Cyclophilin 40 (CyP-40), Mapping of Its hsp90 Binding Domain and Evidence That FKBP52 Competes with CyP-40 for hsp90 Binding*

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The structurally related immunophilins cyclophilin 40 (CyP-40) and FKBP52 have been identified as components of the unactivated estrogen receptor. Both immunophilins have a similar molecular architecture that includes a C-terminal segment with a tetratricopeptide repeat (TPR) domain predicted to mediate protein interaction. hsp90 is a common cellular target for CyP-40 and FKBP52. Deletion mutants of CyP-40 fused to glutathione S-transferase were immobilized on glutathione-agarose, and then used in a rapid hsp90 retention assay to define regions of the CyP-40 C-terminal that are important for hsp90 binding. Our evidence suggests that the TPR domain is not sufficient for stable association of CyP-40 with hsp90 and requires the participation of flanking acidic and basic residues clustered at the N- and C-terminal ends, respectively. Both microdomains are characterized by α-helical structures with segregated hydrophobic and charged residues. Corresponding regions were identified in FKBP52. By preincubating myometrial cytosol with lysates containing bacterially expressed FKBP52, we have shown that FKBP52 competes with CyP-40 for hsp90 binding. Our results raise the possibility of a mutually exclusive association of CyP-40 and FKBP52 with hsp90. This would lead to separate immunophilin-hsp90-receptor complexes and place the estrogen receptor under the control of distinct immunophilin signaling pathways.

The immunophilin components of the unactivated estrogen receptor, cyclophilin 40 (CyP-40),1 and FKBP52, share significant sequence homology in their C-terminal regions (1) and represent separate classes of peptidylprolyl cis-trans-isomerases with binding specificities for the immunosuppressants cyclosporin A and FK506, respectively (2). These immunophilins display a similar structural organization of their functional domains characterized by an N-terminal region with overlapping isomerase and ligand binding domains and a conserved C-terminal segment that incorporates a 3-unit tetratricopeptide repeat (TPR) domain terminated by a potential site for calmodulin binding (1). We have previously speculated that the TPR domain may mediate the protein interaction properties of CyP-40 and FKBP52 (1). This is consistent with evidence that similar repeat units in members of the TPR gene family are involved in functional association with target proteins (3).

FKBP52 binds hsp90 within steroid receptor complexes (4) and also exists in association with hsp90 in the absence of receptor (5, 6). The interaction of FKBP52 with hsp90 has been studied extensively (7), and there is recent evidence that the TPR domain, localized in the C-terminal region of FKBP52, is fundamentally important for hsp90 binding (8). The structural similarity between CyP-40 and FKBP52 has led several groups to propose that the immunophilins may have a similar or perhaps competing role in cellular function (8–10). In this regard, a recent report describes the association of human CyP-40 with hsp90 and provides evidence that the C-terminal, FKBP52-like domain determines this interaction (11).

We have previously described the bacterial overexpression of bovine CyP-40 (bCyP-40) as a glutathione S-transferase (GST) fusion protein and have shown the recombinant protein to be bioactive through a display of isomerase activity that is inhibitable by cyclosporin A (10). In preliminary studies we have confirmed that, like FKBP52 (12), CyP-40 is a calmodulin-binding protein (10). Here we describe the use of a rapid affinity chromatography-based method, with recombinant bCyP-40 and its derivatives immobilized as GST fusion proteins on glutathione-agarose, to define regions within CyP-40 critical for hsp90 interaction. Our results reveal that the TPR domain is not sufficient for stable association of CyP-40 with hsp90 and suggest that acidic and basic regions adjacent to the N- and C-terminal ends of the TPR domain, respectively, might be involved in hsp90 binding. Using the same CyP-40-hsp90 retention assay, we show for the first time that FKBP52 competes with CyP-40 for hsp90 binding. This raises the possibility that the immunophilins may bind hsp90 in a mutually exclusive fashion leading to the formation of separate CyP-40 and FKBP52-hsp90 complexes.

EXPERIMENTAL PROCEDURES

Expression Vectors—The expression plasmid for GST-bCyP-40 fusion protein has been described previously (10). At its C terminus, GST-bCyP-40 contains an additional 6 amino acids derived from the pGEX-2T expression vector sequence (13) (Fig. 1). The full-length cDNA of bCyP-40 was excised from expression plasmid by BamHI/Xmal digestion, cleaved at the unique DraI site, and the resulting BamHI to DraI fragment was inserted back into the parent vector to give GST-bCyP-40 1–213. The expression plasmid for GST-bCyP-40 was cleaved with XhoI, and the DNA was blunt-ended by S1 nuclease treatment. Xmal digestion released the XhoI to Xmal insert which was further cleaved with DraI. The DraI to Xmal fragment was then ligated into the vector DNA to give GST-bCyP-40 17–213.

