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Multiple Domains of the Co-chaperone Hop Are Important for Hsp70 Binding*

The Hop/Sti1 co-chaperone binds to both Hsp70 and Hsp90. Biochemical and co-crystallographic studies have suggested that the EEVD-containing C terminus of Hsp70 or Hsp90 binds specifically to one of the Hop tetraproteptide repeat domains, TPR1 or TPR2a, respectively. Mutational analyses of Hsp70 and Hop were undertaken to better characterize interactions between the C terminus of Hsp70 and Hop domains. Surprisingly, truncation of EEVD plus as many as 34 additional amino acids from the Hsp70 C terminus did not reduce the ability of Hsp70 mutants to co-immunoprecipitate with Hop, although further truncation eliminated Hop binding. Hop point mutations targeting a carboxylate clamp position in TPR1 disrupted Hop70 binding, as was expected; however, similar point mutations in TPR2a or TPR2b also inhibited Hsp70 binding in some settings. Using a yeast-based in vivo assay for Hop function, wild type Hop and TPR2b mutants could fully complement deletion of TPR1 mutant and TPR2a mutants could partially restore activity. Conformations of Hop and Hop mutants were probed by limited proteolysis. The TPR1 mutant digested in a similar manner to wild type; however, TPR2a and TPR2b mutants each displayed greater resistance to chymotryptic digestion. All point mutants retained an ability to dimerize, and none appeared to be grossly misfolded. These results raise questions about current models for Hop/Hsp70 interaction.

Native interactions of the major molecular chaperones Hsp70 and Hsp90 with known client proteins typically involve several attendant co-chaperone proteins. Among the activities attributed to partner co-chaperones are regulation of chaperone ATPase activity or nucleotide exchange (1, 2), recruitment of additional chaperones and co-chaperones (3), targeting of the client complex for degradation (4), or subcellular localization (5). A commonly studied chaperone/client system is the assembly pathway for steroid receptor complexes in which multiple chaperones and co-chaperones participate in a multistep, dynamic assembly process (6, 7). The co-chaperone Hop, which can bind both Hsp70 and Hsp90, plays a key role in recruiting Hsp90 to preexisting receptor-Hsp70 complexes (3, 8, 9), thus promoting assembly progression and ultimate functional maturation of the receptor.

Similar to many Hsp70 and Hsp90 co-chaperones, tetraproteptide repeat (TPR)1 domains of Hop mediate binding to Hsp (10, 11). Whereas the Hsp-binding co-chaperones most often contain a single TPR domain, Hop contains three distinct TPR domains. The C-terminal region of cytoplasmic forms of Hsp70 and Hsp90, both of which terminate with the amino acid sequence EEVD, have been implicated as the binding sites for Hop and for some other TPR co-chaperones (12–14); however, some TPR co-chaperones clearly interact with other Hsp regions (15, 16). Since Hop contains multiple TPR domains, the opportunity exists for Hop to simultaneously bind the EEVD sites of both Hsp70 and Hsp90.

In a major advance toward understanding how TPR domains interface with the Hsp EEVD region, co-crystal structures were obtained for either of two Hop TPR domains complexed with an EEVD-containing peptide (14). One co-crystal contained the N-terminal TPR domain of Hop, TPR1, and the octapeptide GPTIEEVD that corresponds to the Hsp70 C terminus. The other co-crystal contained one of the central TPR domains, TPR2a, in complex with the pentapeptide MEEVD that corresponds to the C-terminal sequence of Hsp90. Similar to some other reported TPR domains, both of the Hop TPR domains consisted of an antiparallel α-helical stack that forms a large groove along one surface of the domain. The EEVD-containing peptides lodged within this groove but in distinct orientations that related to TPR side-chain differences within the groove and unique amino acids in either peptide. In both co-crystal structures, basic TPR side chains within the groove formed salt bridges with acidic peptide side chains, forming what was termed the carboxylate clamp. Consistent with the TPR2a co-crystal structure, point mutation of a conserved carboxylate clamp position in any of several Hsp90-binding TPR co-chaperones has been shown to disrupt Hsp90 binding (13).

The co-crystal results suggest a straightforward model for an Hsp70-Hop-Hsp90 complex in which the C terminus of Hsp70 binds TPR1 of Hop and the C terminus of Hsp90 binds TPR2a of Hop. However, experimental results suggest that additional Hop domains influence Hsp binding. For instance, mutations within the C-terminal DP-domain of Hop greatly inhibit Hsp70 binding (3, 17), and deletions within TPR2b, the third TPR domain of Hop, can reduce Hsp90 binding (18). Furthermore, as shown below, truncation of the EEVD from Hsp70 had little effect on Hop binding. To help resolve relevant interactions between full-length proteins, we have taken advantage of crystallographic structures and recent insight from sequence comparisons between Hop regions to design novel Hop point mutants that were assessed for Hsp binding, support of steroid receptor function in vivo, and conformational changes.

