A Model of Protein Targeting Mediated by Immunophilins and Other Proteins That Bind to hsp90 via Tetratricopeptide Repeat Domains*

(Received for publication, January 19, 1996, and in revised form, March 21, 1996)

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We have shown recently that the immunophilins CyP-40 and FKBP52/hsp56 bind to a common site on hsp90 and that they exist in separate heterocomplexes with the glucocorticoid receptor (GR). FKBP52/hsp56 binds to hsp90 via its tetratricopeptide repeat (TPR) domains, it is not required for GR-hsp90 heterocomplex assembly, and it is thought to play a role in targeted movement of the GR. In this work we examine the hsp90 binding of four proteins (FKBP52/hsp56, CyP-40, p50, Mas70p) thought to be involved in targeted protein trafficking. FKBP52/hsp56 and CyP-40 (each with three TPRs), localize to the nucleus and nucleoli, respectively, and form relatively weak complexes with hsp90 that are competed by a CyP-40 fragment containing its three TPRs. The p50 component of the Src-hsp90 and Raf-hsp90 heterocomplexes localizes to cytoskeletal fibers extending from the perinuclear region to the plasma membrane and forming a rim under the plasma membrane of endothelial cells. p50, Mas70p (seven TPRs), which is a receptor for mitochondrial import, and the p60 (six to eight TPRs) component of the steroid receptor-hsp90 heterocomplex assembly system bind very tightly to hsp90 in a manner that is not competed by the CyP-40 fragment. However, bacterially expressed p60 blocks the binding of p50, Mas70p, FKBP52/hsp56, and CyP-40 to purified hsp90. The data are consistent with binding of all of these proteins to a site on hsp90 that is a general TPR domain acceptor. Our localization and binding data are used to develop a model in which proteins that are chaperoned by hsp90 move as dynamic complexes to their cellular sites of action, with the TPR-containing protein participating in targeting the movement of the complexes.

Little is known about how proteins that are not conveyed by a vesicle-based protein trafficking system move through the cytoplasm to arrive at their sites of action in organelles, such as the nucleus or mitochondria, or at a cellular locus like the internal surface of the plasma membrane. Steroid receptors are a useful model for studying such targeted protein movement. These ligand-regulated transcription factors must travel through the cytoplasm, traverse the nuclear pores, and then travel within the nucleus to their sites of action. Their localization is mediated by nuclear localization signal (NLS) sequences and cytoplasmic nuclear shuttling of receptors occurs constantly (for review, see Ref. 1). In the case of the glucocorticoid receptor (GR), the NLS is under hormonal control (2), and localization to the nucleus occurs only after hormone binding. In contrast, estrogen and progesterone receptors have constitutively functional NLSs (3) such that the receptors are localized in the nuclei of hormone-free cells (4, 5). Despite their different localizations, all of these receptors are recovered in cytosols in the same receptor heterocomplex, which has been demonstrated by cross-linking to exist in intact cells (6–8). This "core" complex consists of the receptor bound to a dimer of hsp90 and one molecule of the immunophilin FKBP52/hsp56. Some hsp70 and an acidic 23-kDa protein (p23), both of which are required for assembly of the receptor-hsp90 complex (9–12), may also be present (see Refs. 13 and 14 for review of receptor heterocomplex assembly and structure).

In 1992, we proposed that the receptors shuttle through the cytoplasm in the heterocomplex form, with hsp90 and the immunophilin acting as a protein transport unit or transportosome (15). This model of receptor movement was supported by experiments in which hsp90 was targeted to the nucleus by fusion to the nucleoplasmin NLS, and it was shown that coexpression of the hsp90 NLS and cytoplasmic receptor mutants devoid of an NLS resulted in complete nuclear localization of the receptors (16). It is important to note that the complex of steroid receptors and hsp90 is dynamic in the sense that assembly and disassembly occurs constantly (17), and it is possible that this dynamic cycling is required for receptor movement.

In 1993, we (18) proposed that the component of the receptor heterocomplex that targets receptor movement to the nucleus is FKBP52/hsp56. This protein is a member of the FK506- and rapamycin-binding class of immunophilins (19–22), and it is a component of all steroid receptor heterocomplexes (23). FKBP52/hsp56 binds directly to the hsp90 component of the receptor heterocomplex (24, 25) via its 3 TPR (tetratricopeptide repeat) domains.