GST-bCyP-40 WT and the mutant expression plasmids GST-bCyP-40 91–370, GST-bCyP-40 185–370, GST-bCyP-40 1–352, and GST-bCyP-40 185–352 (Fig. 1) were generated from a bCyP-40 cDNA tem-
Cell lysates were prepared as described previously (10) prior to affinity culture. Uninduced cultures grown at 37°C were obtained with 0.5–1 mg/liter expression, but adequate yields of the fusion proteins (0.5–1 mg/liter were overexpressed in E. coli. Insertions released from those plasmids by restriction sites occur at positions identified by amino acids 16 and 213, respectively.

Plate by polymerase chain reaction (PCR) amplification using high fidelity Pfu DNA polymerase (Stratagene) and specific oligonucleotide primers containing built-in BamHI (5' end) and SmaI (3' end) restriction sites. The SmaI site in each was preceded by a TGA stop codon. After BamHI and SmaI digestion, the amplified fragments were ligated into pGEM-3Z. For GST-bCyP-40 WT and GST-bCyP-40 1–352, a SmaI to BclI excision allowed replacement with wild type DNA. A similar approach was used for GST-bCyP-40 91–370, GST-bCyP-40 185–370, and GST-bCyP-40 185–352 to replace the PFM1 to BclI excised fragment with wild type sequence. This strategy minimized PCR-introduced mutations. Sequence fidelity of the remaining PCR-derived DNA was confirmed by automated sequence analysis (Applied Biosystems). Inserts released from those plasmids by BamHI/SmaI digestion were ligated into pGEX-2T.

Expression of Full-length and Truncated GST-bCyP-40 Fusion Proteins—GST-bCyP-40 and the truncated derivatives 1–213 and Δ17–213 were overexpressed in E. coli XL1-Blue bacteria after IPTG induction, as described previously (10). With the remaining constructs (GST-bCyP-40 WT, 91–370, 185–370, and 185–352), IPTG failed to induce expression, but adequate yields of the fusion proteins (0.5–1 mg/liter culture) were obtained with uninduced cultures grown at 37°C over 5 h. Cell lysates were prepared as described previously (10) prior to affinity chromatography on glutathione-agarose (Pharmacia).

Recombinant FKBP52 and hsp90α—Expression vectors for human FKBP52 and hsp90α were gifts from D. Peattie, Vertex Pharmaceuticals (14), and C. T. Walsh (15), respectively. Overexpression of both proteins was achieved by induction with IPTG. Bacterial lysates were prepared by sonication in lysing buffer (16 mM disodium hydrogen orthophosphate, pH 7.4, containing 0.15 mM NaCl, 1% Triton X-100, 5 mM dithiothreitol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 5 mM benzamidine). Renaturation of SDS-PAGE-purified Bovine hsp90—Polyacrylamide gel slices containing bovine hsp90 were isolated after preparative SDS-PAGE of affinity-purified inactivated estrogen receptor extracts (1). After elution, hsp90 was renatured as described previously (16).

hsp90 Binding Studies—GST-bCyP-40 fusion protein and bCyP-40 deletion mutants were absorbed from bacterial lysates onto glutathione-agarose, and contaminating proteins were removed from the gel by washing as described (10). Fusion proteins recovered on the resin were semi-quantitated by SDS-PAGE with Coomassie Blue staining. Dillution of the resin-fusion protein complexes with Sepharose 4B (generally 5-fold), allowed an appropriate equalization of protein content, and the resulting gel mixtures were then equilibrated in binding buffer (10 mM Tris, pH 7.3 buffer containing 100 mM KCl, 5 mM dithiothreitol, and 10% [v/v] glycerol). After centrifugation, the gels were washed repeatedly with the same buffer to remove unbound protein contaminants. Retained proteins (lanes 2, 4, and 6) were extracted from the gels with SDS-PAGE sample buffer and were analyzed by SDS-PAGE on a 12.5% w/v polyacrylamide gel followed by Coomassie Blue staining. Fusion protein-charged gels not exposed to cytosol were used as controls (lanes 1, 3, and 5).

