, pH 7.8, 500 mM KCl, 20% glycerol.

For PR assembly studies, receptor was first isolated from Sf9 cell extract by binding to PR22/protein A-Sepharose. After washing, PR resin was aliquotted for individual assembly reactions. Each assembly mixture contained PR-resin (1 μ g PR-A and 10 μ g PR22 on a 10 μ l resin pellet) plus 150 μ l RL supplemented with an ATPRS and one of the radiolabeled Hsp90 synthesis mixtures (5–15 μ l). In two sets of assembly reactions, one set contained 20 μ g/ml geldanamycin (GA) and the other set lacked GA. All samples were incubated at 30° for 30 min, washed 4 $\times$ with ice-cold WB, and extracted into SDS sample buffer. SDS-PAGE separations were

Fig. 1 Mutant forms of avian Hsp90. The cDNA for chicken Hsp90 α was mutated in several ways to code for the protein products diagramed here. Numbers represent amino acid positions along the 728-amino acid sequence of chicken Hsp90 α . Point mutations are indicated above the bar, and truncated/deleted regions are indicated by lines.

Coomassie stained to visualize total proteins, and the dried gels were autoradiographed to visualize radiolabeled Hsp90 forms.

RESULTS

A diagram of the mutants used in this study is shown in Figure 1. All of these were generated from a chicken Hsp90- α cDNA. The three point mutants and 113-C are novel avian Hsp90 mutants while the other deletion and truncation mutants have been previously described (Sullivan and Toft 1993). Mutant AAVD has alanines substituted for glutamates in the highly conserved EEVD sequence that occurs at the C-terminus of Hsp90 and many Hsp70 forms. Mutants G329N and T541I correspond to two randomly generated point mutants of yeast Hsp90 that in genetic screens for steroid hormone action were unable to complement the loss of yeast Hsp90 (Bohen and Yamamoto 1993).

Each of the Hsp90 forms was generated as a radiolabeled protein by expressing the cDNAs in an *in vitro* transcription/translation system based on rabbit reticulocyte lysate (RL). The radiolabeled products are shown in the autoradiogram in Figure 2. As is typical for proteins synthesized *in vitro*, there are smaller products generated along with the larger desired product. These most often result from initiation of protein synthesis at methionine codons down-stream from the desired start

Fig. 2 Expression of Hsp90 forms. WT Hsp90 and mutated cDNAs were expressed by combined *in vitro* transcription/translation in the presence of [35 S]methionine. Two microliters of each synthesis mixture (4 μ l for 381-C and Δ 381-441 because of their low expression levels) were separated by SDS-PAGE and the radiolabeled products were visualized by autoradiography of the dried gel (1 h exposure).

codon. For the co-precipitation trials shown in later figures, an aliquot of the synthesis mix was added to normal RL supplemented with an ATP regenerating system. All samples in a particular experiment contained an equimolar amount of the appropriate Hsp90 product.

Mutant interactions with progesterin complexes

Most of the mutants examined here had been previously described as having some deficiency in interactions with steroid receptors, so the abilities of mutant Hsp90 forms to enter PR complexes were compared. Hsp90 interactions with PR in intermediate and mature complexes were differentiated by the inclusion of GA in one set of assemblies. Mature PR complexes containing Hsp90, p23, and any one of the three Hsp90-binding immunophilins dominate under normal assembly conditions, but are absent in the presence of GA (Johnson and Toft 1995; Smith et al 1995). GA does not block formation of intermediate complexes containing Hsp90, Hsc70, Hip and Hop (Smith et al 1995).

Following *in vitro* assembly reactions in the presence of one radiolabeled Hsp90 form, PR complexes were separated by SDS-PAGE, and the gels were Coomassie-stained and autoradiographed. Radiolabeled mutant Hsp90 bands were quantitated and compared with the binding level of wt Hsp90 to provide a relative measure of their abilities to enter PR complexes (Fig. 3).

