Carboxy-Terminal Sequences Outside the Tetratricopeptide Repeat Domain of FKBP51 and FKBP52 Cause Differential Binding to Hsp90

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SUMMARY

Hsp90 assembles with steroid receptors and other client proteins in association with one or more Hsp90-binding co-chaperone, some of which contain a common tetratricopeptide repeat (TPR) domain. Included in the TPR co-chaperones are the Hsp70-Hsp90 organizing protein Hop, the FK506-binding immunophilins FKBP52 and FKBP51, the cyclosporin A-binding immunophilin CyP40, and protein phosphatase PP5. The TPR domains from these proteins have similar X-ray crystallographic structures and target co-chaperone binding to the MEEVD sequence that terminates Hsp90. However, despite these similarities, the TPR co-chaperones have distinctive properties for binding Hsp90 and assembling with Hsp90-steroid receptor complexes. To identify structural features that differentiate binding of FKBP51 and FKBP52 to Hsp90, we generated an assortment of truncation mutants and chimeras that were compared for co-immunoprecipitation with Hsp90. Although the core TPR domain (approximately amino acids 260-400) of FKBP51 and FKBP52 is required for Hsp90 binding, the C-terminal 60 amino acids (~400-end) also influence Hsp90 binding. More specifically, we find that amino acids 400-420 play a critical role for Hsp90 binding by either FKBP. Within this 20-amino acid region, we have identified a consensus sequence motif that is also present in some other TPR co-chaperones. Additionally, the final 30 amino acids of FKBP51 enhance binding to Hsp90 whereas the corresponding region of FKBP52 moderates binding to Hsp90. Taking into account the X-ray crystal structure for FKBP51, we conclude that the C-terminal regions of FKBP51 and FKBP52 outside the core TPR domains are likely to assume alternative conformations that significantly impact Hsp90 binding.
INTRODUCTION

Typically the most abundant cytoplasmic chaperone in vertebrate cells, Hsp90 serves a vital role in cellular signaling by regulating the folding, activity, and stability of a wide range of client proteins, as exemplified by steroid receptors and protein kinases (1). Client protein complexes contain not only Hsp90 but also one or more cochaperones that partner with Hsp90. One class of Hsp90-binding cochaperone is comprised of proteins with a characteristic tetratricopeptide repeat (TPR) domain that forms an Hsp90-binding site (2). Among the TPR cochaperones of Hsp90 are Hop/Sti1, an adaptor chaperone that also binds Hsp70 (3,4), the protein phosphatase PP5 (5), and members of both the FK506- and cyclosporin A-binding families of immunophilins (6-9). The TPR cochaperones compete for binding the C-terminal region of Hsp90 (10-12), and the highly conserved MEEVD sequence that terminates eukaryotic Hsp90 is a common target for TPR interactions (13-16). Mutation of the MEEVD sequence typically inhibits binding by a TPR cochaperone, although mutations in other C-terminal sequences differentially impact binding by TPR cochaperones (13,17).

The immunophilin-related TPR cochaperones contain peptidylprolyl isomerase domains that also serve as the binding site for immunosuppressive drugs, yet immunophilins can have differential effects on the function of Hsp90 client proteins. In particular, FKBP52 and FKBP51, which share approximately 70% amino acid sequence similarity, affect hormone binding by the glucocorticoid receptor (GR) in opposing manners. A study in the yeast *Saccharomyces cerevisiae*, which lacks endogenous counterparts to FKBP52 or FKBP51, has shown that FKBP52, specifically, can elevate GR responsiveness to hormone (18). Although FKBP51 alone does not reduce GR activity in yeast, it can effectively block the potentiation mediated by FKBP52 when co-expressed (18). Scammell and his colleagues have shown that cortisol
insensitivity observed in New World primates is facilitated by a constitutive overexpression of FKBP51 (19-21), suggesting a physiological relevance for FKBP interactions with GR-Hsp90 complexes. Bourgeois and colleagues first noted that the gene for FKBP51 is up-regulated by glucocorticoids (22), and this observation has been repeatedly confirmed by recent gene expression profiles of steroid responsive genes. The inducibility of FKBP51 by glucocorticoids could provide a mechanism for partial desensitization of cells subsequent to an initial exposure to hormone (23).

