Identification of Conserved Residues Required for the Binding of a Tetratricopeptide Repeat Domain to Heat Shock Protein 90*

(Received for publication, April 28, 1999, and in revised form, May 25, 1999)

Lance C. Russell, Sherry R. Whitt, Mei-Shya Chen‡, and Michael Chinkers§

From the Department of Pharmacology, University of South Alabama, Mobile, Alabama 36688

The sequential binding of heat shock protein 90 (hsp90) to a series of tetratricopeptide repeat (TPR) proteins is critical to its function as a molecular chaperone. We have used site-directed mutagenesis to clarify the structural basis for the binding of hsp90 to the TPR domain of phosphoprotein phosphatase 5 (PP5). This TPR domain was chosen for study because its three-dimensional structure is known. We examined co-immunoprecipitation of hsp90 with wild type and mutant TPR constructs from transfected cells. Only mutations located on one face of the TPR domain affected hsp90 binding. This allowed the identification of a binding groove. Three basic residues that are highly conserved in hsp90-binding TPR proteins extend prominently into this groove. Lys-97 and Arg-101 were absolutely required for hsp90 binding, while mutation of Arg-74 diminished, but did not abrogate, hsp90 binding. Mutation of Lys-32, another conserved basic residue in the binding groove, also blocked hsp90 binding. The TPR domain of PP5 bound specifically to a 12-kDa C-terminal fragment of hsp90. This binding was reduced by mutation of acidic residues in the hsp90 fragment. These data suggest conservation, among hsp90-binding TPR proteins, of a binding groove containing basic residues that interact with acidic residues near the C terminus of hsp90.

Heat shock protein (hsp)1 90 is a molecular chaperone necessary for viability (1) and for the proper folding, processing, and function of proteins involved in several signal transduction pathways (2). In perhaps the best characterized of these pathways, association of steroid receptors with a series of hsp90 complexes is required for their acquisition of hormone-binding ability (3). After hormone binding, steroid receptors are released from hsp90 and bind to DNA, activating transcription.

* This work was supported by National Institutes of Health Grants HL 47063 and DK 55877 (to M. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Dept. of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO 63110.

§ To whom correspondence should be addressed. Tel.: 334-460-6782; Fax: 334-460-6788; E-mail: michaelc@jaguar1.usouthal.edu.

† The abbreviations used are: hsp, heat shock protein; TPR, tetratricopeptide repeat; PP5, phosphoprotein phosphatase 5; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase.

hsp90 complexes are also important for the folding of a variety of protein kinases and other signal transducing proteins (2, 4–7). Recent data suggest that hsp90 may play an important role in facilitating evolution (8). Advances in our understanding of the mechanisms of hsp90 action have raised the possibility of using hsp90 and associated proteins as targets for pharmacological intervention (9, 10).

Tetratricopeptide repeat (TPR) domains consist of tandem repeats of a 34-amino acid consensus sequence. These domains mediate protein-protein interactions, and different TPR domains have different protein-binding specificities (11). Several TPR proteins that bind to hsp90 play a critical role in steroid receptor assembly (3). After newly synthesized receptors bind to hsp70, Hop, a protein that binds to both hsp70 and hsp90 via separate TPR domains, recruits hsp90 to the receptor complex. Hop and hsp70 are then displaced by a large immunophilin (FKBP51, FKBP52, or CyP-40) (12) that contains a TPR domain that binds to the carboxyl end of hsp90 (13–15). These large immunophilins have co-chaperone activity in vitro that does not involve their peptidylprolyl isomerase activity and have biological activity intrinsic to the TPR domain (16–18). Hop, in contrast, inhibits the ATPase activity of hsp90 (19). Like hsp90, yeast homologues of Hop and CyP-40 are required not only for optimal cell growth, but for optimal signaling by recombinant steroid receptors and tyrosine kinases (18, 20, 21).