In the presence of GA (hatched bars), the amount of radiolabeled wt Hsp90 recovered in PR complexes is reduced approximately 50% from levels in the absence of

Fig. 3 Association of Hsp90 forms with progesterone receptor. PR complexes were assembled in vitro using RL containing one of the radiolabeled Hsp90 forms and either lacking or containing GA (20 μ g/ml final concentration). Radiolabeled forms were present in each assembly mixture at the same molar concentration. PR-associated components were resolved by SDS-PAGE, and the gels were both Coomassie stained and autoradiographed. Bands on the gels and X-ray films were quantitated by densitometry; the O.D. values for radiolabeled Hsp90 forms were normalized to the amount of Coomassie-stained native Hsp90 in each lane. The adjusted values were plotted relative to the level of radiolabeled WT in the minus-GA sample. For deletion mutants, the plot labels contain only the first portion of each name.

GA (open bars). This is fully consistent with earlier results and reflects the loss of mature complexes and a partially compensating increase in intermediate complexes (Smith et al 1995). Compared to wt Hsp90, mutant AAVD was somewhat reduced in its ability to associate with mature PR complexes but was more markedly impaired in its association with intermediate complexes. Mutant T541I was recovered at approximately 40–50% of wt levels in both mature and intermediate complexes. All other mutants were present in either mature or intermediate PR complexes at less than 20% of wt's level.

In establishing why a particular Hsp90 mutant associates poorly with PR, one must account for various biochemical interactions of Hsp90 during assembly. Since Hsp90 binds directly to PR, at least in mature complexes, there could be a defect in Hsp90's target-binding site. On the other hand, formation of mature complexes follows an ordered pathway requiring the function of several Hsp90-associated proteins. Defects in Hsp90's interaction with one or more accessory proteins might also disfavor its association with PR, even if Hsp90's target-binding site is intact. Furthermore, Hsp90 normally functions as a dimer, so a defect in dimerization may impair its binding to PR or to accessories.

There are also some technical factors in the methodologies used here that must be considered. First, the radiolabeled Hsp90 forms added to RL are competing with a much larger pool of unlabeled, endogenous wt Hsp90.

Fig. 4 Dimerization of Hsp90 forms. Radiolabeled Hsp90 synthesis mixtures and purified WT Hsp90 (Pur. WT) were treated either with the homobifunctional crosslinker Bis (sulfosuccinimidyl) suberate or with the monofunctional control compound sulfo-N-hydroxysuccinimide. Reaction mixtures contained 0.5 M NaCl to dissociate heteromeric Hsp90 complexes. Samples from two sets of reactions (A and B) were separated by SDS-PAGE and visualized by autoradiography or Coomassie blue stain (Pur. WT). Crosslinked ('Xlink') or control-treated samples are indicated above the lanes.

Therefore, the values shown in Figure 3 represent the relative abilities of mutant Hsp90 forms to compete with wt Hsp90 for PR association and do not directly address whether mutants would be efficiently incorporated into PR complexes if wt Hsp90 were absent. Another consideration, which will be addressed further below, is that the results in Figure 3 probably reflect the behaviors of wt: mutant heterodimers rather than the potentially distinct behaviors of mutant:mutant homodimers.

Dimerization of Hsp90 forms

Dimerization of Hsp90 mutants is addressed in Figure 4. Each mutant was expressed and radiolabeled in vitro, and aliquots of the synthesis mixtures were treated either with the crosslinking agent Bis (sulfosuccinimidyl)

suberate or the monofunctional non-crosslinking compound sulfo-N-hydroxysuccinimide. To prevent crosslinking of Hsp90 with associated proteins, heteromeric complexes were dissociated by adjusting samples to 0.5 M NaCl prior to addition of crosslinking agents. An aliquot of each treated sample was separated by SDS-PAGE and autoradiographed. The final two lanes in Fig. 4B are Coomassie-stained samples of highly purified Hsp90.