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The abbreviations used are: TPR, tetraproteptide repeat; GST, glutathione S-transferase; RL, reticulocyte lysate; DOC, deoxy cortico-sterone; PR, progesterone receptor; GR, glucocorticoid receptor.
**EXPERIMENTAL PROCEDURES**

*Hop Interactions with Hsp70*

**cDNA Mutagenesis and Construction of Expression Plasmids—** A series of Hsp70 C-terminal truncation mutants was generated by introducing corresponding cDNA into the rat Hsp70 cDNA. Mutant cDNAs were generated by site-directed mutagenesis (QuickChange™ kit, Stratagene, La Jolla, CA) using the *in vitro* expression plasmid rat Hsc70/pSPUTK as template. The following mutants were generated, according to the final amino acid encoded: N642, N614, N608, N604, N595, N575, N534, and N425.

Mutant cDNAs were generated in a similar manner using human Hop/pSPUTK as template. Mutations in the following domains were identified: TRP1, Y27A, K73E, and K73A; TRP2a, K301E, K301A, R305E, and R305A; TRP2b, K429E, K429A, R433E, and R433A; DP1, D140A and D149A; AP1, D140A/D149A. A mutant in the DP2 domain (AP2) was constructed previously (3). Sequences for mutagenic primers are available on request. All mutant cDNA sequences were verified by automated sequencing.

For bacterial expression of recombinant proteins, mutant and wild type cDNA were cloned into PET28 for expression of untagged proteins in bacteria, pET30 for expression of His-tagged proteins, and pGEX-5X-3 for expression of GST-tagged proteins. When mutagenesis or PCR products were employed for subcloning, all insert sequences were verified by automated sequencing.

*In Vitro Binding Assays—* Radiolabeled Hop70 or Hop forms were generated by *in vitro* expression ( TNT Kit; Promega, Madison, WI) in the presence of [35S]methionine. A 5-μl aliquot of each synthesis reaction was separated by SDS-PAGE and visualized by autoradiography; the labeled products were quantitated by densitometry (Fluor-2 Multi-Image Software). A pool of cellular equivalents of each radiolabeled product were added to rabbit reticulocyte lysate (1:1 lysate, Green Hectors, Oregon, WI) for immunoprecipitation and receptor assembly trials. Mouse monoclonal antibodies specific for Hop (F5), Hsp70 (BB70), or Hsp90 (H90-10) were preadsorbed to protein G-Sepharose (1 μg of antibody/μl of packed resin). For each immunoprecipitation reaction, 100 μl of reticulocyte lysate (RL) supplemented with a radiolabeled product was added to a 10-μl immunoresin pellet. The mixtures were incubated for 30 min at 30 °C with brief vortexing every 5 min. Resin-bound complexes were washed four times in 1 ml of wash buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5% (v/v) Tween 20), resuspended in 20 μl of 2× SDS-PAGE sample buffer, and separated by SDS-PAGE. Gels were Coomassie-stained to visualize total proteins and then dried and autoradiographed to visualize bound radiolabeled proteins.

Comparisons of wild type and mutated Hop forms assembling with PR complexes were performed as described previously (3). Briefly, recombinant chicken PR was immunopurified from Sf9 insect cell extracts, washed cell pellets were resuspended in cracking buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, and 20 mM β-mercaptoethanol), and incubated for 1 h at 4 °C. Resin-bound complexes were washed three times in 1 ml of wash buffer, and bound proteins were separated by SDS-PAGE. Gels were stained with Coomassie Blue followed by autoradiography of dried gels. Bands on x-ray film were quantitated by densitometry.

**YEAST ASSAYS**

*Yeast Cell Extracts and Western Immunobots—* To prepare whole cell extracts, washed cell pellets were resuspended in cracking buffer (8 m urea, 5% (w/v) SDS, 40 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.04% (w/v) bromphenol blue) at 4 mg/l of cells. Cells were homogenized with glass beads in a Mini Bead Beater (Biospec Products, Bartlesville, OK). Cell homogenates were centrifuged to remove nuclei and debris, and separated at 95 °C for 5 min. Lysate aliquots (10–15 μl) were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and immunostained for Sti1p (mouse monoclonal IgG ST-2), Hop (mouse monoclonal IgG F5), or the yeast ribosomal protein L5 (mouse monoclonal IgG anti-L3).