It is not clear what structural features are responsible for the differential effects of FKBP52 and FKBP51. Three-dimensional crystallographic structures for FKBP51 have recently been solved (24), but there is no corresponding structure for FKBP52. Based on the 70% amino acid sequence similarity of FKBP51 and FKBP52 and the conservation of apparent domain sequences, there is no reason to suspect a dramatic structural difference. Nonetheless, previous studies from us showed that mutations in the C-terminal half of Hsp90 have different effects on binding by FKBP52 versus FKBP51 (13). Also, we observed that an exchange of sequences between the FKBP TPR domains had no effect on Hsp90 binding by FKBP52 but blocked binding by FKBP51 (25). These observations suggested that each FKBP has a distinctive interaction with Hsp90. In the current study, we have taken advantage of the X-ray crystallographic structure of FKBP51 to functionally map sequences of FKBP51 and FKBP52 that are important for Hsp90 binding. We find that sequences in the C-terminal region, both inside and outside the TPR domain, greatly influence FKBP binding to Hsp90.
EXPERIMENTAL PROCEDURES

Preparation of mutant cDNAs

*In vitro* expression plasmids containing either human FKBP51 or FKBP52 cDNA inserted into pSPUTK (Stratagene, La Jolla, CA) were used to generate mutants used in these studies. To construct the C-terminal truncations, stop codons were introduced by site-directed mutagenesis (QuickChange kit, Stratgene, La Jolla, CA). To help clarify our naming convention for many of the mutants generated for this study, it is helpful to note that FKBP52, relative to FKBP51, contains a 2-amino acid insert in the loop connecting FK1 and FK2 domains; thus, the position of corresponding mutations in the C-terminal halves of either FKBP will differ by two residues. For example, the FKBP51 truncation mutant N-404 contains an engineered stop codon at position 405; the equivalent FKBP52 truncation mutant, N-406, contains a stop codon at position 407. The FK mutants were generated by introducing stop codons at position 258 or 260, respectively, which lies in the linker region between FK2 and the TPR domain. The TPR mutants were created by first introducing an F252M or F254M substitution and then removing all upstream coding sequences. FKBP chimeric cDNAs were constructed by two different approaches. The FKBP51 395 (positions 1-395 code for FKBP51 and positions 396-end code for FKBP52) and the converse FKBP52 397 chimeric cDNA were originally cloned into yeast expression vectors (18). These cDNA were PCR-amplified from yeast vectors and subeloned into pSPUTK. Other chimeras were created as follows. A gene fragment encoding the N-terminal portion of the desired chimeric protein was generated by PCR. A second gene fragment encoding the C-terminal portion of the desired chimera was separately generated by PCR. Primers for the two fragments were designed such that sequences surrounding the desired fusion site were complementary. The resulting DNA products were gel-purified and used as mega-
primers in a reaction with the appropriate 5’ and 3’ primers to generate the full length chimeric cDNA. The final PCR product was subcloned into pSPUTK. Sequence changes in mutated cDNAs and all PCR-generated products were confirmed by automated DNA sequencing.

**In vitro Hsp90 binding and PR complexes assembly**

The Hsp90- and PR-binding abilities of wildtype proteins and mutants were analyzed by a co-immunoprecipitation approach as described previously (25). Briefly, radiolabeled immunophilins were synthesized from plasmid DNA templates in an *in vitro* transcription/translation system (TnT lysate, Promega). Radiolabeled products were quantitated by densitometry of gel autoradiographs, and an equimolar amount of each radiolabeled product was added separately to 100 µl rabbit reticulocyte lysate (RL; Green Hectares, Oregon, WI).

Each RL sample was added to a 10 µl pellet of protein G-Sepharose (Amersham Pharmacia) pre-bound with 10 µg H90-10, a specific anti-Hsp90 mouse monoclonal antibody. After incubation on ice for 1 hr, unbound proteins were removed by washing 3 times in 1 ml wash buffer (20 mM Tris-HCl pH 7.4, 50 mM KCl, 1% Tween-20). Bound proteins were then extracted with SDS sample buffer and separated by SDS-PAGE. The assembly of PR complexes was analyzed in a similar manner, except 200 µl RL was supplemented with an ATP regenerating system, samples were incubated with 1 µg recombinant PR bound to PR22-protein A-Sepharose, and incubations were at 30°C for 30 min. After electrophoresis, gels were stained with Coomassie brilliant blue to visualize total proteins. Gels were then dried and autoradiographed to visualize radiolabeled proteins, and bands were quantitated by densitometry using a Fluor-S Imager (BioRad).

**Yeast Strains, Plasmids and Methods**

FKBP functional assays were performed in *Saccharomyces cerevisiae* essentially as described previously (18). All experiments were performed using a GR reporter strain in the...
parental cells were transformed with a plasmid that constitutively expresses rat GR, a second plasmid that expresses β-galactosidase from a glucocorticoid-regulated promoter, and a third plasmid constitutively expressing one of the human immunophilins. Cells were grown in minimal medium containing 0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose, the appropriate SC supplement mixture (Q-biogene, Carlsbad CA); for growth on plates the culture medium was supplemented with 1.6% w/v agar.