In the radiolabeled crosslinked sample lanes, wt Hsp90 and all mutants except $\Delta 661-677$ could be found in a slowly migrating complex. For wt Hsp90 and the larger mutant forms, the crosslinked complex has an apparent molecular mass of approximately 250 kDa. The apparent size variance from the predicted 180 kDa is probably an artifact due either to the chemical modification of Hsp90 or to conformational restrictions imposed by crosslinking. Note that the crosslinked dimer using purified Hsp90 (Fig. 4B, penultimate lane) has exactly the same apparent size of 250 kDa. In the crosslinked lane for $\Delta 521-540$, an additional band larger than the dimer is observed, but the nature of this complex has not been further characterized. The results in Figure 4 are consistent with an earlier study (Meng et al 1996) in which many of these same mutants were tested by alternate means, and $\Delta 661-677$ alone was shown to lack dimerization.

Some proportion, perhaps most, of the crosslinked dimers generated in Figure 4A and B are likely heterodimers between the low abundance radiolabeled chicken Hsp90 form and the much more abundant rabbit Hsp90 endogenous to RL. This is evident for mutant 381-C where the most prevalent crosslinked complex has the slower mobility expected of a heterodimer. Heterodimerization of other mutants was confirmed by co-precipitation assays using an antibody specific for mammalian Hsp90. Monoclonal antibody H90-10, which was prepared against human Hsp90, efficiently binds rabbit Hsp90 in RL but reacts very weakly with Hsp90 in chicken oviduct cytosol (not shown). Radiolabeled chicken Hsp90 forms, except for $\Delta 661-677$, were efficiently recovered in H90-10 immunoprecipitates from RL, indicating an interaction between chicken and rabbit Hsp90s (results not shown).

Co-precipitations with Hsp90 accessories

The abilities of Hsp90 mutant forms to associate with the Hsp90-associated proteins Hop, Cyp40, FKBP51, FKBP52 and p23 were assessed by co-immunoprecipitation assays. To measure Hsp90 interactions with Hop, an affinity resin containing the anti-Hop monoclonal antibody F5 was used to purify Hop complexes from RL supplemented with radiolabeled Hsp90 forms (Fig. 5A). As seen in the Coomassie-stained gel (upper panel), equal

amounts of Hop and the associated proteins Hsp90 and Hsc70 were recovered in each sample. Bear in mind that the Hsp90 seen in the stained gel is the endogenous rabbit protein. Autoradiography of the dried gel for 7 h (lower panel) revealed the presence of radiolabeled chicken Hsp90 forms associated with Hop complexes. A negative control for non-specific adsorption of Hsp90 forms to affinity resin was performed using monoclonal anti-PR antibody PR22 (Fig. 5B). No radiolabeled Hsp90 forms were detected in the control precipitations following a 4-day exposure on X-ray film.

A similar analysis was performed in triplicate for each of the 5 Hsp90 accessories (Fig. 6). A representative Coomassie-stained lane for each immunoprecipitation reaction is shown in Figure 6A. Endogenous Hsp90 from RL can be seen coprecipitating with each accessory but is absent from the control lane. As previously seen in Figure 5A, Hop complexes contain both Hsp90 and Hsc70. In the FKBP lanes, the native FKBP51 are not evident due to their low abundance and comigration with heavy chains, but His-tagged versions of FKBP51 (comigrating with Hop in the adjacent lane) and FKBP52 (the doublet migrating just above heavy chain) that were added to RL can be seen. Native Cyp40, the most abundant of the Hsp90-associated immunophilins in RL (Nair et al 1997), is seen below the anti-Cyp40 heavy chains. The gel region containing p23 was cropped from the image.

Autoradiographs of the full gels were generated, and bands representing co-precipitating Hsp90 forms were quantitated by densitometry. The O.D. values were normalized to the amount of stained Hsp90 present in each lane, adjusted to reflect molar equivalents, and the mean and standard error for each set of triplicates was calculated. These values are plotted relative to wt Hsp90 for each set of immunoprecipitations (Fig. 6B-F).

There was reduced co-precipitation of AAVD and $\Delta 661-677$ with all accessories, suggesting global conformational defects in these mutants. As shown above, $\Delta 661-677$ lacks dimerization, so it appears that the dimerized conformation of Hsp90 is important for many protein interactions. Interestingly, the reduced association of AAVD with various accessory proteins suggests that the highly conserved EEVD at the C-terminus of Hsp90 also influences Hsp90 conformation in a global manner, though not at the level of dimerization.