1 The abbreviations used are: NLS, nuclear localization signal; CyP-40, the 40-kDa cyclosporin A-binding protein; FKBP, FK506-binding protein; hsp, heat shock protein; Mas70p, 70-kDa mitochondrial import receptor; PAGE, polyacrylamide gel electrophoresis; GR, glucocorticoid receptor; TPR, tetratricopeptide repeat; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline.
kinase c-Raf, which is involved in normal mitogenic signal transduction, is also in a heterocomplex with p50 and hsp90 (55).

Both the Src and Raf heterocomplexes can be assembled under cell-free conditions with the same reticulocyte lysate system that assembles steroid receptor heterocomplexes (55–57). Like the immunophilins FKBP52/hsp56 and CyP40, p50 exists in cytosolic complexes with hsp90 (38, 39). However, the steroid receptors and protein kinases make different choices of hsp90-associated protein, in that native steroid receptor-hsp90 complexes contain FKBP52/hsp56 but not p50 (39), whereas the protein kinase heterocomplexes contain p50 but not FKBP52/hsp56 (55). The receptor and protein kinase heterocomplexes are similar in the respect that the presence of p50 in the kinase-hsp90 complexes (55–57) and immunophilin in receptor-hsp90 complexes (25, 36) is stabilized by molybdate, vanadate, and tungstate. We have proposed previously (55) that p50 may play a role in targeted movement of the protein kinases to their sites of action at the plasma membrane, much as FKBP52/hsp56 may participate in targeted movement of receptors to their sites of action in the cell nucleus.

In this paper we show that CyP40 and FKBP52/hsp56 (each with three TPRs) form relatively weak complexes with purified hsp90 that are competed by a purified fragment containing the three TPR domains of human CyP40. In contrast, p60 (with six to eight TPRs) and p50 bind very tightly to hsp90 and their binding is not competed by the CyP40 TPR fragment at the concentrations we can achieve. Native p60/hsp90 complexes do not contain FKBP52/hsp56, CyP40, or p50, and, consistent with a common binding site for all the proteins on hsp90, bacterially expressed human p60 inhibits the binding of each to purified hsp90. With its seven TPR domains, Mas70p binds tightly to purified hsp90 in a manner that is competed by the tight binder p60 but not by the weakly binding CyP40-TPR fragment. From these data we predict that hsp90 has a universal TPR domain binding region that permits it to bind to multiple proteins. Although the gene for p50 is not yet cloned, we predict that, like the others, it will encode TPR domains. We show by indirect immunofluorescence that each of these hsp90-associated proteins localizes to different organelles in a manner that is consistent with their predicted role in targeted protein movement.

EXPERIMENTAL PROCEDURES

Materials

Untransformed rabbit reticulocyte lysate was from Green Hectares (Oregon, WI). 125I-Conjugated goat anti-mouse and anti-rabbit IgGs were from DuPont NEN. Iron-supplemented bovine calf serum was from HyClone Laboratories, Inc. (Logan, UT). Trypsin, powdered Dulbecco's modified Eagle's medium (high glucose), goat anti-mouse IgG-horseradish peroxidase conjugate, monodonal non-immune IgG and IgM, non-immune rabbit serum, TUB2.1 monoclonal anti-jt-tubulin IgG, anti-nuclear antibody (nuclear positive control) and the fluorescein isothiocyanate (FITC)-conjugated antihuman IgG were from Sigma. Actigel ALD (activated aldehyde agarose) affinity support for protein immobilization was purchased from Sterogene Biochemicals (San Gabriel, CA). Goat anti-mouse IgM, donkey anti-rabbit IgG-horseradish peroxidase conjugate, and protein A-agarose were from Pierce. The ACB8 monoclonal IgG against hsp90 and the N27F 3-4 anti-72/73-KDa heat shock protein monoclonal IgG (anti-hsp70) were from StressGen (Victoria, Canada). The anti-cyclipholin 40 (COOH-terminal peptide) antibody and the 3G3 monoclonal anti-hsp90 IgM were from Affinity Bioreagents (Golden, CO). FITC-conjugated donkey anti-mouse IgG and IgM and rhodamine-conjugated donkey anti-rabbit IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA). The UPJ56 rabbit antiserum against FKBP52/hsp56 (58) was a gift from Drs. Karen Leach and Martin Debel (The Upjohn Company). The J 5 monoclonal antibody against p23 (59) was a gift from Dr. David Toft (Mayo Clinic, Rochester, MN) and the D554 monoclonal antibody against p60 (42) was kindly provided by Dr. David Smith (University of Nebraska, Omaha, NE). The anti-Mas70p rabbit antiserum (44) was a gift from Dr. Gottfried Schatz (Biozentrum, University of Basel).