Hormone induction assays were performed as described previously (18). Briefly, yeast strains were grown in selective medium at 25°C, and the optical density at 600 nm (OD600) of the culture was monitored to ensure exponential growth. Reporter gene expression was induced by adding deoxycorticosterone (25 nM final concentration) to log phase cultures. Starting 70 min later, cells were sampled at 10 min intervals over the next 40 min for reporter activity. β-galactosidase activity was measured by adding 100 µl culture to an equal volume of Gal-Screen assay solution (Tropix, Bedford, MA) according to manufacturers instructions. Reporter expression rate was calculated as the linear slope of relative light units versus OD600/1,000. For each strain tested, hormone-induced reporter expression rate was determined with 2-4 independent transformants to assure consistency of results.

RESULTS

The three-dimensional crystallographic structure for FKBP51 depicted in Figure 1A reveals three major structural domains. Of the two FKBP12-like domains, PPIase and FK506-binding activities reside in FK1 (24). The Hsp90-binding TPR domain has a similar structure to other described TPR domains, in particular the Hsp90-binding domains from PP5 (26), Hop (16),
and CyP40 (27). The consensus TPR motifs terminate with helix 6 (H6, Figure 1B); however, similar to structures for PP5 and CyP40, there is a seventh helix that extends beyond the core TPR domain for a minimum of 23 amino acids. How much further H7 may extend is unclear since the final 36 amino acids could not be resolved from crystal data. Although a crystal structure for FKBP52 has not been reported, it seems likely that it will share the same overall domain organization as FKBP51.

**FKBP regions required for Hsp90 and receptor interactions**

As presented in Figure 2, an initial set of FKBP51 and FKBP52 mutants (Fig. 2A) were surveyed to compare interactions with Hsp90 and steroid receptor complexes. Co-immunoprecipitations of radiolabeled FKBP51 mutants (Fig. 2B) and FKBP52 mutants (Fig. 2C) were used to monitor Hsp90 binding (left-hand panels) and assembly into PR complexes (right-hand panels). We consistently find that incorporation of FKBP51 exceeds that of FKBP52 by 2 to 3-fold in Hsp90 complexes (compare lanes 1 and 2 in each data set). In line with previous observations (25), FKBP51 recovery in PR complexes exceeds the recovery of FKBP52 by 5-fold or greater (compare lanes 8 and 9 in each set). The TPR truncation mutants, which include the TPR domain plus C-terminal sequences, are sufficient for binding Hsp90 (lane 3) and assembling with PR complexes (lane 10). The TPR region is necessary for binding Hsp90 and assembling with PR since the FK domains show no interactions (lanes 4 and 11 in each set). The importance of the TPR domain is further demonstrated by point mutation at one of the carboxylate clamp residues (K352A for FKBP51 and K354A for FKBP52) that abrogates Hsp90 binding and PR association (lanes numbered 5 and 12). To test whether C-terminal sequences influence protein interactions, truncation mutants (N404 and N406) were generated that lacked sequences within H7 extension (see Fig. 1B) and beyond. Despite retention of the core TPR
domain in these constructs, Hsp90 binding (lane 6 in both sets) and PR association (lane 13 in both sets) were largely abrogated. The final samples in this mutant series are a pair of chimeric constructs (395 for FKBP51 and 397 for FKBP52) in which the region from H7 extension through the C-terminus was exchanged between FKBPs. This exchange had little effect on FKBP52 (Fig. 2C, lanes 7 and 14), but greatly diminished interactions of FKBP51 with Hsp90 and PR (Fig. 2B, lanes 7 and 14). In an earlier study (25) in which the C-terminal region was retained but TPR motifs were exchanged, we observed a similar phenomenon; the FKBP52-based construct functioned normally, but the FKBP51-based construct lost the ability to bind Hsp90 or assemble with PR complexes.

Taking advantage of the yeast model for FKBP52-dependent potentiation of GR signaling (18), we further tested FKBP52 constructs for function in vivo (Fig. 2D). First, note that FKBP52 significantly enhances hormone-induced β-galactosidase activity compared to yeast expressing FKBP51 or lacking either FKBP (Vector). None of the FKBP52 mutants displayed potentiation of GR signaling except the C-terminal chimera 397. This is consistent with the dual requirement for Hsp90-binding and FK1 PPIase activity in order for FKBP52 to elevate GR hormone binding affinity (18). Since 397 retains wildtype activity in this assay, it appears that specific sequences in the tail region of FKBP52 are not critical for its function in GR potentiation.