**Protein Expression and Purification—** BL21 bacterial cells were transformed with each of the various expression plasmids for protein expression. Briefly, 1 l of LB medium plus 100 μg/ml ampicillin was inoculated 1:100 with an overnight culture of transformed bacteria. Cells were grown at 37 °C until the A600 reached 0.5, at which time 100 μl of isopropyl-β-D-galactopyranoside was added to a final concentration of 0.4 mM. Cells were incubated for an additional 3 h at room temperature. Cell homogenates were resuspended in 10 ml of buffer A (20 mM Tris, pH 7.5, 1 mM EDTA, and 5 mM dithiothreitol plus Complete Mini protease inhibitor mixture (Roche Applied Science)) and sonicated to generate cell extracts.

His-tagged proteins were purified by metal affinity chromatography according to manufacturer’s instructions (Qiagen, Valencia, CA). Extracts were applied to Ni2+-nitrilotriacetic acid affinity resin and incubated for 1 h at 4 °C. Resins were washed three times in 1 ml wash buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, and 20 mM imidazole). Protein was eluted at 250 mM imidazole.

GST-tagged bacterial extracts were rocked batchwise with glutathione-4B resin (Amersham Biosciences) for 1 h at 4 °C. Proteins were eluted from the glutathione resin with 50 mM Tris-HCl containing 10 mM reduced glutathione.

Untagged Hop forms were purified by three-step chromatography (Akta FPLC, Amersham Biosciences). First, bacterial extracts containing untagged proteins were loaded on a HisPrep16/10 Heparin-Sepharose column equilibrated in buffer A, and proteins were eluted with a salt gradient of 0–0.5 mM KCl in buffer A. Peak fractions containing Hop were identified by gel electrophoresis and pooled. For the next purification step, samples were loaded on a Resource Q column and eluted with 0–500 mM KCl. Peak fractions were collected and then applied to a 16/60 Superdex 200 column equilibrated with 20 mM Tris, pH 7.5, 150 mM NaCl, and 5 mM MgCl2. Final peak fractions were collected and concentrated using Amicon Centricon-10 filters (Millipore Corp., Billerica, MA). The purity of all Hop forms was judged to be greater than 95%.

**GST-Hop Pull-down Assays—** Prior to purification and quantification GST-tagged Hop forms were incubated batch-wise with glutathione-Sepharose 4B (5 μg of protein/μl) for 20 μl of bed volume on a rotator at room temperature on a rocking platform and washed three times in 1 ml of incubation buffer (20 mM Tris, pH 7.5, 100 mM KCl, 5 mM MgCl2, and 2 mM dithiothreitol). Resin pellets were resuspended in 150 μl of incubation buffer plus 10 μg of purified Hop70 for 30 min at 30 °C. Resin pellets were subsequently washed three times in 1 ml of ice-cold incubation buffer containing 0.01% Nonidet P-40. Resin pellets were eluted with SDS sample buffer, separated on gels, and visualized by Coomassie Blue staining.

**Protease Digestion of Recombinant Hop Forms—** Purified His-tagged Hop forms were subjected to partial proteolytic digestion (21) using...
known structural/functional domains of Hsp90, Hsp70, and Hop are illustrated in Fig. 1A. Hsp90 contains an N-terminal ATPase domain (22), a large middle domain (23), and a C-terminal region of unknown conformation that contains a strong dimerization site. There is evidence for peptide binding by both the ATPase and middle domains of Hsp90 (24). Hsp70 contains an N-terminal ATPase domain (25) unrelated to the corresponding Hsp90 domain, a central peptide binding domain (26), and a C-terminal 10-kDa domain (27). Hop contains three TPR domains plus a C-terminal DP domain and a putative central DP domain (further described below). As indicated, TPR1 is thought to bind the C-terminal PTIEEVD sequence of Hsp70, whereas Hop TPR2a is thought to bind the C-terminal MEEVD sequence of Hsp90 (14).

In Fig. 1B, the TPR2a/MEEVD co-crystal structure is depicted in two alternate views. The TPR motifs in antiparallel α-helices form the peptide binding groove. Positively charged side chains of Arg305 and Lys301 that form the TPR2a carboxylate clamp are highlighted, as are the negatively charged side chains of the MEEVD peptide. The TPR1/GPTIEEV sequence of Hsp70, whereas Hop TPR2a is thought to bind the C-terminal MEEVD sequence of Hsp90 (14).