2 The consensus sequence AEAKGLGHYEKFLGLDQLKDALAFOQ-KALLDPNN for a TPR domain was determined by Sikorski et al. (70) from five proteins (CDC23, Nuc22, CDC16, S5N6, SK13), each with multiple TPR units. The residues in bold are present in 40% or more of the TPRs.

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clonal antibody against p50 (39) has been described previously. CyP-4059 is a bacterially expressed human CyP-40 COOH-terminal fragment containing the FKBP52-like TPR domain, but not the CyP-18-like domain, and it was purified by Ni2+ affinity chromatography and thrombin cleavage.3

Methods

Cell Culture—Rat pulmonary endothelial cells (60) were cultured in T25 flasks in Dulbecco’s modified Eagle medium with 10% iron-supplemented calf serum. At least 2 days prior to immunofluorescent staining, cells were lifted from the flasks using 0.05% trypsin, 0.5 mM EDTA in calcium-free and magnesium-free Hanks’ buffered saline and plated onto 11 × 22-mm glass coverslips (10100-mm dish) in medium containing 10% iron-supplemented serum.

Immunofluorescence—Rat pulmonary endothelial cells were fixed for 1 h in 1.37% formaldehyde and permeabilized by 5 min incubation in −20°C methanol prior to staining for hsp56, CyP-40, tubulin, or the nucelar positive control. Prior to staining for p50 or Mas70p, cells were fixed and simultaneously permeabilized in −20°C methanol and then incubated in −20°C acetone for 1 min. All cells were washed with phosphate-buffered saline (PBS) and then incubated for 45–60 min with primary antibody or mixtures of primary antibodies as noted in the figure legends. The cells were washed again with PBS and incubated in secondary antibody or mixtures of secondary antibodies for 30 min. The secondary antibodies used were rhodamine-conjugated donkey anti-rabbit for labeling with preimmune rabbit serum, UP) 56, anti-CyP-40 or p50, FITC-conjugated donkey anti-mouse IgG for TUB2, rhodamine-conjugated donkey anti-mouse IgM for anti-p50, or FITC-conjugated goat anti-human IgG for anti-nucleolar antibody. The cells were washed with PBS a final time and the coverslips mounted on slides with p-phenylendiamine mounting medium (61). The cells were viewed on a Leitz Aristoplan epifluorescence microscope equipped with a Leitz Vario-Orthomat camera and photographed with T-Max400 film (Leitz, Rockleigh, NJ). Confocal microscopy was viewed on a Bio-Rad MRC-600 laser scanning confocal microscope (Bio-Rad).

Immunoadsorption—Aliquots of rabbit reticulocyte lysate (100 μl), purified hsp90 (75 μl, 0.5 mg/ml), or rabbit brain cytosol (25 μl) were immunoadsorbed to 7.5-μl pellets of Actigel ALD precoupled with either nonimmune mouse ascites or 3G3 anti-hsp90 IgM or to 8 μl of protein A-agarose prebound with DS14F5 antibody against p60 (5%), UP) 56 antisera against FKBP52/56 (4%), anti-CyP-40 (10%), JJ5 antibody against p23 (5%), or nonimmune mouse IgG (5%) or nonimmune rabbit serum (2.5%). Immunoadsorptions were performed with the samples rotating at 4°C for 2 h. Immunopellets were washed twice by suspension in 1 ml of HEG buffer (10 mM Hepes, pH 7.4, 1 mM EDTA, 10% methanol) and centrifugation.

Gel Electrophoresis and Western Blotting—Immunopellets were boiled in SDS sample buffer, and proteins were resolved on 10% SDS-polyacrylamide gels. Proteins were transferred to Immobilon-P membranes and probed with 1 μg/ml AC88 for hsp90, 1 μg/ml N27F3–4 for hsp70, 0.1% anti-Mas70p, 0.1% DS14F5 for p60, 0.1% UP) 56 for FKBP52/56, 0.3% anti-p50, or 0.1% anti-CyP-40. The immunoblots were then incubated a second time with the appropriate affinity-conjugated counterantibody to visualize the immunoreactive bands.