The TPR-containing proteins offer some striking and surprising differences in their co-precipitation patterns. Looking at just the pattern for Hop (Fig. 6B), note how association with the N-terminal truncation 113-C is quite poor while 381-C, lacking a greater portion of the N-terminus, binds better than 113-C. The internal deletion $\Delta 381-441$ binds poorly to Hop complexes, but co-precipitation of $\Delta 548-567$ is actually enhanced to approximately 150% of wt's level.

Fig. 5 Specific co-precipitation of Hsp90 forms with Hop. (A) Monoclonal antibody F5 adsorbed to protein A-Sepharose was used to precipitate Hop complexes from RL supplemented with an ATP regenerating system and containing one of the radiolabeled Hsp90 forms (an equimolar amount in each sample). After 45 min at 30%, resin pellets were washed, and bound proteins were extracted with SDS sample buffer. Proteins were separated by SDS-PAGE and Coomassie blue-stained (upper panel). Radiolabeled Hsp90 forms were visualized by autoradiography (lower panel; 7 h exposure). Migration positions for Hop, associated proteins, and the F5 heavy chain (HC) are indicated on the right. (B) Identical to (A), except antibody PR22 was used as a negative control and film was exposed for 4 days.

To more easily compare the 4 TPR proteins, some of the data from Figure 6B–E were reorganized (Fig. 7). The patterns for Cyp40, FKBP52 and FKBP51 vary in several ways from Hop. First, note for all three immunophilins that co-precipitation of mutant 113-C is elevated 2- to 3-fold over wt Hsp90 while 113-C binding to Hop is barely detectable. Δ381–441, whose recovery with Hop is poor, is recovered at 60%, 130%, and 300% of wt Hsp90 levels with FKBP51, Cyp40, and FKBP52, respectively. The two remaining deletions shown in Figure 7, Δ548–567 and Δ601–620, are deficient in binding to FKBP51, but bound the other TPR proteins near or above wt's level. Finally, the point mutant T541I binds normally to Hop, Cyp40 and FKBP52, but is recovered in FKBP51 complexes at only 10–20% of wt levels. Overall, Cyp40 and FKBP52 have the most similar patterns of Hsp90 co-precipitations, with FKBP51 and Hop each differing from these in distinctive ways.

Co-precipitations with p23 (Fig. 6F) differed significantly from the TPR-containing accessories. All mutants were deficient in p23 binding, with the least affected being AAVD. The N-terminal truncations 113-C and 381-C completely lost p23 binding, but each deletion in the C-terminal region also impaired p23 binding.

DISCUSSION

Hsp90 assembly with steroid receptors is an ordered, dynamic process involving a number of molecular chaperone components. Five of these components are

commonly found associated with Hsp90 and may serve as accessories that modulate the activity of Hsp90. In this report, we examined the ability of various Hsp90 mutants to interact with these partner proteins by expressing radiolabeled Hsp90 forms in vitro and comparing co-precipitation patterns using antibodies specific for each Hsp90 partner. There are several advantages to this experimental approach. First, Hsp90 forms are being compared in a complex, cell-free environment that retains efficient assembly of PR complexes and the requisite side-assembly pathways. This lends an important degree of physiological relevance to the assay system. Second, multiple Hsp90 mutants can be readily compared for several functional criteria in a common assay. Third, mutant proteins are generated in RL which is more conducive to Hsp90 folding than bacterial expression. The major limitation with this assay is the inability to produce pure homodimers of mutant Hsp90 subunits. In Hsp90 mutants retaining dimerization, functional defects may be partially suppressed by, or partially arise from, heterodimerization of the recombinant mutant with a normal Hsp90 subunit in RL.

Each of the mutant Hsp90s examined here displayed some relative deficiency in associating with PR (Fig. 3), but in no instance could this deficiency be unambiguously linked to a defect in Hsp90's poorly defined target-binding site. The confounding factor for each mutant is its poor interaction with one or more Hsp90 accessory that participates in PR assembly (Fig. 6).