Mechanisms controlling which TPR proteins bind to hsp90, and when, are clearly important. Binding of TPR proteins during protein folding occurs in an ordered manner, and different large immunophilins associate preferentially with hsp90 complexes containing different steroid receptors (2). This has functional consequences, e.g. glucocorticoid receptor complexes containing FKBP51 have lower binding affinity than complexes containing FKBP52 (22). Understanding the structural basis for the binding of different TPR proteins to hsp90 at different times will be important for understanding its function as a molecular chaperone.

We have shown that PP5, a protein-serine phosphatase containing a TPR domain at its N terminus (23–25), binds to hsp90 via its TPR domain (26). PP5 is a major component of mature glucocorticoid receptor complexes (27) and appears to regulate glucocorticoid receptor function in vivo (26). These observations suggest that PP5 may act as a co-chaperone for hsp90 and raise the possibility that protein dephosphorylation may play a role in protein folding. The three-dimensional structure of the TPR domain of PP5 was recently described (28). As predicted by the original papers describing TPR domains (29, 30), the TPR domain of PP5 consists of six amphipathic a helices bundled together to form a globular domain. It has been speculated that a groove on one face of this domain could form a binding site for other proteins (28).

We report here that four basic residues in the TPR domain, which are conserved among PP5 and other hsp90-binding proteins, are critical for binding to hsp90. These residues all lie along what we now definitively identify as a binding groove. We also show that acidic residues in hsp90 are required for optimal binding to the TPR domain of PP5.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis of the TPR Domain of PP5—Mutagenesis with the Stratagene QuikChange kit was performed according to the manufacturer’s instructions, using the previously described pCMV8-
FLAG-TPR (26) as a template. Mutants were sequenced to confirm the presence of the desired mutations and the absence of additional mutations. As an aid in choosing residues to mutate, the three-dimensional structure of the PP5 TPR domain (28) was visualized using the RasMol program.

Plasmid Construction—Sequences encoding residues 628–732 of human hsp90α, designated C90, were amplified by polymerase chain reaction and cloned into pGEX-4T-1 (Amersham Pharmacia Biotech) as an EcoRI/BamHI fragment, essentially as described by Young et al. (31). Mutants of C90 in which the C-terminal sequence was changed from EEVD to AAVA (C90—A3) or KKVK (C90—K3) were prepared in a similar manner, using 3′ primers encoding these mutations. Each clone was sequenced to confirm the presence of the desired mutations and the absence of additional mutations. C90 and the two C90 mutants were also subcloned into pET30a (Novagen) as EcoRI/BamHI fragments.

For expression as a fusion to the N terminus of GFP, sequences encoding all but the C-terminal four amino acids (LGMM) of PP5 were excised from pCMV6-FLAG-PP5 (26) as an EcoRI (blunted)/PstI fragment and cloned into the Nhel (blunted)/PstI sites of pEGFP-N1 (CLONTECH). Similarly, the FLAG-tagged TPR domain was excised from pCMV6-FLAG-PP5 as an EcoRI (blunted)/HindIII fragment and cloned into the Nhel (blunted)/HindIII sites of pEGFP-N1.

Tissue Culture and Transfections—All cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. COS-7 cells were transfected using a DEAE-dextran method (32).

Immunoprecipitation and Immunoblotting—COS-7 cells in 35-mm wells were transfected with the indicated plasmids. One day later, cultures were incubated overnight in 1 ml of medium (90% methionine-free, cysteine-free Dulbecco’s modified Eagle’s medium, 10% growth medium) containing 25 μCi of [35S]methionine/cysteine (NEN Life Science Products, 1175 Ci/mmol). Cells were washed in ice with 20 ml HEPES, pH 7.4, 150 mM NaCl (HBS), and lysed in 1 ml of HBS containing 1% Triton X-100 and 10 μg/ml aprotinin. Lysates were clarified by centrifugation at 2 °C for 15 min at 21,000 g. Clarified lysates were then subjected to immunoprecipitation using 1 μg of the monoclonal anti-FLAG antibody M2, followed by 20 μl of goat antimouse IgG beads to collect immune complexes, as described previously (26). After washes with lysis buffer, beads were heated in SDS sample buffer, and samples were analyzed by SDS-PAGE and fluorography (33). Aliquots of the same samples were analyzed by immunoblotting with a monoclonal antibody to hsp90 as described previously (26).