To test directly which C-terminal sequences of Hsp70 are required for Hop binding, a series of C-terminal truncation mutants was prepared (Fig. 2A), and each mutant was compared with full-length Hsp70 in a Hop co-immunoprecipitation assay (Fig. 2B). The smallest truncation (N642) lacks the terminal EEVD site, and each mutant progressively removes upstream highly conserved sequence patches (solid underlined regions). Equivalent amounts of radiolabeled Hsp70 forms were added to rabbit reticulocyte lysate, and the Hsp90-Hop-Hsp70 complex was immunoprecipitated using an anti-Hop antibody. Proteins were separated by SDS gel electrophoresis and visualized by Coomassie staining for total proteins (top panel) or by autoradiography to detect bound radiolabeled Hsp70 forms (middle panel). Stained bands, which represent rabbit Hsp90 and Hsp70 that co-precipitate with Hop and the anti-Hop heavy chain (HC), are indicated on the left.
Contrary to expectations, truncation of the C-terminal EEVD (N642) or more extensive truncations (N614 and N608) had little effect on recovery of Hsp70 in Hop complexes. Only when the final 40–50 amino acids were truncated (N604 and N595) was there a major loss of Hop binding. An additional truncation mutant that terminates at position 575 also failed to bind Hsp70 (not shown), thus minimizing the likelihood that N604 and N595 artifactually block Hop access to upstream binding sites. On the other hand, in probing the region from 590 to 606 that contains sequences necessary for Hop binding, several single and double point mutations were generated, but none of these inhibited Hop binding (not shown). Point mutant results argue against the presence of a discrete Hop binding site in this region. Although this limited mutational analysis does not identify sequences in the C-terminal region of Hsp70 that are sufficient for Hop binding, we can conclude that the EEVD and adjacent GGMP repeat region are not required for Hop binding in our assay.

Mutagenic Analysis of Hop Domains—To further explore Hop/Hsp70 interactions, we generated domain-specific point mutants of Hop to verify that TPR1 is required and to test whether other Hop domains participate in Hsp70 binding. Careful sequence analysis of Hop revealed a previously overlooked symmetry between the N-terminal and C-terminal regions of Hop. Fig. 3 shows an alignment of TPR1 plus downstream sequences compared with TPR2b and downstream sequences. We have previously shown that sequences downstream from TPR2b contain a DP-repeat motif (underlined sites) that contributes to an independent structural domain (17). Truncation or point mutations within the so-called DP domain lessens binding of Hop to Hsp70 without affecting Hop binding to Hsp90. As the alignment in Fig. 3 reveals, there is similarity between the C-terminal DP domain and sequences correspondingly positioned downstream from TPR1. We refer to this recently recognized N-terminal DP region as DP1 and the original DP domain as DP2.

Point mutants were generated for each of the three TPR domains as well as the two DP regions. Within the TPR domains, point mutations targeted basic residues in the carboxylate clamp. For TPR2b, whose structure has not been solved, basic amino acids corresponding to the carboxylate clamp positions in TPR1 were mutated (double underlines in Fig. 3). In other Hsp90-binding TPR proteins, point mutations of basic amino acids that form the carboxylate clamp have been shown to efficiently disrupt Hsp90 binding (13, 28–30). In the present study, two alternative rationales were used for point mutagenesis of carboxylate clamp positions. First, either Lys or Arg was converted to glutamic acid, thus reversing the charge at this position. Since the charged side chain projects into the solvent space of the binding groove, we reasoned that reversing the charge would not impact overall domain folding while maximizing inhibition of interactions with a negatively charged substrate. Second, alanine substitution was used to neutralize the basic charge while minimizing the likelihood for unintended conformational changes. Both carboxylate clamp positions were separately mutated for TPR2a (Lys173 or Arg305) and TPR2b (Lys429 or Arg433). A single carboxylate clamp position was mutated in TPR1 (Lys170), and we used a previously reported (31) TPR1 point mutant that disrupts Hsp70 binding (Y27A). For the DP2 domain, we used the previously reported AP2 construct (3) in which Asp of both DP repeats was substituted with Ala. For DP1, we generated single alanine mutants (D140A and D149A) plus the combined double mutant (AP1).

Wild type and mutant cDNAs were expressed in vitro to generate radiolabeled products. Molar equivalents of each radiolabeled form were added to RL and tested for co-immunoprecipitation with Hsp90 or Hsp70 or tested for assembly with progesterone receptor (PR) complexes (Fig. 4). For the TPR carboxylate clamp mutants, data are only shown for the glutamic acid substitutions; however, the alternative alanine substitutions gave essentially identical results (not shown).