Fig. 6 Relative binding of Hsp90 forms to accessory proteins. Using conditions identical to those in Figure 5, antibodies specific for each of 5 Hsp90-associated accessory proteins were used to co-precipitate radiolabeled Hsp90 forms. For each antibody, reactions were performed in triplicate. (A) A representative Coomassie-stained gel lane from each set of immunoprecipitations. Migration positions for co-precipitating endogenous Hsp90 and antibody heavy chains (HC) are indicated. Each lane is labeled to indicate the Hsp90-associated protein being targeted. Precipitation with an anti-progesterone receptor monoclonal antibody ('Control') was used to monitor non-specific resin binding of proteins to immunoaffinity resins. (B–F) Bands on autoradiographs were quantitated and the O.D. values normalized to the amount of Coomassie-stained endogenous Hsp90 in each lane. Triplicate values were averaged and the standard errors determined. The values are plotted relative to the level of radiolabeled WT Hsp90 in each set.

Fig. 7 Comparison of mutant Hsp90 interactions with TPR proteins. Results selected from Figure 6B–F were reorganized to more easily compare Hsp90 associations of the four TPR proteins.

Some novel conclusions can be drawn from these studies. First, TPR proteins do not bind Hsp90 in an identical manner, supporting the notion that each TPR accessory may modulate Hsp90 function in a distinctive way. Second, the highly conserved MEEVD sequence at Hsp90's C-terminus is important for maintaining normal interactions with several Hsp90 accessories. This is the first demonstration to our knowledge that altering this sequence in any way influences Hsp90 behavior. Finally, interactions between Hsp90 domains in the monomer and/or between dimeric partners have an important impact on Hsp90's overall conformation and function.

The cartoon in Figure 8 summarizes some of the interactions between Hsp90 domains and Hsp90-binding proteins. Briefly, dimerization of Hsp90 appears to occur primarily through a C-terminal site (Fig. 4; Minami et al 1994; Meng et al 1996), but there may be additional, weaker interactions stabilizing the dimer such as one observed between N-terminal fragments of yeast Hsp90 (Prodromou et al 1997b). Some data suggest that p23 binds in or near the ATP-binding domain, but this remains an open question. Hop and the TPR-containing immunophilins interact with the C-terminal half of Hsp90, localizing TPR recognition to this region, but there are also apparent interactions of Hop with Hsp90's N-terminal region. Contemporaneous with Hop's binding, Hsp90 can also bind Hsc70 in an ADP-dependent manner (Chen et al 1996). It is still unresolved where Hsp90 targets such as steroid receptors bind.

Distinctive interactions of TPR proteins with Hsp90

Competition between immunophilins for binding Hsp90 has been demonstrated (Owens-Grillo et al 1995; Nair et al 1997), and the mutual exclusion of Hop and immunophilins from Hsp90 complexes (Owens-Grillo et al 1996) argues that Hsp90 can accommodate only a single TPR protein. Consistent with this possibility, in every instance where there was an increased recovery of an Hsp90 mutant with one or more TPR proteins, there was a corresponding decrease in another TPR protein (Fig. 7).

It is likely that TPR recognition resides in Hsp90's C-terminal half, since binding of TPR proteins is retained when the N-terminal 380 amino acids of Hsp90 are removed. Mutants AAVD and dimerization-deficient Δ661–677 were the only ones having reduced interactions with all four TPR proteins; however, various deletion mutants between positions 380 and 620 and the point mutant T541I are selectively deficient in binding TPR proteins. The TPR recognition site is probably complex, with non-contiguous sequences in the C-terminal half contributing to the binding of each TPR protein. Moreover, patterns of binding to Hsp90 mutants do not strictly correlate with TPR sequence similarity. Cyp40 and FKBP52 have quite similar behaviors with Hsp90 mutants (Fig. 7), but their TPR regions are less than 30% identical. FKBP51's TPR region shares 60% identity with FKBP52, yet FKBP51 differs markedly from FKBP52 and Cyp40 in its interactions with Hsp90 mutants.