FIG. 1. Co-immunoprecipitation of hsp90 with mutants of the FLAG-tagged TPR domain of PP5. COS-7 cells were transfected with the indicated constructs, and the FLAG-tagged TPR domains were immunoprecipitated with M2 antibody. Immunoprecipitates from cells labeled with [35S]methionine/cysteine were analyzed by SDS-PAGE and fluorography (top) or by immunoblotting with an antibody to hsp90 (bottom).

Identification of TPR Residues Required for Binding of hsp90—Guided by the three-dimensional structure of the TPR domain of PP5 (28), we performed charged-to-alanine scanning mutagenesis. The scanning was not random in that we chose residues whose side chains extended into the solvent, and that seemed unlikely, therefore, to be critical for protein folding. In the first round of mutagenesis (Fig. 1, left), we mutated seven residues that are oriented along what has been suggested to be a possible binding groove (28). COS-7 cells were transfected with plasmids encoding the wild-type FLAG-tagged TPR domain or with plasmids encoding the indicated mutant FLAG-tagged TPR domains. We then examined co-immunoprecipitation of hsp90 with the various FLAG-tagged TPR proteins from extracts of cells labeled with [35S]methionine/cysteine. Extracts were analyzed by SDS-PAGE and fluorography (Fig. 1, top left). As we have shown previously, hsp90 co-immunoprecipitated specifically with the wild-type TPR domain. The identity of hsp90 was confirmed by immunoblotting with a monoclonal antibody (Fig. 1, bottom left). Mutating Glu-29, Lys-40, Lys-120, or Glu-149 to alanine had no significant effect on hsp90 co-immunoprecipitation. The latter two residues lie beyond the TPR domain but are on the same face of the protein as the putative binding groove (28). In contrast, mutation of Arg-74, Lys-97, or Arg-101, which are highly conserved among hsp90-binding TPR proteins and extend prominently into the putative binding groove (28), led to markedly decreased hsp90 binding. No co-immunoprecipitation of hsp90 was observed with the Lys-97 or Arg-101 mutants, and co-immunoprecipitation was dramatically reduced with the Arg-74 mutant. This
Identification of TPR Residues Required for Binding of hsp90

The above experiment suggested that hsp90 binds to a groove in the TPR domain and that three basic residues in this groove that are conserved among hsp90-binding TPR proteins are critical for binding.

The above experiment tested only the importance of residues lying within the groove in the TPR domain. In a second round of mutagenesis, we also examined residues on different faces of the TPR domain (Fig. 1, right). Mutation of Lys-32, located at one end of the binding groove, completely abrogated hsp90 co-immunoprecipitation. Mutation of Ile-63, which is on the surface of the binding groove, reduced but did not eliminate hsp90 binding. This mutant was expressed at dramatically lower levels than the wild-type TPR domain and may be improperly folded. Mutations of residues on the surface of the TPR domain that are not in the groove (Glu-56, Cys-77, Tyr-80, Arg-113, and Arg-117) had no effect on hsp90 co-immunoprecipitation. Thus, four conserved basic residues in a groove within the TPR domain of PP5 are essential for hsp90 binding, while other residues in this groove, and residues on other faces of the TPR domain, are not required for this function. Binding of the TPR Domain of PP5 to the C Terminus of hsp90—Several studies have shown an interaction between the C-terminal domain of hsp90 and large immunophilins or Hop (13–15). As a first step toward identifying binding determinants in hsp90 that interact with the TPR domain of PP5, we tested the ability of the C-terminal 12-kDa domain of hsp90, designated C90 (as per Young et al. (31)), to bind to this TPR domain. Extracts of COS cells transfected with the wild-type FLAG-tagged TPR domain of PP5, or with two mutants that failed to bind full-length hsp90, were incubated with glutathione beads to which either control GST or a GST-C90 fusion was bound. After washing away unbound material, samples were analyzed by immunoblotting (Fig. 2A). The wild-type TPR domain of PP5 bound specifically to GST-C90, indicating that this portion of hsp90 is sufficient for binding. The two PP5 mutants that did not bind to full-length hsp90 were also unable to bind to the C90 fragment (Fig. 2A).