**Hsp90 binding by C-terminal truncation mutants**

The defect in Hsp90 interactions apparent with FKBP51-N404 and FKBP52-N406 was unexpected and raised the question of what sequences downstream from the core TPR domain are minimally required for Hsp90 binding. To begin addressing this question, a series of FKBP truncation mutants were generated that focus on this region, and these were tested for co-
immunoprecipitation with Hsp90. Densitometric measurements were taken from gel autoradiographs similar to those in Figure 2, and these data were plotted as shown in Figure 3. Significant recovery of FKBP51 mutants in Hsp90 complexes (solid circles) began with constructs that included residues 415-420, near the down-stream end of H7. Full Hsp90 binding was only observed with constructs containing sequences beyond 430.

Truncations of FKBP52 (open circles) differed from FKBP51 mutants in two ways. First, note that there is a leftward shift in the Hsp90 binding curve for the FKBP52 series as compared to the FKBP51 series. Thus minimally sized FKBP52 truncations that retain measurable levels of Hsp90 binding are 5-8 amino acids shorter than the smallest FKBP51 constructs that retain Hsp90 binding. The second difference pertains to the influence of sequences beyond position 430. Whereas the C-terminal region heightened Hsp90 binding by FKBP51, there appears to be a modest inhibition of Hsp90 binding by the corresponding region of FKBP52. Mutants terminating at approximately position 430 display equivalent binding to Hsp90. However, the inclusion of sequences beyond this point moderates binding of FKBP52 to Hsp90 but enhances binding by FKBP51.

**Hsp90 binding by FKBP51 chimeras containing C-terminal portions of FKBP52**

In marked contrast to the corresponding FKBP52 chimeras, swapping either the TPR domain (25) or the adjacent C-terminal tail (chimera 395 in Fig. 2B) abrogates FKBP51 binding to Hsp90. Swapping both regions together has no effect on Hsp90 binding. These observations suggest important intramolecular interactions between the C-terminal tail region and the TPR domain that are distinctive in FKBP51 and FKBP52. As shown in Figure 4, additional FKBP51 chimeras focusing on the TPR domain boundary from H5 through H7 were constructed to more thoroughly probe for potential interactions. Comparing hydrophobicity patterns in this region
from FKBP51 and FKBP52, the two proteins are similar (Fig. 4A), although there is only 50% amino acid sequence identity. Another series of FKBP51 chimeras was generated in which an exchange occurred at one of 8 positions along H6, H7, or just beyond H7 (7 of these sites are indicated in Fig. 4A). Radiolabeled products were synthesized and compared for co-immunoprecipitation with Hsp90 (Fig. 4B). As consistently observed, recovery of wildtype FKBP51 in Hsp90 complexes exceeds that of wildtype FKBP52 (compare first two lanes). Chimeras containing the H6 region and beyond from FKBP52 (365, 374, and 378) retain Hsp90 binding. When the exchange occurs within H7 (395, 404, and 413), there is a complete loss of Hsp90 binding. Hsp90 binding is again observed when the exchange is restricted to sequences beyond 422. To reiterate, we have observed no defect in Hsp90 binding by FKBP52 chimeras that contain similar tail regions from FKBP51 (for example, see Fig. 2C for results with FKBP52 chimera 397). Collectively, these observations suggest that there is an interaction within the region from H6 through H7-extended that is uniquely required by FKBP51 for Hsp90 binding.

An additional analysis was undertaken in which a series of C-terminal truncation mutants was generated from the defective chimera FKBP51-395. Similar to previous binding assays, truncations of 395 were examined for co-immunoprecipitation with Hsp90 on gel autoradiographs. The quantitated data were plotted in Figure 4C (solid triangles). Also plotted in this figure are the quantitated data for FKBP51 chimeras (solid circles) that were examined in Fig. 4B and for FKBP52 and the FKBP52-397 chimera (open circles). Consistent with the behavior of FKBP52 truncation mutants (Fig. 3), the FKBP52-397 chimera displays greater binding than wildtype FKBP52 (compare open circles), presumably due to the loss of inhibitory sequences beyond position 430 of FKBP52. As seen from the gel data in Fig. 4B, the FKBP51 chimeras (solid circles) had a pattern of loss then recovery of Hsp90 binding as the fusion point
in chimeras progressed from H6 through H7. Interestingly, truncations of FKBP51-395 (triangles) showed that Hsp90 binding could be partially restored in constructs that terminate between 410 and 430. The sharp boundary at approximately 410 corresponds well with the Hsp90 binding boundary observed with FKBP52 truncation. Likewise, the trailing boundary near 430 corresponds to the inhibition boundary deduced from FKBP52 truncation mutants.