Looking first at Hsp90-binding properties of the mutants (Fig. 4, upper set of panels), only mutations in TPR2a were defective for binding Hsp90. This finding is consistent with earlier reports that have linked TPR2a with Hsp90 binding. Normal Hsp90 binding was observed with TPR2b point mutants, which contrasts somewhat with an earlier report that deletions within TPR2b inhibit Hsp90 binding (18). However, weakened binding by the earlier deletion constructs may be attributable to unintended alterations in Hop conformation that impacted TPR2a activity.

Multiple domain mutants affected Hsp70 binding (Fig. 4, middle set of panels). First, TPR1 mutants lost binding, an observation consistent with earlier indications that this domain plays a key role in Hsp70 binding. The DP2 mutant (AP2), as seen before, also had greatly reduced binding to Hsp70. Unexpectedly, however, we observed that both TPR2a and TPR2b mutants failed to bind Hsp70, indicating for the first time that these domains somehow influence Hsp70 interactions. DP1 mutants were the only domain mutants that retained normal binding to Hsp70.

The ability of Hop mutants to assemble with PR complexes (Fig. 4, lower set of panels) mostly corresponded with deficiencies in Hsp90 or Hsp70 binding. Exceptions are TPR2b mutants that fail to bind Hsp70 yet appear to assemble normally with the PR complex. Note that TPR1 mutants retain a partial ability to assemble with PR complexes despite our observation that these mutants bind poorly to Hsp70. Therefore, it is still possible for Hop mutants that are defective in Hsp70 binding to assemble with receptor complexes, presumably reflecting inter-
actions with receptor-associated Hsp90. Finally, in this particular data set, the DP1 mutant D140A appears to assemble less well with PR, but this apparent defect was not reproducible in replicate experiments.

Due to the unexpected finding that point mutants of TPR2a and TPR2b, as well as the TPR1 mutants, failed to co-purify with Hsp70 complexes from RL, these mutants were directly tested for Hsp70 binding by a GST pull-down approach (Fig. 5). A series of GST fusion proteins was prepared in which glutathione S-transferase was fused to the N terminus of wild type Hop or Hop mutants. Either GST alone or one of the GST-Hop constructs was purified in which glutathione S-transferase was fused to the N terminus of wild type Hop or Hop mutants. Either GST alone or one of the GST-Hop constructs was purified in which glutathione S-transferase was fused to the N terminus of wild type Hop or Hop mutants. Either GST alone or one of the GST-Hop constructs was purified. Specific binding of Hsp70 to each Hop form was estimated from densitometric analysis of gel bands from four separate experiments after subtracting the level of nonspecific Hsp70 (GST alone) from each sample. As seen in a summary of these data (Fig. 5B), Hsp70 bound R305E (TPR2a mutant) equally as well as wild type Hop, in contrast with reduced co-immunoprecipitation from RL (Fig. 4). However, consistent with the RL co-precipitation results, K73E (TPR1) and K429E (TPR2b) were both defective for binding Hsp70. Therefore, it consistently appears that TPR2b along with TPR1 is somehow involved in Hsp70 binding. TPR2b, on the other hand, impacts Hsp70 binding in a context-dependent manner that is evident only in the more complex RL environment.

**Fig. 4. Interaction of Hop point mutants with Hsp90, Hsp70, and progesterone receptor.** Radiolabeled wild type and mutant forms of Hop were synthesized in vitro. A molar equivalent of each form (Input; autoradiograph in uppermost panel) was added to aliquots of reticulocyte lysate prior to immunoprecipitations of Hsp90 (top panel pair) or Hsp70 (middle panel pair) or assembly of PR complexes (bottom panel pair). The upper panel of each pair is a Coomassie-stained gel to visualize total proteins, and the lower panel is the corresponding autoradiograph that shows the level of each radiolabeled Hop form recovered in respective complexes.

**Fig. 5. Direct interactions between purified Hsp70 and Hop forms.** A, purified GST fusion proteins (5 μg) or a molar equivalent of GST alone was immobilized on glutathione resin (20 μl pellet) and incubated with purified Hsp70 (20 μg) for 30 min at 30°C (total volume 150 μl). After washing, bound proteins were eluted from resin with SDS sample buffer, separated on gels, and visualized by Coomassie staining. The region of the stained gel containing Hsp70 and the GST-Hop fusion (~80 kDa) is shown. A corresponding image of the smaller GST alone band is inserted in the first lane. B, gel bands from four replicate experiments were quantitated by scanning densitometry. The OD value of Hsp70 bound nonspecifically to GST alone was subtracted from other Hsp70 OD values to obtain specific binding levels. The average and S.D. values for Hsp70 recovered in each sample are plotted.