Tris, pH 7.3, containing 100 mM KCl, 5 mM dithiothreitol, and 10% [v/v] glycerol, hsp90 retention studies were carried out initially with bovine myometrial cytosol prepared in binding buffer as described previously (17). Aliquots (15–30 μl) of the fusion protein-charged resins were incubated with cytosol (200 μl) by rotation over 6 h at 4°C. After centrifugation, the gels were washed repeatedly with binding buffer (8 × 500 μl aliquots). Retained proteins were recovered by boiling the gels with 50 μl of 2 × SDS-PAGE sample buffer and were then analyzed by SDS-PAGE on 12.5% w/v polyacrylamide gels. Resins with immobilized fusion proteins not exposed to cytosol were used as controls. Similar methodology was applied for interaction studies of GST-bCyP-40 fusion protein with an IPTG-induced, bacterial lysate preparation of human recombinant hsp90α and SDS-PAGE purified, renatured bovine hsp90.

bCyP-40-hsp90 Interaction Studies in the Presence of FKBP52—0.5, 10, and 100 μM aliquots of induced FKBP52 bacterial lysate were added to separate 200-μl aliquots of bovine uterine cytosol, and the total volume was brought to 400 μl with lysing buffer. The mixtures were allowed to incubate for 3 h at 4°C. Glutathione-agarose charged with GST-bCyP-40 fusion protein was then added to each, and incubation was continued with rotation for an additional 5 h at 4°C. Proteins retained on the resin were recovered by boiling with 2 × SDS-PAGE sample buffer and analyzed by SDS-PAGE with Coomassie Blue staining. A parallel study with 100 μl of uninduced lysate in cytosol was used as control. In a reciprocal experiment, cytosol (200 μl) was first incubated with gel-immobilized GST-bCyP-40 (3 h at 4°C). The mixtures were supplemented with buffer or induced or uninduced FKBP52 lysate (100 μl), and, after an additional 5-h incubation at 4°C, gel-retained proteins were analyzed as described above.

RESULTS

The C-terminal Domain of CyP-40 Mediates hsp90 Interaction—We have previously described the expression of bCyP-40 as a fusion protein with glutathione S-transferase (10). The fusion protein, GST-bCyP-40 (Fig. 1), includes an additional six amino acids incorporated from the pGEX-2T expression vector sequence (13). Selective absorption of GST-bCyP-40 onto glutathione-agarose allowed the resin to be used in affinity chromatography mode to screen for protein targets for CyP-40 in bovine uterine cytosol. Consistent with results published for human cyclophilin 40 (11) we were able to demonstrate the specific interaction of bCyP-40 with hsp90 (Fig. 2, lane 2). bCyP-40-hsp90 binding was confirmed by incubation of gel-immobilized GST-bCyP-40 with recombinant human hsp90α (15) and with bovine hsp90 renatured from preparative SDS-PAGE of estrogen receptor extracts isolated by ligand affinity...
chromatography (17) (results not shown). By manipulating unique XhoI and DraI restriction sites within bCyP-40 cDNA in the expression plasmid, we prepared the truncated GST-bCyP-40 fusion proteins 1–213 and Δ17–213 (Fig. 1), which separately contain the N- and C-terminal domains of bCyP-40, respectively. Using a similar approach, we then assessed the contribution of these regions to hsp90 binding. We observed hsp90 binding only with the C-terminal domain of bCyP-40 (Fig. 2). However, in comparison with the parent fusion protein, the binding efficiency of the C-terminal construct for hsp90 was substantially reduced (Fig. 2), suggesting that additional regions toward the N terminus might contribute significantly to the interaction.

An Acidic Region Separates the N- and C-terminal Domains of CyP-40 and FKBP52—Examination of the intervening protein sequence between the ligand binding/somerase and the TPR domains in bCyP-40 (1) revealed the 41-residue region to contain a predominance of acidic residues (14 acidic Asp, Glu versus 5 basic Lys, His) (Fig. 3A). Within this acidic domain, eight charged amino acids (6 acidic, 2 basic) are located in a 13-residue, α-helical segment (18, 19) proximal to the TPR domain (Fig. 3A). Helical wheel representation showed a segregation of hydrophobic and charged residues within this region (Fig. 3B). Most of the acidic domain, including the first eight residues of the α-helical segment, is deleted in the low efficiency hsp90-binding mutant, Δ17–213 (see Fig. 3A), prompting speculation that this region might play a role in hsp90 interaction.