**Functional analysis of FKBP52 sequences in the 400-420 region of H7**

Experimental results with FKBP52 and FKBP51 mutants point to the extended portion of H7 (amino acids 400-420) as being important for Hsp90 binding and FKBP function. This region of FKBP52 was analyzed further, as shown in Figure 5. Alignment of FKBP sequences in this region highlights the conservation of amino acids 406-415 (Fig. 5A, FKBP52 numbering), a 10-amino acid stretch consisting of a highly charged segment followed by YANMF. A series of FKBP52 truncation mutants that target this region were generated and tested for Hsp90 binding and assembly with PR complexes (Fig. 5B). As seen previously (Fig. 2), binding to Hsp90 and assembly into receptor complexes are greatly reduced with N406, but both interactions are restored when the C-terminus is extended to 414 and beyond. Yeast GR reporter strains were generated to correlate protein-protein interactions *in vitro* with FKBP52 function *in vivo* (Fig. 5C). Corresponding with weak Hsp90 and PR interactions, N406 and N410 lack the ability to potentiate GR signaling; however, potentiation is significantly boosted with N414 and larger mutants in parallel Hsp90 and PR interaction patterns.

Sequence databases were accessed to determine whether other TPR-dependent Hsp90 cochaperones share a motif similar to the 406-415 segment. For each co-chaperone we selected sequences that lie in the same relative downstream juxtaposition to TPR motifs. These juxta-TPR sequences are aligned in Figure 6. Included in the comparison are the Hsp90-binding
FKBP family members FKBP52, FKBP51, FKBP36 (28), and Xap2/AIP (29,30). CyP40 and PP5 sequences are present, as well as sequences from Hop (31) and CHIP (32). As shown above the alignment, an 11-amino acid motif, what we term the charge-Y motif, was identified with the consensus organization -+-+xΦYxxMF, where “-” represents E or D, “+” represents K or R, “Φ” represents a hydrophobic amino acid, and “x” represents any amino acid. There is also a negatively charged amino acid 5 positions further downstream that may relate to the consensus. Six of the 9 Hsp90-cochaperones match the consensus in at least 4 of 9 positions (indicated by left arrowhead).

**DISCUSSION**

FKBP52 and FKBP51 are closely related Hsp90-binding co-chaperones, yet they have distinctive patterns of interaction with Hsp90 and Hsp90 client proteins such as steroid receptors. In an effort to better understand the structural basis for distinctive interactions with Hsp90, we performed a mutagenic analysis of sequences in FKBP51 and FKBP52 that impact Hsp90 binding. Both immunophilins have a core TPR domain that is necessary for binding to Hsp90, but this core domain is not sufficient for full binding to Hsp90 (Fig. 2). We have identified a conserved region, the charge-Y motif, which lies immediately downstream from the TPR domain and is required for Hsp90 binding (Figs. 3 and 5). A similar sequence is found in some other Hsp90-binding TPR proteins (Fig. 6). Further downstream in either FKBP, unique sequences within the final 30 amino acids appear to distinguish the relatively higher Hsp90-binding affinity of FKBP51 as compared to FBP52 (Figs. 3 and 4). The FKBP5s are further distinguished by an intramolecular interaction peculiar to FKBP51 that involves H7, the final helix in the core TPR domain, and the adjacent charge-Y motif (Fig. 4). Thus, the Hsp90 binding properties of these
two TPR co-chaperones result from a combination of the core TPR domain and the influence of C-terminal sequences outside this domain. Consistent with the notion of alternative modes of interaction with Hsp90, point mutations in the C-terminal region of Hsp90 have distinct effects on the binding of individual TPR co-chaperones (13,17). This suggests that co-chaperones might interface with distinct structural features of Hsp90 in addition to a common interaction with the C-terminal MEEVD.

**Jack-knife model for charge-Y motif participation in Hsp90 binding**

According to the crystal structure for FKBP51 (Fig. 1), the charge-Y motif lies in a portion of H7 that extends beyond the core TPR domain. As depicted in Figure 7, we hypothesize two alternative conformational states of H7 sequences that could account for our mutagenic data. In the first state (Fig. 7A), H7 exists in the extended conformation that is consistent with the FKBP51 crystal structure. Here, charge-Y could directly contact Hsp90 and complement or facilitate binding through the core TPR domain. However, it is difficult to reconcile this conformational state with data from FKBP51 chimeras that argue for matching of sequences between the core and extended portions of H7 (Figs. 2 and 4). Yet based on the FKBP51 crystal structure, there is unlikely to be direct contact between these regions. We propose the alternative possibility (Fig. 7B) that H7 may be disrupted in some circumstances, forming an eighth helix that continues the anti-parallel pattern of interactions observed with H1 through H7. In this conformational state, the putative H8 may contribute to core TPR domain interactions that enhance TPR affinity for Hsp90.