Hydroxyapatite Chromatography of Rabbit Brain Cytosol—Rabbit brain cytosol (20 ml) was diluted with an equal volume of 10 mM K2HPO4, 1 mM EDTA, pH 7.4, and then chromatographed on a 2 ml column equilibrated to the same conditions. Proteins were eluted with a 300-ml gradient of 0–500 mM K2HP04, pH 7.4, and then dialyzed against HKD buffer. The sample was contracted to 1 ml, and dialyzed against H2O buffer to remove reagents. The resulting supernatant was clarified by centrifugation at 100000 g for 10 min. The soluble supernatant fraction was dialyzed into a 10% sucrose buffer. The sample was assayed for hsp56, CyP-40, and p23—Rabbit brain cytosol (20 ml) was adsorbed to a 2.5 × 20-cm column of DE52 equilibrated with HE buffer, the column was washed with 150 ml of HE buffer, and the proteins were eluted with a 400-ml gradient of 0–0.5 M KCl. hsp90, p60, FKBP52/56, p50, CyP-40, and p23 were detected by resolving an aliquot of each fraction by SDS-PAGE and Western blotting with appropriate antibodies. Fractions containing p50 and hsp90, but not p60, FKBP52/56, p50, CyP-40 or p23, were pooled, concentrated to 1 ml, and dialyzed against H2O buffer. The sample was adjusted to 0.5 M KCl and rotated at 4°C for 2 h prior to chromatography through a 1.5 × 120-cm column of Sepharose CL-6B in HE buffer containing 0.5 M KCl. p50 binds tightly to hsp90 and 0.5 M KCl is required to dissociate the complex. Fractions containing hsp90 and p50 were identified by SDS-PAGE and Western blotting with appropriate antibodies. The p50-containing, hsp90-free fractions were pooled, contacted to 0.5 M dialyzed against H2K buffer, flash-frozen, and stored at −70°C.

RESULTS

The TPR-containing Proteins Exist in Different Heterocomplexes with hsp90—As shown in Fig. 1, immunoadsorption of hsp90 from rabbit reticulocyte lysate with the 3G3 monodonal IgM is accompanied by immunoadsorption of hsp70, p60, FKBP52/56, p50, CyP-40, and p23. We have indicated previously that p23 dissociates rather easily during washing of the immunopellet (12), but some immune-specific p23 is clearly detectable in the 3G3 immunoadsorption of Fig. 1. Immunoad-
that there is some similarity between the two hsp90-associated proteins, p50 and FKBP52/hsp56. This cross-reactivity suggests that the antibody against p50 does not recognize FKBP52/hsp56 or p60. Thus, the three hsp90-immunoassociates of p23, CyP-40, and p50 likely exist in separate native heterocomplexes with hsp90.

Because p60 has six to eight TPR domains (41) and bacteriophage T4 lysozyme (42, 43), that hsp90, hsp70, and p60 form a major complex, and in Fig. 1 it is shown that immunoadsorption of p60 is not accompanied by any coimmunoadsorption of FKBP52/hsp56, p50, CyP-40, or p23. As we have reported previously (36), immunoadsorption of FKBP52/hsp56 does not yield coimmunoadsorption of CyP-40, but as shown in Fig. 1, there is also no coadsorption of p60. Similarly, immunoadsorption of CyP-40 does not yield coimmunoadsorption of FKBP52/hsp56 or p60. Thus, the three hsp90-associated proteins that are known to have TPR domains appear to exist in separate native heterocomplexes with hsp90. p50 could not be immunoadsorbed from reticulocyte lysate, because the anti-p50 IgM only recognizes the denatured protein on Western blot. We reported previously (55) that immunoadsorption of FKBP52/hsp56 from reticulocyte lysate with the UPJ56 antiserum yields coimmunoadsorption of p50, leading us to conclude that the two proteins may exist in the same heterocomplex with hsp90. As shown in Fig. 1, immunoadsorption of either FKBP52/hsp56 or CyP-40 is accompanied by the immune-specific presence of some p50. Immunoblottting of aliquots of reticulocyte with the antibodies against FKBP52/hsp56, CyP-40, and p50 suggests a lack of cross-reactivity between them (Fig. 2A). However, both UPJ56 and anti-CyP-40 immunoadsorb some p50 after it has been separated from hsp90, hsp56, and CyP-40 (Fig. 2B), implying direct recognition of p50 by both antibodies. When p50 is concentrated by adsorption to hsp90 such that much more of the protein is present in each lane than was present in Fig. 2A, reaction with both UPJ56 and anti-CyP-40 can be demonstrated by immunoblotting (Fig. 2C). On Western blotting of the denatured proteins, the antibody against p50 does not recognize FKBP52/hsp56 or CyP-40, UPJ56 recognizes p50 but not CyP-40, and the antibody against the COOH-terminal peptide of CyP-40 recognizes both p50 and FKBP52/hsp56. This cross-reactivity suggests that there is some similarity between the two hsp90-associated immunophilins and p50. Also, it is likely that p50 is present in UPJ56 and anti-CyP-40 immunopellets as a result of direct immunoadsorption, rather than being present because it exists in the same hsp90 heterocomplex with each immunophilin and is coimmunoadsorbed with it. Thus, p60, FKBP52/hsp56, CyP-40, and p50 likely exist in separate native heterocomplexes with hsp90.