A considerably longer (134 amino acid residues) intervening sequence separates the ligand binding/catalytic domain from the C-terminal TPR domain in FKBP52 (14). Although bCyP-40 and FKBP52 do not share sequence homology in this region, there are notable similarities. As in bCyP-40, the intervening sequence in FKBP52 contains a net surplus of acidic amino acid residues (29 acidic, 21 basic Arg, Lys, His) (14). Additionally, FKBP52 has a corresponding α-helical microdomain with a similar segregated distribution of hydrophobic and charged residues (Fig. 3B). These observations identify additional structural similarities between the CyP-40 and FKBP52 gene products and lend support to accumulating evidence that they may have similar or competing modes of action in cellular processes.

Acidic and Basic Regions Flanking the TPR Domain in CyP-40 Are Essential for Efficient hsp90 Binding—To test our hypothesis that the acidic domain of bCyP-40 is involved in hsp90 binding, we constructed two N-terminal deletions, 91–370 and 185–370 (Fig. 1). These constructs, together with a fusion protein representing full-length bCyP-40 (GST-bCyP-40 WT) (Fig. 1), were prepared without the 6-residue, vector-related peptide to ensure that our study was not compromised by undefined contributions from this region. A C-terminal deletion mutant, 1–352 (Fig. 1), was also prepared to assess the participation of the putative calmodulin binding site (1, 20) in hsp90 interaction. A final construct, 185–352 (Fig. 1), deleted in both the N-terminal functional domain and the proposed calmodulin binding site, contained only the acidic and TPR domains. Exposure of these constructs and the original deletion mutant, Δ17–213, to bovine myometrial cytosol provided a comparison of their ability to bind hsp90 (Fig. 4). Our binding profiles showed that inclusion of an intact acidic domain (as in constructs 91–370 and 185–370) restored high efficiency hsp90 binding, consistent with the proposed involvement of this region in hsp90 interaction (Fig. 4). Surprisingly, removal of the 18-residue segment at the C terminus of bCyP-40, corresponding to a suggested site for calmodulin interaction, resulted in a markedly decreased level of hsp90 binding (Fig. 4). The results suggest that this deleted region, consisting of an α-helical domain with segregated hydrophobic and positively charged residues (10), contains additional features important for efficient hsp90 binding.

FKBP52 Competes for Binding of CyP-40 to hsp90—hsp90 is a common target for CyP-40 and FKBP52 protein interaction. The results we have presented for bCyP-40 and those already published for FKBP52 (8) suggest that elements which mediate hsp90 binding are located within the C-terminal regions of both proteins. To further study the functional relatedness of CyP-40 and FKBP52, we tested the possibility that they might compete with one another for hsp90 binding. Fig. 5B shows that preincubation of bovine myometrial cytosol with increasing volumes of IPTG-induced lysate containing bacterially expressed FKBP52 resulted in a progressive reduction in the amount of hsp90 retained by the GST-bCyP-40 resin. In a parallel control
The hsp90 Binding Domain of Cyclophilin 40

experiment, uninduced lysate was unable to influence the level of bCyP-40-hsp90 binding (Fig. 5B). The reciprocal study, in which FKBP52 lysate was added to cytosol containing hsp90 already prebound to resin-immobilized GST-bCyP-40, also resulted in a diminution of gel-retained hsp90 (Fig. 5C). The results raise the possibility of a mutually exclusive association of CyP-40 and FKBP52 with hsp90. Additionally, they suggest the existence of a dynamic system in which the composition of the immunophilin-hsp90 complexes might be determined by the relative abundance of CyP-40 and FKBP52 in the cell.

DISCUSSION

We have used a rapid, glutathione-agarose affinity chromatography-based method with GST fusion proteins containing wild type bCyP-40 and bCyP-40 deletion mutants, to identify regions within the CyP-40 C terminus that are important for hsp90 binding. The critical regions include acidic and basic peptides located at the N- and C-terminal ends of the TPR domain, respectively. Both microdomains are characterized by amphiphilic α-helical structures suggesting that hydrophobic and electrostatic interactions might contribute significantly to hsp90 binding. We have previously proposed that the 3-unit TPR domain might mediate the protein interaction properties of CyP-40 (1). Disruption of the acidic domain (as in construct Δ17–213) or complete removal of the C-terminal basic region flanking the TPR domain (as in construct 1–352) leads to a sharp reduction in hsp90 binding efficiency. The result suggests that for CyP-40, an intact TPR domain is not sufficient for stable association with hsp90, but is consistent with a requirement for multiple elements present in the C-terminal half of the protein. In such a model it is possible that the TPR domain, stabilized by internal contacts between individual repeat units, maintains an appropriate spatial orientation of both charged regions relative to hsp90. Alternatively, the charged domains might act coordinately enabling the TPR domain to form a suitable binding conformation.