Although an anti-parallel H8 is not observed in the static FKBP51 crystal structure, crystal packing may not have favored a conformation of FKBP51 that exists in solution or that is induced by binding to Hsp90. Furthermore, there are precedents for alternative conformational
states in TPR domains such as proposed in Fig. 7. Walkinshaw and colleagues obtained two crystal forms for CyP40 from which two TPR conformations were resolved (27). One structure contained a TPR domain similar to PP5, Hop-TPR2a, and FKBP51. In the alternate structure, the loops separating H2-H3 and H3-H4 have been reorganized into alpha helical forms, thus resulting in a single, greatly extended H2. A similar phenomenon was observed in the crystal structures for peroxisomal TPR protein PEX5. The structure for human PEX5 has the canonical anti-parallel helix arrangement (33). In contrast to this structure, Kumar et al. (34) obtained a structure for trypanosome PEX5 in which H5 and H6 were fused into a single extended helix. They proposed the possibility that certain TPR domains may naturally assume alternative conformations through extension versus folding-back of helices, what they termed the “jackknife model” for TPR motif rearrangement (34). The alternate FKBP structures proposed in Fig. 7 are analogous to the open and closed states of the TPR jackknife model, except in this case the sequences involved are not within the consensus TPR motifs that form H1 through H6.

Chimeric data suggest that FKBP51 may assume the closed conformation (Fig. 7B) while FKBP52 binds Hsp90 in an open conformation (Fig. 7A). One can visualize in the closed conformation how the charge-Y motif and adjacent amino acids may interact with amino acids in the core portion of H7 or even with side chains extending from H6. The C-terminal 30-35 amino acids that are unresolved in the FKBP51 crystal structure are not illustrated in Figure 7. However, this region also impacts Hsp90 binding (Figs. 3 and 4), enhancing binding by FKBP51 and moderating binding by FKBP52. In the jackknife model, this tail region would have to undergo a dramatic positional swing, but such a difference perhaps contributes to influences of the tail on Hsp90 binding.
Further structural studies are needed to test the jackknife model, and alternative explanations for our observations remain viable. For example, the charge-Y motif lies within a region that is purported to be a calmodulin binding site in FKBP52 (35). We considered whether Ca\(^{2+}\)/calmodulin interactions could either influence the conformational state of this region or provide an alternative mechanism for distinguishing FKBP51 and FKBP52 interactions with Hsp90. However, we think a role for calmodulin is unlikely for several reasons. First, the putative calmodulin binding motif scores very weakly for FKBP52 and FKBP51 when analyzed in the Calmodulin Target Database (http://calcium.uhnres.utoronto.ca/ctdb/flash.htm). Second, we have never observed calmodulin in FKBP complexes with Hsp90 or steroid receptors. Finally, neither Ca\(^{2+}\) nor the Ca\(^{2+}\) chelators EGTA or EDTA alter FKBP binding to Hsp90 (results not shown).

**General significance of TPR co-chaperone interactions with Hsp90 and client proteins**

Hsp90 serves a large number of client proteins that regulate cellular pathways, and in every case examined one or more co-chaperones accompany Hsp90 in client protein complexes. Individual clients display distinct preferences for certain Hsp90 co-chaperones. For example, among the TPR co-chaperones, Xap2/AIP is found in arylhydrocarbon receptor complexes but not steroid receptor or kinase complexes (29,30). FKBP51, FKBP52, PP5, and CyP40 associate differentially with progesterone, estrogen, and glucocorticoid receptor complexes in a receptor-specific manner (25,36,37). Preferential assembly with a client may be due to direct interactions between client and the Hsp90 co-chaperone (24), but the co-chaperone may also influence how Hsp90 interfaces with client, either stabilizing or destabilizing client interactions relative to other Hsp90/co-chaperone pairs. Thus, the TPR co-chaperones that competitively interact with the
MEEVD terminus of Hsp90 might, through unique contacts with Hsp90, induce distinct structural/functional changes in Hsp90 that elaborate the chaperoning of client proteins.

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FIGURE LEGENDS

Figure 1. FKBP51 structure. A. FKBP51 contains two FKBP12-like domains; FK1 has PPIase activity and provides the binding site for immunosuppressive drugs; FK2 has a similar fold, but differs at several amino acid positions important for PPIase activity and drug binding. The TPR domain forms an Hsp90-binding site and is structurally similar to other Hsp90-binding TPR domains. For each domain, the putative or known binding site is indicated by label positions. The N-terminal 27 amino acids and C-terminal 36 amino acids of full-length FKBP51 were unresolved in this structure. B. The three repeat motifs in the TPR domain form 6 alpha helices (H1-H6). A seventh helix has a core region that forms interactions with H6 and a region that extends beyond the TPR domain. The complete extent of H7 is unknown since the observed structure terminates at position 421 out of 457. The locations of Lys-352 and Arg-404 are indicated; these positions are relevant to mutations described in later figures.