Binding of p60, FKBP52/hsp56, p50, and CyP-40 to Purified hsp90—To assay directly the binding of each protein to purified hsp90, rabbit brain cytosol was chromatographed on hydroxyapatite as described under “Methods” (solid line, absorbance at 280 nm; dotted line, K2HPO4 gradient). An aliquot from each fraction was analyzed for hsp90, hsp70, p60, FKBP52/hsp56, p50, CyP-40, and p23 by SDS-PAGE and Western blotting. Fractions that did not contain hsp90 but did contain the other proteins were pooled (indicated by the thick solid line under the Western blots), concentrated, and dialyzed against HKD buffer. This hydroxyapatite packing hsp90 was used in subsequent experiments.

To ask if TPR domains are involved in the protein binding to hsp90, in the experiment of Fig. 5A we added CyP-4059 to the hydroxyapatite pool prior to incubation with the purified hsp90. The CyP-4059 fragment contains the three TPR domains of CyP-40 and its COOH-terminal calmodulin binding domain (63). CyP-4059 prevents the binding of CyP-40 and almost completely inhibits the binding of FKBP52/hsp56 to hsp90 (cf. lane 2 and lane 3 of Fig. 5A). However, the binding of p60 and p50 to hsp90 is not inhibited by the 60 μg of CyP-4059 present in this experiment or by the highest concentrations we could achieve (300 μg, data not shown).

Because p60 has six to eight TPR domains (41) and bacteri-
ry endothelial cells are an optimal system for examining cytосkeletal structure and detecting cytосplasmic organelles by indirect immunofluorescence (60). We have reported previously colocalization of cytосplasmic immunofluorescence by the UPJ 56 antibody against FКBP52/hsр56 and the TUB2.1 antibody against β-tubulin (31) and that observation is repeated in B and C of Fig. 6 to permit comparison with the localization of anti-Cyр-40 and anti-р50 immunofluorescence in the same figure.

The localization of the three hsp90-binding proteins is quite different. The majority of FКBP52/hsр56 is localized in the nucleus, and the nucleolar shadows (Fig. 6B and Ref. 31) suggest that this immunohitin is not present, or is present at much lower concentration, in nuclei. In contrast, virtually all of the nuclear Cyр-40 immunofluorescence (Fig. 6E) is localized in nuclei, as verified by colocalization with anti-nuclear antibody (Fig. 6F). The cytосplasmic Cyр-40 immunofluorescence localizes to small punctate, and often oblong, bodies located throughout the cytосplasm. Although these cytосplasmic bodies look like mitochondria, this has not been established.

In quite a different pattern, immunofluorescence due to the anti-р50 antibody extends on cytосkeletal fibrils from a perinuclear region of intense signal out to the cell periphery (Fig. 6H). To demonstrate that the sharp fluorescence defining the cell periphery is not an artifact of ruffling of the cell margins, we show a confocal image through a single plane of the cells in Fig. 6i. This sharply defined peripheral immunofluorescence is consistent with a localization of some of the р50 at the inner surface of the plasma membrane. We were unable to obtain any distinct pattern of immunofluorescence with the DS14F5 antibody against р60.

Mas70p Binds to hsp90—The immunofluorescence patterns shown in Fig. 6 were consistent with the notion that FКBP52/ hsр56, Cyр-40, and р50 might serve to target protein movement to different sites, such as the nucleus, nucleoli, the internal surface of the plasma membrane, and perhaps to mitochondria. In considering a model of targeted protein movement in which a protein moves to an organelle in a transport complex, there must be some way to “hand-off” the protein to the organelle. In this kind of a model, it might be important that the hsp90 component of the complex bind tightly to the protein import receptor upon arrival at the organelle. There is solid evidence that Mas70p is a component of the mitochondrial receptor machinery for protein import (46), and we asked if Mas70p would bind to hsp90.