Role of Acidic Residues in the C Terminus of hsp90 in Binding of the TPR Domain of PP5—Because basic residues in the TPR domain of PP5 are critical for binding to hsp90, we hypothesized that acidic residues in hsp90 would be important for its binding. Acidic residues are distributed throughout the C90 fragment, but previous studies suggested that the C-terminal EEVD sequence might be of particular interest. This sequence is required for binding to Hop or CyP-40, and its mutation reduces binding to FKBP51 or FKBP52 by one-half (13–15). We tested, therefore, the effects of mutating these three acidic residues to Ala or Lys. His-tagged C90 or C90 mutants were adsorbed to beads and tested for their ability to bind to the FLAG-tagged TPR domain. As controls, the Lys-97 and Arg-101 mutants of the TPR domain were also tested, as was a double mutant in which both Lys-97 and Arg-101 were mutated to Glu (Fig. 2B). The wild-type TPR domain bound to His-tagged C90, as expected (Fig. 2B). Binding to the C90 mutants was significantly reduced but not eliminated (Fig. 2B). The TPR mutants did not bind to C90 or to either C90 mutant. Thus, while C-terminal acidic residues of hsp90 are important for optimal binding to the TPR domain of PP5, additional binding determinants must exist. These results suggest that complex formation occurs as a result of multiple interactions between basic residues in the TPR domain of PP5 and acidic residues in the carboxyl domain of hsp90.

Cytoplasmic Localization of PP5—We performed the above experiments based on our data suggesting that hsp90 and PP5 interact in vivo (26). Chen et al. (24), however, have suggested that PP5 is almost exclusively a nuclear protein, whereas hsp90 is cytoplasmic. To address this discrepancy, we attempted to reproduce the subcellular fractionation and immunofluorescence experiments of Chen et al. (24). When examined by subcellular fractionation, PP5 was overwhelmingly cytoplasmic in either HeLa cells or L929 cells (Fig. 3A). When examined by immunofluorescence, PP5 was primarily cytoplasmic in HeLa cells (Fig. 3B), as well as in L929 and COS-7 cells (not shown). This was not due to cross-reactivity of our antibody with cytoplasmic phosphatases, because preadsorption of anti-PP5 with purified PP5 reduced cytoplasmic immunostaining, whereas preadsorption with purified PP1 did not (Fig. 3B). Also, our PP5 antibody did not cross-react with microgram amounts of PP1 or PP2A in immunoblotting experiments (not shown). We conclude that PP5 is primarily a cytoplasmic pro-
tein, and therefore that it is in the correct cellular compartment to interact with hsp90 in vivo.

To examine this question further, GFP fusions of PP5 or its TPR domain were expressed in COS-7 cells and examined by confocal microscopy (Fig. 3C). Control GFP was distributed uniformly throughout the cell, but GFP fused to the TPR domain of PP5 or to full-length PP5 was excluded from the nucleus. These results suggested that PP5 does not contain a nuclear localization sequence.