**Cellular Activity of Hop Mutants—**A physiological role for Hop in steroid receptor signaling was first demonstrated in a yeast model, where Lindquist and co-workers (32) showed that deletion of the gene for STI1, the close S. cerevisiae ortholog of vertebrate Hop, impaired the transcriptional activity of glucocorticoid receptor. Sti1p and human Hop share ~50% amino acid sequence similarity, and we have shown that Sti1p can functionally substitute for Hop in a cell-free assay for steroid receptor assembly (3). To test the functional ability of Hop mutants in vivo, we first established that human Hop could functionally replace Sti1p in a yeast model for steroid receptor function. Wild type or a strain lacking the STI1 gene (sti1Δ0) was transformed with a plasmid that constitutively expresses rat GR and a lacZ reporter plasmid under control of glucocorticoid-responsive elements. Additionally, sti1Δ0 cells were transformed with an empty vector or a plasmid constitutively expressing human Hop. Hormone-induced β-galactosidase activity was measured in cells treated or not with 50 nM DOC, a preferred GR agonist in the yeast model. As shown in Fig. 6A, DOC stimulated a 15-fold increase in enzymatic activity in wild type cells. Deletion of STI1 abrogated hormone-induced reporter activity, but stimulation was fully restored in sti1Δ0 cells expressing human Hop. The ability of Hop mutants to rescue the sti1Δ0 phenotype was tested in similar assays (Fig. 6B). Mutants in TPR1 (K73E) and TPR2a (R305E) could partially restore GR activity but only to ~50% the level of wild type Hop. No significant loss of activity was observed with the TPR2b mutant (K429E), and the DP-1 double mutant (AP1) consistently displays somewhat enhanced activity when compared with wild type Hop. The only mutant with a complete lack of activity is the DP2 mutant (AP2).

Since reduced function in this assay could be due to differences in protein expression levels rather than inherent differences in activity, yeast culture extracts were assayed for relative Hop protein levels by Western immunoblotting (Fig. 6C).
this modest difference is unlikely to account for the complete loss of activity in the AP2 strain.

Conformational Analysis of Hop Mutants—We have previously reported that AP2 and other mutations in the DP2 region display heightened sensitivity to proteolytic digestion and altered partial proteolytic patterns. As shown in Fig. 7, Hop domain mutants were analyzed by partial proteolysis for indications that mutant conformation differs from wild type Hop. Purified recombinant proteins were digested with chymotrypsin, which cleaves at positions with large hydrophobic side chains or with subtilisin Carlsberg, which cleaves more nonspecifically. Trypsin, which cleaves at basic amino acids, was not employed, since mutation of basic amino acids in the TPR mutants would confound interpretation of digestion patterns. For subtilisin and chymotryptic digests, 2–4 replicate digests were analyzed for pattern reproducibility, and, in the case of TPR mutants, both glutamic acid and alanine substitutions were tested. The fragment patterns obtained were consistent between replicates and between the alternative TPR mutants.

In comparing chymotryptic digestion patterns (Fig. 7A), there were only a few qualitative differences in band patterns that we consistently observed. The most notable difference is the resistance of certain mutant forms to initial cleavage. This is perhaps best illustrated by comparing half-lives of full-length proteins (Fig. 7B). Bands corresponding to full-length protein were quantitated by densitometry, and values were plotted versus duration of digestion. From these data, the half-life (i.e. time at which one-half the original amount of full-length protein remains) was calculated for each Hop form. Full-length wild type was degraded with a half-life of ~40 s. In contrast, the TPR2a and TPR2b mutants (R305E and K429E, respectively) had half-lives of 100–120 s, indicating that these mutants are more resistant to chymotryptic cleavage. The TPR1 and DP mutants varied less from wild type.

Hop forms were similarly subjected to limited digestion with subtilisin, but, in contrast to chymotryptic digests, full-length wild type and mutant proteins were cleaved at similar rates. As an example, the rates of subtilisin digestion for wild type and R305E were identical as were the major fragment patterns (Fig. 7C), although differences in less abundant fragments were observed. The subtilisin results suggest that overall conformations between mutants are similar, whereas the chymotryptic results suggest a change in the TPR2a and TPR2b mutant conformations such that enzyme access is restricted at one or more particularly sensitive cleavage sites.