Rabbit brain was used as a source of Mas70p, and as shown in Fig. 7A, a mitochondrial pellet prepared from a brain homogenate contains both 70- and 60-kDa bands (lane 1) that immunoblot with anti-Mas70p antiserum. Both species are also present in a detergent extract of the mitochondrial pellet (lane 3). When the anti-Mas70p serum is used for immunofluorescence in rat pulmonary endothelial cells, it localizes mitochondria in the cytoplasm (Fig. 7B), but it also produces a nuclear immunofluorescence, which may suggest cross-reaction of the antiserum with a nuclear protein (Fig. 7B). Fortunately, the high speed supernatant of brain homogenate contains a lot of the 60-kDa fragment of Mas70p (Fig. 7A, lane 4), and this cytosolic fraction could be directly immunoadsorbed with 3G3 antibody to determine if Mas70p was present in native complexes with hsp90. Because other organs of the rabbits were being used for intact physiological preparations, the brains were not removed and placed in ice until ~20 min after death. This delay before tissue cooling and homogenization may account for the extensive, but useful, cleavage of Mas70p to its 60 kDa cytosolic fragment. Immunoadsorption of brain cytosol with 3G3 antibody resulted in coadsorption of Mas70p 60-kDa fragment with
hsp90 (Fig. 7C, lanes 1 and 2). Preincubation of the brain cytosol with the CyP-4059 fragment eliminates binding of CyP-40 to hsp90 but does not affect the amount of hsp90-Mas70p complex that is immunoadsorbed (cf. lanes 2 and 4 of Fig. 7C).

Like the immunophilins and p50, the Mas70p 60-kDa fragment is present in the hydroxylapatite pool of brain cytosol proteins, and we could assay its binding to purified hsp90 as we did with the other proteins in Fig. 4. In the experiment of Fig. 7D, aliquots of the hydroxylapatite pool were incubated with 3G3-Actigel (lane 1) or with 3G3-Actigel prebound with purified hsp90 (lane 2), and it was shown by immunoblotting that the TPR-containing Mas70p 60-kDa fragment bound to hsp90. As described above for p50, binding of Mas70p to purified hsp90 was not competed by 60 μg of CyP-4059 (data not shown). However, the bacterially expressed p60 does compete for binding of Mas70p (cf. lanes 3 and 4 of Fig. 7D), suggesting that these two TPR-containing proteins may bind to the same region on hsp90.

DISCUSSION

hsp90 has been reported to be in association with at least 9 transcription factors, 10 protein kinases, the G protein β/γ subunit and some other regulatory proteins (see Ref. 64 for review). Additionally, hsp90 complexes containing receptors and protein kinases have been reported to contain various amounts of hsp70, p60, immunophilins, p50, and p23 in various combinations and depending upon the conditions of the assay. An ability to associate with multiple proteins is consistent with the role proposed for this ubiquitous, abundant, and conserved protein as a member of a cytosolic superchaperone system (see Ref. 65 for review). Although there are multiple protein interactions and it is likely that many more will be reported, there are potentially four categories of protein interaction sites on hsp90 that have been established or can be reasonably predicted.