**DISCUSSION**

In conjunction with the known three-dimensional structure of the TPR domain of PP5 (28), our experiments have identified a binding groove through which this domain binds to hsp90. Four basic amino acids that protrude into this groove are essential for binding to either full-length hsp90 or a 12-kDa C-terminal fragment of hsp90. This carboxyl fragment of hsp90 is highly acidic; in the simplest model, basic residues in the binding groove of the TPR domain of PP5 would be predicted to interact with acidic residues at the carboxyl end of hsp90. Because the basic residues important for hsp90 binding are conserved in the TPR domains of other hsp90-binding proteins, they are likely to be of general importance for the binding of TPR proteins to hsp90. The observation that several acidic residues at the very C terminus of hsp90 are important for maximal binding of several TPR proteins including PP5 further supports a general role for conserved basic residues in binding of TPR proteins to hsp90.

In the past, the significance of interactions between PP5 and hsp90 has been questioned based on the cytoplasmic localization of hsp90 and the apparent nuclear localization of PP5 (24). We were unable to reproduce subcellular fractionation and immunofluorescence experiments suggesting that PP5 is a nuclear protein; we find PP5 to be predominantly cytoplasmic. In addition, fusions of PP5 or of its TPR domain to GFP are excluded from the nucleus, indicating that PP5 lacks a nuclear localization signal.

The TPR domain of PP5 is autoinhibitory (34), suggesting that it may bind to the catalytic domain of PP5 in the basal state. In that case, PP5 could be activated by a mechanism in which binding of hsp90 competitively displaced the TPR domain from the phosphatase catalytic domain. If binding of the TPR domain to the catalytic domain involves the same residues as binding to hsp90, then mutations in the TPR domain that interfere with hsp90 binding would be predicted to result in release of the TPR domain from the catalytic domain and increased basal activity. Alternatively, the catalytic domain of PP5 may interact with other residues in the binding groove or may bind to a different face of the TPR domain. We are currently testing these hypotheses.

In addition to helping to identify the general mechanisms by which TPR domains bind to hsp90, our results may be useful in the development of compounds that disrupt hsp90 binding to specific TPR proteins. Determination of the three-dimensional structures of hsp90 complexes with these proteins will be necessary to determine the details of their molecular interactions. Our identification of the hsp90-binding site in the PP5 TPR domain, however, combined with the known structure of this domain, may allow molecular modeling of TPR domains whose structure is not yet known, and the rational synthesis of compounds that can compete with hsp90 for binding to particular TPR proteins. This could be important not only for hsp90-binding TPR proteins known to regulate various signaling pathways, but also for less characterized TPR proteins such as SGT/UBP, which regulates the assembly and release of HIV and may also be involved in the parvovirus life cycle (35, 36). Based on the sequence of this TPR protein, we would predict that its effects on viral pathogenesis involve binding to hsp90. Having identified the location of the residues involved in PP5 binding to hsp90 may also be helpful in modeling how other TPR proteins interact with their non-hsp90 partners.