Hop is reported to exist in a dimeric state (33, 34), although the structural basis and functional significance of dimerization is unknown. To test whether TPR or DP1 mutants might affect the Hop dimeric state, purified proteins were analyzed by gel filtration (Fig. 8). All forms eluted with similar retention volumes corresponding to the anticipated dimeric size of ~120 kDa. None of the forms displayed a tendency toward aggregation, yet a minor fraction of each sample migrated as a small peak that corresponds in predicted size to a tetrameric complex. The similarity of profiles among all Hop forms suggests that a dimeric state is retained and that gross misfolding is not occurring with any mutant.

DISCUSSION

These studies were intended to experimentally address the nature of interactions between Hsp70 and the co-chaperone Hop. Previous findings have suggested that the TPR1 domain of Hop binds to C-terminal sequences in Hsp70 (35, 36), and a co-crystal structure for TPR1 complexed with an octapeptide that corresponds to the EEVD-containing C terminus of Hsp70 further supported this view (14). However, several observations in the present study suggest that Hop/Hsp70 interactions...
are more complex and involve additional sequences in both Hop and Hsp70.

The Hop Binding Site in Hsp70—Contrary to expectations from earlier studies, truncation of the Hsp70 EEVD sequence plus as many as 34 upstream amino acids (N608) failed to significantly reduce the ability of mutant Hsp70 forms to bind Hop (Fig. 2). However, since extending the truncation through position 605 (N604) or beyond resulted in a dramatic reduction in Hop binding, some sequences in the C-terminal region of Hsp70 are clearly necessary for Hop binding. Crystal structures for a peptide corresponding to amino acids 542–646 of the rat Hsc70 C terminus have recently been solved (27). The portion resolved in the crystal structures spanned amino acids 542 to –610. Four slightly variant structures were observed, but each revealed a helix-loop-helix structure in which amino acids 542–553 formed one helix and 564–610 formed the much longer helix. It is perhaps noteworthy that we observed loss of Hop binding as we began truncating sequences (residues 608–604) corresponding to the end of this long helix. Unfortunately, the GPTIEEVD (residues 639–646) and GGMP-repeat (residues 615–635) portions of the crystallized peptide were not resolved in x-ray diffractions, suggesting at least that these motifs do not form a stable interaction with other C-terminal sequences.

In an attempt to refine localization of minimal C-terminal sequences necessary for Hop binding, we generated N-terminal truncations of Hsp70 that lacked the well defined ATPase and peptide binding domains, but none of these constructs displayed significant binding to Hop (not shown). The ADP-bound state of Hsp70 is required for efficient binding to Hop (37, 38). Therefore, the ATPase domain of Hsp70 somehow communicates with C-terminal sequences to which Hop binds. Although we can conclude that the GGMP-EEVD region is not strictly required for Hop binding, we nonetheless suspect, as discussed below, that direct interactions between the Hsp70 EEVD tail and the Hop TPR1 domain are physiologically relevant.

What Roles do Hop Domains Play in Hsp70 Binding?—Consistent with predictions from current models, point mutation of carboxylate clamp residues in TPR1 disrupts Hsp70 binding (Figs. 4 and 5) and Hop function (Fig. 6), so the TPR1 domain plays a critical role. There have been clear indications, however, that other regions of Hop can have a profound influence.
on Hsp70 binding. Mutations of the C-terminal DP2 domain inhibit Hsp70 binding and Hop function equally as well as TPR1 point mutants (Fig. 4) (3, 17). Furthermore, we report here that carboxylate clamp mutations in TPR2a and TPR2b can also inhibit Hsp70 binding in vitro (Figs. 4 and 5). The TPR2a mutants are defective in vivo, but this may largely reflect a primary role for TPR2a in Hop90 binding. The function of TPR2b and a potential ligand for this domain have not been identified, so it was intriguing to observe that carboxylate clamp clamp mutations diminish Hsp70 binding in both complex (Fig. 4) and purified (Fig. 5) systems. On the other hand, the TPR2b mutants were fully competent for supporting GR function in vivo (Fig. 6) and assembled with receptor complexes in vitro similar to wild type Hop (Fig. 4).

The newly recognized DP1 domain (Fig. 3) was a compelling target for mutagenesis, since the related DP2 domain is important for Hsp70 binding and Hop function. Mutations in this domain did not alter Hop70 binding (Fig. 4); curiously, however, DP1 mutants displayed a reproducible, albeit modest increase in Hop function in vivo (Fig. 6). These findings do not suggest a critical function for DP1 in Hop70 or Hop90 binding, yet neither do they exclude an influence of DP1 on Hop function at some level.