There is a region of hsp90 that interacts with the proteins that are being chaperoned (e.g. steroid receptors, protein kinases). This region appears to be located in the COOH-terminal half of hsp90 (71, 72), and it binds proteins of different structure and function without any specific binding motif having been detected. It is also thought that there is a region of hsp90 that binds hsp70, which is required for at least some hsp90 chaperone functions, such as hsp90 binding to steroid receptors (9, 10). However, we have been unable to demonstrate direct binding of purified hsp90 to purified hsp70 (25), and when the hydroxylapatite pool of brain cytosol is incubated with purified...
Fig. 7. Binding of Mas70p 60-kDa fragment to hsp90. A, Western blot of Mas70 and its 60-kDa fragment in mitochondrial pellet (12,000 \times g pellet) and cytosolic fraction (100,000 \times g supernatant) of homogenized rabbit brain. Lane 1, 10 \mu l of mitochondrial pellet; lane 2, mitochondrial pellet after extraction with Triton X-100; lane 3, Triton X-100 extract of mitochondrial pellet; lane 4, 10 \mu l of rabbit brain cytosol. B, Immunolocalization of Mas70p. Rat pulmonary endothelial cells on coverslips were fixed in -20°C methanol and incubated with anti-Mas70p diluted 1:30. The cells were washed with PBS, incubated with rhodamine-conjugated donkey anti-rabbit IgG diluted 1:60, and visualized as described under “Methods.” C, communiaoddsorption of Mas70p 60-kDa fragment in a native complex with hsp90. Brain cytosol containing the 60-kDa fragment of Mas70p (10 \mu l) was incubated for 1 h at 4°C with (40 \mu l) K_2HPO_4 buffer, pH 4.2, or with buffer containing 60 \mu g of CyP-4059, and the mixture was adsorbed with nonimmune IgG-Actigel or 3G3-Actigel. After washing, the pellet-associated hsp90, CyP-40, and the 60-kDa fragment of Mas70p were resolved by SDS-PAGE and Western blotting. Lanes 1 and 2, nonimmune (lane 1) and 3G3 (lane 2) pellet of samples without CyP-4059; lanes 3 and 4, nonimmune (lane 3) and 3G3 (lane 4) pellet of samples with CyP-4059. D, Binding of the 60-kDa fragment of Mas70p to purified hsp90 is competed by bacterially expressed p60. 3G3-Actigel or 3G3-Actigel prebound with purified hsp90 was incubated at 4°C with the rabbit brain hydroxyapatite pool (in this case, pooled to contain Mas70p and CyP-40, but not p60) and 0.1% Nonidet P-40 in the presence or absence of lysate from control or p60-expressing bacteria. After washing, the pellet-bound proteins were resolved by SDS-PAGE and Western blotting. Lane 1, 3G3-Actigel pellet without hsp90, but with hydroxyapatite pool; lane 2, 3G3-Actigel pellet with hsp90 and hydroxyapatite pool; lane 3, 3G3-Actigel with hsp90 incubated with control bacterial lysate and hydroxyapatite pool; lane 4, 3G3-Actigel with hsp90 incubated with lysate from bacteria expressing p60 and hydroxyapatite pool.

hsp90, we do not recover an hsp90-hsp70 complex (Fig. 4). As shown previously by Smith et al. (42), p60 binds to both hsp90 and hsp70, and large amounts of hsp70 are only obtained in native hsp90 heterocomplexes when p60 is present (repeated in Fig. 1, p60 immunoadsorption). It is likely that the presence of p60, and perhaps other lysate factors, generates a direct interaction between hsp70 and hsp90. This predicted hsp70 interaction site on hsp90 could lie proximate to the region that interacts with the chaperoned proteins; this is inferred from the fact that hsp70 binds to steroid receptors during receptor-hsp90 heterocomplex assembly (see Refs. 13 and 14 for review of heterocomplex assembly).

A third protein interaction site on hsp90 is likely required for binding of p23, which binds to hsp90 by an ATP-dependent, but apparently not hydrolysis-dependent, mechanism (62). This p23 interaction is required for proper receptor heterocomplex assembly (11, 12). Preformed complexes that contain hsp90, hsp70, and p60 but have been washed free of a component we have identified as p23 (12) will form a glucocorticoid receptor-hsp90 heterocomplex, but that complex does not bind to steroid unless p23 is present (66). The glucocorticoid receptor must be bound to hsp90 to be in high affinity steroid binding conformation (13); thus, in this instance at least, p23 would seem to be required for a “conformationally productive” receptor-hsp90 interaction to occur (66). As with the predicted hsp70 interaction site, we would predict that the p23 binding site might be located within an active chaperoning center of hsp90 involved in heterocomplex assembly with receptors and other proteins.