Fig. 8 Summary of protein interactions with an Hsp90 dimer. Dimerization occurs primarily through the C-terminal region of Hsp90 (solid bar), but portions of the N-terminal domain (dashed bar) may also contribute to dimer stabilization and conformation. Accessory p23 binds Hsp90 in an ATP-dependent manner, and this binding may localize to the N-terminus. The TPR-containing immunophilins (Imm.) and Hop bind the C-terminal region, but Hop may have additional interactions with Hsp90's N-terminal domain. Hop is also capable of binding Hsc70 in an ADP-dependent manner. The site or sites on Hsp90 for binding targets such as steroid receptors have not been resolved.

The unique interactions of FKBP51 with Hsp90 mutants can be added to a list of behaviors distinguishing FKBP51 and FKBP52. FKBP51's association with PR was found to be particularly sensitive to hormone binding and sulfhydryl modifying agents (Smith et al 1990, 1993c). FKBP51 in complexes with Hsp90 was found to bind an FK506 affinity resin much more weakly than FKBP52-Hsp90 complexes even though both free FKBP51 and FKBP52-Hsp90 complexes bound the resin efficiently (Smith et al 1993b). Recently, we have observed that in vitro association of FKBP51 with PR and GR complexes is greatly favored over FKBP52 and Cyp40 (Nair et al 1997; Barent et al 1998). Mutational analyses of the FKBP51 revealed that FKBP51 has interactions between its TPR region and unique C-terminus that were not observed with FKBP52 (Barent et al 1998). These biochemical differences belie the overall sequence similarity of FKBP51 and FKBP52 and argue for significant conformational differences in the two FKBP51s and potential differences in how they influence Hsp90•target interactions.

Relationship between p23 and Hop binding to Hsp90

As mentioned above, p23 may bind in or near the N-terminal ATP-binding domain of Hsp90. Consistent

with this possibility, truncation of Hsp90's N-terminus (113-C or 381-C) resulted in a complete loss of p23 co-precipitation (Fig. 6F). Countering the possibility of an N-terminal p23 binding site is the failure of N-terminal fragments to bind p23 independently (J. P. Grenert, W. P. Sullivan, D. O. Toft, unpublished observations) and the deleterious effect of C-terminal truncations on p23 binding. Perhaps p23 recognizes an N-terminal conformation that, in addition to ATP-dependent influences, is highly sensitive to interactions with downstream sequences, including but not restricted to the strong dimerization domain.

In contrast to p23, Hop binding to Hsp90 is less sensitive to nucleotides (Chen et al 1996) and unaffected by GA (Smith et al 1995). Similar to p23, however, mutant 113-C exhibited a greater than 90% decrease in its association with Hop. Correlating with the effect of the N-terminus on both p23 and Hop binding, there is a mutually exclusive relationship in the binding of Hop and p23 to Hsp90 (Dittmar et al 1997; Johnson et al 1998). This does not hold for immunophilins since they are readily isolated in co-complexes with Hsp90 and p23 (Johnson and Toft 1994).

Hop thus appears to interact with both N- and C-terminal sites in Hsp90 while immunophilin binding is restricted to the C-terminal region. Binding of Hop to Hsp90 could preclude binding of p23 to Hsp90's N-terminus, and mutants favoring Hop binding might have reduced p23 binding as a consequence. For example, mutant $\Delta 548-567$ displays enhanced binding to Hop and poor binding to p23 (Fig. 6B and F); however, p23 binding remains depressed in other C-terminal deletions having reduced Hop binding. Conversely, binding of p23 could occlude or alter an N-terminal site that helps stabilize Hop binding. Without its N-terminal interactions, Hop binding would be weakened, which would favor, in turn, binding of immunophilins to the C-terminal region of Hsp90, just the behavior seen with 113-C and 381-C (Fig. 7).

Hsp90's EEVD C-terminus

Many eukaryotic forms of Hsp70 and Hsp90 share the C-terminal amino acids EEVD. Mutation of this sequence in Hsc70 was shown to alter its function in several ways, indicating perhaps that its EEVD tail helped to establish Hsc70's global conformational status (Freeman et al 1995). A functional consequence from mutating Hsp90's EEVD has not been previously reported. Truncation of the final 20 amino acids from Hsp90 did not prevent, qualitatively, association of mutant N-708 with PR complexes (Sullivan and Toft 1993). A similar truncation of yeast Hsp90 had little consequence on steroid receptor function in a mutant yeast strain (Louvion et al 1996). Finally, truncation of EEVD has no effect on the ability of

purified recombinant Hsp90 to stabilize misfolded substrates in an in vitro refolding assay (R. I. Morimoto, personal communication).