Figure 2. Mutant FKBP interactions with Hsp90 and steroid receptor. A. The diagrams depict a series of mutant forms generated for either FKBP51 or FKBP52, respectively. In descending order, these are the full-length wildtype protein (wt), a truncation mutant containing the TPR domain through the C-terminus (TPR), a truncation mutant containing the N-terminus through the FK domains (FK), a point mutant in the TPR domain (K352A or K354A), a truncation mutant that terminates just beyond the TPR domain (N404 or N406), and a chimera in which the C-terminal sequences were exchanged (395 or 397). B. The ability of mutant forms of FKBP51 to bind Hsp90 (left-hand panels) and assemble in vitro with PR complexes (right-hand panels) are shown in comparison with human FKBP52. Radiolabeled proteins were generated by in vitro expression, and equivalent amounts of each form were added separately to RL prior to immunoprecipitation of Hsp90 complexes or assembly of PR complexes. The top
panel in each set is a Coomassie-stained gel image of total proteins recovered in each sample. Major protein bands, including antibody heavy chains (HC), are identified. The middle panel (^35S bound) is an autoradiograph of the stained gel that reveals bound FKBP forms, and the lower panel (^35S input) is an autoradiograph of a separate gel that demonstrates the relative level of radiolabeled protein added to each sample. C. Same as B, except the corresponding FKBP52 mutants were substituted for FKBP51 mutants. D. The ability of FKBP52 mutant proteins to enhance GR signaling was measured in a yeast strain that constitutively expresses rat GR and expresses β-galactosidase in a hormone-inducible manner. The reporter strain was transformed with an empty vector (Vector) or vector constitutively expressing FKBP51 or the indicated FKBP52 form. For each strain the linear rate of increase in β-galactosidase activity was measured over a 40-min period beginning 70 min after addition of deoxycorticosterone (25 nM final concentration). Reporter activities were normalized to that seen with yeast expressing FKBP51, and the results shown are typical for each of 4 independent isolates of each strain. Western immunostains confirmed that each FKBP form was present at approximately equivalent levels in total cell extracts (not shown). The results shown are typical measurements from 4 independent isolates for each strain.

**Figure 3.** Binding of FKBP truncation mutants to Hsp90. Truncation mutants of FKBP51 (solid circles) and FKBP52 (open circles) terminating at the indicated amino acid were compared for interactions with Hsp90. Radiolabeled protein products were added to RL prior to immunoprecipitation of Hsp90 complexes. Binding levels were quantitated by autoradiography of gel-separated samples and plotted relative to full-length FKBP51 (defined as 100% binding). The diagram above the plot illustrates the corresponding positions for H7 and C-terminal sequences of FKBP51.
Figure 4. Binding of FKBP chimeric proteins to Hsp90. A. The H5-H7 region (amino acids 348-421, which is the final resolved residue in the crystal structure) of FKBP51 and FKBP52 are illustrated with coloring to denote hydrophobicity (blue indicates hydrophobic side chains, red hydrophilic). The FKBP52 structure is modeled on the solved structure for FKBP51. The numbers on FKBP51 indicate 6 of the 8 positions at which C-terminal sequences from FKBP52 were placed to generate chimeric proteins. Downstream chimeras started at 422 and 438. B. FKBP51 chimeras containing the tail sequences from FKBP52 were compared with wildtype FKBP52 for co-immunoprecipitation with Hsp90. Radiolabeled products were added to RL prior to immunoprecipitation of Hsp90 complexes. The top panel is an image from a Coomassie-stained gel separation of each immunoprecipitate. The number above lanes indicates the final FKBP51 residue prior to the switch to FKBP52 sequences. The middle panel (Bound) is an autoradiograph of the stained gel used to detect bound FKBP forms. The lower panel (Input) is a separate autoradiograph that demonstrates the relative levels of radiolabeled protein added to each sample. C. The Hsp90 binding data were plotted for FKBP51 chimeras terminating with FKBP52 sequences (solid circles) and for FKBP52 and the FKBP52 397 chimera (open circles). Also plotted are binding data for truncation mutants of the FKBP51 395 chimera (solid triangles). The level of Hsp90 binding to full-length FKBP51 was defined as 100%. The diagram above the plot illustrates the corresponding positions of H5, H6, and H7 in the FKBP51 crystal structure.