**Acknowledgments**—We thank Drs. Mark Gillespie and Andrew Ramsey for careful reading of the manuscript.

**REFERENCES**

1. Parsell, D. A., and Lindquist, S. (1993) Annu. Rev. Genet. 27, 437–496
2. Pratt, W. B. (1996) Proc. Soc. Exp. Biol. Med. 217, 420–434
3. Pratt, W. B., and Toft, D. O. (1997) Endocrinol. Rev. 18, 396–396
4. Chen, C.-F., Chen, Y., Dai, K., Chen, P.-L., Riley, D. J., and Lee, W. H. (1996) Mol. Cell. Biol. 16, 4691–4699
5. Whittles, L., Surpin, P. D., Polcin, E. J., Martinez, J. D., and Cook, P. H. (1998) Mol. Cell. Biol. 18, 1517–1524
6. Garcia-Cardena, G., Fan, R., Shah, V., Sorrentino, E., Cirino, G., Pappan, W. C. (1998) Nature 392, 821–824
7. Loo, M. A., Jensen, T. J., Cui, L., Hou, X.-Y., Chang, X.-B., and Riordan, J. R. (1998) EMBO J. 17, 6879–6887
8. Rutherford, S. L., and Lindquist, S. (1998) Nature 396, 336–342
9. Smith, D., Whitesell, L., and Katzenia, E. (1998) Pharmacol. Rev. 50, 493–513
10. Scheibl, T., and Buchner, J. (1998) Biochem. Pharmacol. 56, 675–682
11. Lamb, R. J., Tugendreich, S., and Heter, P. (1995) Trends Biochem. Sci. 20, 257–259
12. Frydman, J., and Höhfeld, J. (1997) Trends Biochem. Sci. 22, 87–92
13. Chen, S., Sullivan, W. P., Toft, D. O., and Smith, D. F. (1998) Cell Stress & Choreoneres 3, 118–129
14. Young, J. C., O’Beirne, W. M. J., and Hartl, F. U. (1998) J. Biol. Chem. 273, 18007–18010
15. Carello, A., Ingley, E., Minchin, R. F., Tsai, S., and Ratajczak, T. (1999) J. Biol. Chem. 274, 663–669
16. Bose, S., Weikl, T., Bugl, H., and Buchner, J. (1996) Science 274, 1715–1717
17. Freeman, B. C., Toft, D. O., and Morimoto, R. I. (1996) Science 274, 1718–1720
18. Duina, A. A., Marsh, J. A., Kurtz, R. B., Chang, H.-C. J., Lindquist, S., and Gaber, R. F. (1998) J. Biol. Chem. 273, 10819–10822
19. Prodromou, C., Siligardi, G., O’Brien, R., Woolfson, D. N., Regan, L., Panaretou, B., Lasbury, J. E., Piper, P. W., and Pearl, H. L. (1999) EMBO J. 18, 754–762
20. Dolinski, K. J., Cardenas, M. E., and Heitman, J. (1998) Mol. Cell. Biol. 18, 7344–7352
21. Marsh, J. A., Kalson, H. M., and Gaber, R. F. (1996) Mol. Cell. Biol. 16, 7353–7359
22. Reynolds, P. D., Ruan, Y., Smith, D. F., and Scammell, J. G. (1999) J. Clin. Endocrinol. Metab. 84, 2381–2388
23. Chinkers, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11075–11079
24. Men, W. C., McFarland, A. E., Brown, L., Chen, Y. H., Barker, H. M., and Cohen, P. T. W. (1994) EMBO J. 13, 4275–4280
25. Becker, W., Kastrop, H., Klaupp, S., Schultz, J. E., and Joost, H. G. (1994) J. Biol. Chem. 269, 22586–22592
26. Chen, M.-S., Silverstein, A. M., Pratt, B. W., and Chinkers, M. (1996) J. Biol. Chem. 271, 32215–32220
27. Silverstein, A. M., Galagnian, M. D., Chen, M.-S., Owens-Grillo, J. K., Chinkers, M., and Pratt, W. B. (1997) J. Biol. Chem. 272, 16224–16230
28. Das, A. K., Cohen, P. T. W., and Barford, D. (1998) EMBO J. 17, 1192–1199
29. Skovits-L., Boguski, M. S., Goelz, M., and Heter, P. (1990) Cell 60, 307–317
30. Hirano, T., Kinoshita, N., Morikawa, K., and Yanagida, M. (1990) Cell 65, 319–326
31. Young, J. C., Schneider, C., and Hartl, F. U. (1997) FEBS Lett. 418, 139–143
32. Cullen, B. R. (1987) Trends Biochem. Sci. 12, 257–259
33. Chamberlain, J. P. (1979) Anal. Biochem. 98, 132–135
34. Papam, Y. H., and Cohen, P. T. W. (1997) FEBS Lett. 400, 136–140
35. Czepiegl, C., Kordes, E., Poirey, R., Greenwic, A., Rommelas, J., and Jauniaux, J.-C. (1998) J. Virol. 72, 1419–1456
36. Callahan, M. A., Handley, M. A., Lee, Y.-H., Talbot, K. J., Harper, J. W., and Panganiban, A. T. (1998) J. Virol. 72, 5189–5197