In the present studies, recovery of the AAVD mutant was inhibited by more than 90% in Hop and Cyp40 complexes, 70–75% in FKBP complexes, and 50% in p23 complexes (Fig. 6). Contrasting with AAVD's deficiency in accessory protein interactions, its recovery in mature PR complexes was reduced by only 25% (Fig. 3). From this it would appear that the EEVD sequence is not absolutely required for PR association. On the other hand, AAVD was decreased by 75% relative to wt Hsp90 in intermediate receptor complexes where Hop function is important (Chen et al 1996; Dittmar et al 1996; Dittmar and Pratt 1997), suggesting that there may be a defect in AAVD's ability to support intermediate complex formation. With the in vitro assembly assay used here, native Hsp90 in RL could support formation of intermediate complexes while AAVD might subsequently enter mature complexes in a functionally passive manner.

In light of our findings with AAVD, the failure of a C-terminally truncated yeast Hsp90 to impair steroid receptor function (Louvion et al 1996) is somewhat surprising. One resolution to these seemingly conflicting observations may relate to the efficiency of Hsp90 function within the chaperone machinery. Sti1, a yeast homolog of Hop, is not absolutely required for GR function in yeast, but Sti1 does facilitate short-term responses to hormone administration (Chang et al 1997). The genetic study using a yeast Hsp90 lacking its EEVD terminus did not address the short-term efficiency of receptor assembly and function.

Hsp90 — general structural considerations

A recent report (Nemoto et al 1997) used proteolytic sensitivity to map three structural domains of human Hsp90 comprised of residues 1–400 (domain A), 400–620 (domain B), and 620–732 (domain C). Comparing with the present results, the structural domains roughly correspond with functional domains for ATP/p23 binding (domain A), TPR-protein binding (domain B), and dimerization (domain C). However, this is clearly a simplistic view of Hsp90 structure/function.

Interpretation of our results and other published results leads to the conclusion that the global conformation of Hsp90 is influenced by many factors. Hsp90 predominantly exists as a dimer, and it is clear from the general functional impairment of the dimerization-deficient mutant $\Delta 661$ –677 that dimerization has important conformational consequences for multiple Hsp90–protein interactions. ATP binding clearly influences the conformation of Hsp90 as judged by its affinity for hydrophobic resins (Sullivan et al 1997), yet little conformational differ-

ence was noted in the crystal structures for N-terminal fragments of Hsp90 occupied with ATP, ADP, or no nucleotide (Prodromou et al 1997a); however, due to the similarity of the ATP-binding domains of Hsp90 and DNA gyrase B, it was proposed that there might be a 'lid-closing' event in soluble Hsp90 that is constrained in the crystal matrix (Prodromou et al 1997a). Conformational changes in the N-terminal ATP-binding domain may be amplified into more profound conformational changes through interactions between the N- and C-terminal regions of Hsp90. Binding of p23 to Hsp90 appears to be a particularly sensitive indicator of Hsp90 conformational status, since it is dependent on the ATP-bound conformation (Grenert et al 1997) and is negatively influenced by a variety of mutations throughout Hsp90 (Fig. 6F). The EEVD terminus of Hsp90, analogous to conclusions drawn from mutations of Hsc70's EEVD terminus (Freeman et al 1995), potentially interacts with upstream sequences, either within a monomer or perhaps between dimer partners, and has a global influence on Hsp90 interactions with accessory proteins. Clearly, there are multiple structural factors determining Hsp90's ability to associate with each of its various partner proteins. Understanding Hsp90 actions on target proteins will necessitate a better understanding of Hsp90 structure at the intramolecular and dimeric levels and the influence of accessory proteins on Hsp90 structure and function.

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