Is there a second Hop70 binding site in Hop apart from TPR1? Hernandez et al. (38) reported that Hop70 binds Hop in a 2:1 molar ratio in the absence of Hop90 and a 1:1 ratio in the presence of Hop90, which might indicate that Hop90 binding to Hop occludes a second Hop70 binding site. In our GST pull-down assay, we did not observe a molar excess of Hop70 binding to Hop. On the other hand, none of the Hop point mutations reduced Hop70 binding to background levels, suggesting that there might be separate sites for Hop70 binding (Fig. 5). In an attempt to detect directly an alternative Hop70 binding site, various truncation mutants lacking TPR1 were tested, but with each truncated construct we observed only increased levels of nonspecific Hop70 binding, presumably due to misfolding of the truncation mutations (results not shown). We also tested a triple point mutant in which carboxylate clamp positions in each of the three TPR domains were mutated, but the triple mutant bound Hop70 at the same residual level as either TPR1 or TPR2b single mutants (comparison not shown). Therefore, if there are two Hop70 binding sites, they are somehow interdependent, or one of the sites does not involve carboxylate clamp interactions.

**Conformational Roles for Hop Domains**—Based on structural studies of various TPR domains, we did not anticipate that point mutations in the TPR domain carboxylate clamp positions would significantly alter Hop conformation. Conformational effects could result either from misfolding of individual mutant domains or from loss of domain/domain interactions. By gel filtration analysis (Fig. 8), each of the Hop mutants migrated as a homogeneous peak that closely resembled wild type Hop. From this we conclude that mutants retain their propensity for dimer formation and do not display gross misfolding that would favor aggregation. Still, we did infer conformational differences based on limited chymotryptic digests (Fig. 7). TPR1 and DP1 mutants yielded fragment patterns much like wild type Hop. In contrast, TPR2a and TPR2b mutants consistently displayed a similar resistance to proteolysis compared with other Hop forms (Fig. 7B). This finding is suggestive of domain/domain interactions involving TPR2a and TPR2b, either between dimeric pairs or within a single Hop polypeptide. Perhaps apparent defects in these mutants for Hop70 binding in vitro are a consequence of conformational alterations that can be reversed in vivo.

**Hsp70/Hop Interactions and Progressive Client Maturation**—

During the progressive assembly of steroid receptor complexes, Hop plays an important, transient role, but little is known about the molecular mechanisms that direct receptor transit from intermediate to mature complexes. ATP hydrolysis and changes in the nucleotide-bound states of Hsp70 and Hsp90 are important, probably because these lead to changes in Hop conformation and co-chaperone interactions. Hop, whose binding to either Hsp is nucleotide-sensitive and can affect ATPase activities (37–39), is in position to stimulate and coordinate Hop structural changes and help translate these into remodeling of receptor complexes.

In order to reconcile our present studies of Hop/Hsp70 interactions with previous reports and to place these findings in the context of receptor maturation, the following speculative model is proffered. We propose that a region of Hop70 upstream from the EEVD terminus binds TPR1 and that this non-EEVD interaction is critical for initial Hop70 binding to Hop. Subsequently, within the context of the receptor heterocomplex and ATP hydrolysis, the EEVD tail displaces the upstream Hop70 site from TPR1. This putative rearrangement of Hop/Hsp70 interactions would in turn stimulate a change in Hop70 or Hop90 behavior that promotes re-engineering of the receptor heterocomplex. In this conceptual model, EEVD/TPR1 interaction serves as a secondary regulatory signal rather than a primary binding interface.

Freeman et al. (40) published evidence that the EEVD tail can interact with other regions of Hop70, so there may be intramolecular interactions of EEVD that change with Hop70 status such that the EEVD tail becomes available for TPR1 binding in a regulated manner. Structural data on the peptide binding and C-terminal domains of Hop70 have also suggested domain/domain interactions (27). Recall also that Hop binding is sensitive to the nucleotide-bound state of Hop70. Therefore, the ATPase domain of Hop70 communicates with C-terminal sequences of Hop70 to which Hop binds. In addition to Hop’s adaptor role in bringing together Hop70 and Hop90 in a common complex, we think our results add a novel structural dimension to a model (38) in which Hop also monitors change in Hop70 status and stimulates additional change that bears on the chaperone machinery and modeling of steroid receptor complexes.

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Hop Interactions with Hsp70

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