The importance of the TPR domain for the interaction of FKBP52 with hsp90 has been demonstrated clearly (8). FKBP52 derivatives, altered within the third unit of the TPR domain, either by a 2-amino acid residue insertion or by a C-terminal truncation, failed to bind hsp90 (8). The result supports a structural role for the TPR domain. The basic region located at the C-terminal end of the TPR domain is highly conserved between CyP-40 and FKBP52 and is a likely site for calmodulin interaction (1, 20). Removal of this domain from FKBP52 appears not to disrupt hsp90 binding, and the complex remains stable during nondenaturing gel electrophoresis (8). This contrasts with our observation of low level hsp90 retention by the corresponding bCyP-40 1–352 mutant. We have used an extensive washing procedure with buffer containing 100 mM KCl to minimize nonspecific protein interaction with gel-immobilized GST-bCyP-40 constructs. It is possible that the binding of the 1–352 derivative to hsp90 is sensitive to these washing conditions.

The nonhomologous, intervening sequences which separate the N-terminal ligand/isomerase domain from the C-terminal TPR region in CyP-40 and FKBP52 differ considerably in length (CyP-40, 41 residues; FKBP52, 134 residues), but display some common features. Both are characterized by an excess of negatively charged amino acid residues and contain similar amphipathic α-helical regions located close to the TPR domain. We speculate that, as in CyP-40, the acidic domain in FKBP52 may participate in hsp90 binding. Identification of this common acidic region in CyP-40 and FKBP52 extends the remarkable structural similarity between these proteins and suggests that they may have closely related roles in cellular function.

We have shown for the first time that FKBP52 competes with CyP-40 for hsp90 binding. Our findings complement those reported by Owens-Grillo et al. (22) and suggest that CyP-40 and FKBP52 share an identical interaction site within the hsp90 protein. The reversible nature of this interaction is consistent with the presence of a dynamic system in which the
immunophilin component of immunophilin-hsp90 complexes might be determined by the tissue distribution and the relative cellular concentrations of CyP-40 and FKBP52 (9). The mutually exclusive association of CyP-40 and FKBP52 with hsp90, leading to the formation of separate hsp90-receptor complexes, would place steroid receptors under the control of distinct immunophilin signaling pathways. This raises the possibility of a modulating role for putative endogenous immunophilin ligands in receptor activity. However, although FK506 has been shown to enhance glucocorticoid and progesteronereceptor-mediated gene expression in intact cells (23, 24), in vitro studies indicate that the drug does not influence hsp90 association with the glucocorticoid receptor (25). In our own studies, 20 μM cyclosporin A had no effect on hsp90 retention by gel-immobilized GST-CyP-40 fusion protein (not shown). Nevertheless, a model in which the immunosuppressants, acting via CyP-40 and FKBP52, induce a succession of conformational changes that result in altered hsp90 and receptor function, cannot at this stage be discounted. This proposal is supported by additional evidence that cyclosporin A can potentiate glucocorticoid- (26), progestin- (27), and estradiol-induced gene expression.2

The major molecular chaperones hsp90 and hsp70 are key participants in steroid receptor activation (28), and the proper interaction of receptors with hsp90 is essential for efficient ligand binding and response (29, 30). Recent evidence suggests that receptor-hsp90 complexes exist in dynamic equilibrium with hsp70 (31) and that the folding of receptors to a hormone-activatable conformation also involves DnaJ and p23, together with additional components (9, 32–34). The presence of p23 with hsp90 and the immunophilins, CyP-40 and FKBP52, as the major proteins in progesterone receptor complexes (9), suggests that p23-hsp90-immunophilin heterocomplexes might represent important intermediates in receptor assembly and that they may act together to establish a poised receptor conformation that is optimally responsive to hormone. It has been suggested that DnaJ and hsp70 might also regulate the activities of a wider range of signaling proteins, all of which are targeted by hsp90 (32). Although the cellular functions of CyP-40 and FKBP52 have yet to be defined, a more general role for these immunophilins in modulating the cellular activity of hsp90 chaperone substrates is distinctly possible.

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