Figure 5. Minimal FKBP52 sequences required for protein interactions and function. A. FKBP sequences are aligned that correspond to the extended portion of H7. Arrows denote the positions for a series of FKBP52 truncation mutants that terminate in this region. B. FKBP52 wildtype (wt) and H7 truncation mutants were compared for co-immunoprecipitation with Hsp90
and assembly with PR complexes. Shown are images from Coomassie-blue stained gels (upper panels), the corresponding autoradiographs (middle panels), and separate autoradiographs of synthesis products (bottom panels). **C.** The yeast GR reporter strain was separately transformed with empty vector (Vector) or plasmids expressing each form of FKBP52, as indicated. Hormone-induced reporter gene activity was measured in each strain and results were normalized to the activity observed in the Vector strain. The results are typical for multiple independent strain isolates.

**Figure 6.** Charge-Y motif in the juxta-TPR region of human Hsp90-binding cochaperones. A region identified with FKBP mutagenesis as important for Hsp90 binding is aligned with the corresponding region from other human TPR-containing Hsp90 cochaperones. For Hop, which has multiple TPR domains, the sequence shown lies adjacent to the Hsp90-binding domain TPR2a. An asterisk denotes the stop codon in some sequences. A consensus motif, termed “charge-Y,” is depicted above the sequence alignments. Motif elements are defined as appearing in at least 4 of the 8 co-chaperone sequences and are denoted within the sequences by bold capitalization. Arrowheads on the left indicate those sequences having at least 4 of the motif elements. Accession numbers used for the deduced human amino acid sequences are: FKBP51 – U71321, FKBP52 – M88279, CyP40 – A45981, FKBP36 – NP003593, Xap2/AIP – O00170, Hop – A38093, PP5 – P53041, and CHIP – AF129085.

**Figure 7.** Alternative models for FKBP binding to Hsp90. **A.** Based on the crystal structure for FKBP51, H7 extends beyond the core TPR domain. The charge-Y motif (blue box marked with Y) is important for maximal Hsp90 binding. One possibility is that the charge-Y motif contacts Hsp90 at a site separate from the core TPR domain. **B.** A second possibility is
that a break may form in H7 such that an eighth helix containing the charge-Y motif becomes part of the core TPR domain and enhances binding to Hsp90.
Figure 1
Figure 2

A. Diagram showing the structure of FKBP proteins with various mutations and chimera constructs.

B. Western blots showing Hsp90 Co-IP and PR assembly with different FKBP52 variants.

C. Similar Western blots as in B, focusing on Hsp90 and Hsp70 interactions.

D. Bar graph showing normalized reporter activity with different FKBP52 variants.
Figure 3

[Graph showing % Hsp90 Binding against C-Terminus of Truncation Mutants]
Figure 4
Figure 5

A

\[
\begin{align*}
401-\text{RRQLAREKLYANMFERLAEEEN}-423 & \quad \text{FKBP 52} \\
399-\text{KEHNERDRIYANMFKKFAEQDA}-421 & \quad \text{FKBP 51}
\end{align*}
\]

B

Hsp90 Co-IP

|       | WT | N406 | N410 | N414 | N416 | N420 |
|-------|----|------|------|------|------|------|
| Hsp90- |    |      |      |      |      |      |
| Hsp70- |    |      |      |      |      |      |
| Hop-   |    |      |      |      |      |      |
| HC     |    |      |      |      |      |      |

\[35S\text{ bound} \]

\[35S\text{ input} \]

PR Assembly

|       | WT | N406 | N410 | N414 | N416 | N420 |
|-------|----|------|------|------|------|------|
| Hsp90- |    |      |      |      |      |      |
| Hsp70- |    |      |      |      |      |      |
| HC     |    |      |      |      |      |      |

C

| Normalized Reporter Activity |
|-------------------------------|
| Vector | FKBP 52 | N406 | N410 | N414 | N416 | N420 |
|-------|---------|------|------|------|------|------|
Figure 6

--- Y MF ---

> nERDRrIYanMFkkfaEq FKBP51
> laREKkLYanMFerlaEe FKBP52
> kDKEKaVYakMFa* CyP40
> vDKEKwMrhMFapcgDg FKBP36
> dEeDKarfrgiFsh* Xap2/AIP
> kEqERlaYinpdlaleEk Hop
> kafERaTagdehkrsvvd PP5
> ihqEseLhsylsrliaae CHIP
Figure 7
Carboxy-terminal sequences outside the tetratricopeptide repeat domain of FKBP51 and FKBP52 cause differential binding to Hsp90

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