The fourth protein binding site on hsp90 may very well be shared by immunophilins p60 and p50. The fact that p60, FKBP52/hsp56, and CyP-40 exist in different native heterocomplexes with hsp90 (Fig. 1) and that p60 prevents binding of the immunophilins to hsp90 (Fig. 5B) is consistent with shared or overlapping sites. Because the TPR domains of FKBP52/hsp56 (26) and CyP-40 (67) are required for their binding to hsp90, which itself does not have TPR domains, there is likely a TPR acceptor site on hsp90. The affinity of binding to this site may at least in part reflect the number of repeats in the binding protein. FKBP52/hsp56 and CyP-40, both of which possess three TPR domains, are relatively weakly associated with hsp90 (25, 36), and the binding of both is readily competed by the three TPR-containing CyP-4059 fragment (Fig. 5A). In contrast, the binding of p60, with six to eight TPR domains, and of Mas70p, with seven TPR domains, is not competed by CyP-4059 under the same conditions of competition (Figs. 5A and 7C). Obviously, any of these proteins could also bind to regions of hsp90 outside of its TPR acceptor site, with that non-TPR-mediated binding contributing to the overall affinity. p50 is not yet cloned, but we would suggest from these binding competition studies that it may also possess TPR domains and that it binds to a general TPR acceptor region on hsp90.

It has been shown that the GR becomes bound to hsp90 at the termination of its translation (68) and that p60 dissociates from the progesterone receptor during the process of heterocomplex assembly (17). We propose here a general model in which, upon their translation, multiple proteins may be assembled into complexes with hsp90 by a process involving hsp70, p60, p23, and possibly other components of the chaperone system. When p60 dissociates during the assembly process, the TPR acceptor site on hsp90 is available to interact with a targeting protein, such as an immunophilin or p50. This TPR-containing targeting protein binds to a localization signal on the chaperoned protein, as proposed for the GR NLS binding of FKBP52/hsp56 (30). The TPR-containing protein would then, either directly or through additional protein interactions, determine association of the multiprotein complex to the machinery for movement in the appropriate anterograde or retrograde direction. This is not envisioned as a static “piggyback” movement of the chaperoned protein with hsp90, but as with the steroid receptors, as a dynamic process in which heterocomplex assembly and disassembly may occur continuously (17). Proteins such as the Mas70p mitochondrial import protein may serve to accept the complex by binding the hsp90 chaperone at the site of protein delivery via a tight interaction with the TPR acceptor site. The yeast PAS10 protein, which contains seven TPR domains and is essential for the import of most matrix proteins into peroxisomes (69), might serve the same acceptor function as Mas70p in that organelle. The nuc2p protein is a nuclear protein with TPR domains (43) that is thought to be associated with the nuclear scaffold, and it might serve as a candidate for performing a similar function to that we propose for Mas70p, in that it binds hsp90 when the chaperoned complex arrives at the termini of the nuclear movement machinery.

One question that must be asked is what role does CyP-40 play in such a model of movement for steroid receptors? It is
possible that CyP-40 has no function with respect to steroid receptors. CyP-40 binds much more weakly to the GR than does FKBP52. It is possible that CyP-40 has no function with respect to steroid receptor function.

FKBP52, CyP-40, and possibly other immunophilins can bind to the hsp90 complex than FKBP52/hsp56, there is very little of it irrelevant to receptor function.

REFERENCES

1. Defranco, D. B., Madan, A. P., Tang, Y., Chandran, U. R., Xiao, N., and Yang, P. (1995) Vitam. Horm. 53, 315–338
2. Picard, D., and Yamamoto, K. R. (1987) EMBO J. 6, 3333–3340
3. Picard, D., Kumar, V., Chambon, P., and Yamamoto, K. R. (1990) J. Biol. Chem. 265, 21729–21735
4. King, W. J., and Greene, G. L. (1984) J. Biol. Chem. 259, 9504–9509
5. Perrot-Applanat, M., Logeat, F., Groyer-Picard, M. T., and Milgrom, E. (1985) J. Biol. Chem. 260, 2878–2884
6. Picard, D., and Yamamoto, K. R. (1987) EMBO J. 6, 3333–3340
7. Picard, D., Kumar, V., Chambon, P., and Yamamoto, K. R. (1990) J. Biol. Chem. 265, 21729–21735
8. Segnitz, B., and Gehring, U. (1995) EMBO J. 14, 1418–1429
9. Brugge, J. S., Erikson, E., and Eriksson, R. L. (1981) Cell 25, 363–372
10. Peattie, D. A., Harding, M. W., Fleming, M. A., DeConzo, M. T., Lipsky, J. A., Livingston, D. J., and Benasutti, M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10974–10978
11. Peattie, D. A., Harding, M. W., Fleming, M. A., DeConzo, M. T., Lipsky, J. A., Livingston, D. J., and Benasutti, M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10974–10978
12. Peattie, D. A., Harding, M. W., Fleming, M. A., DeConzo, M. T., Lipsky, J. A., Livingston, D. J., and Benasutti, M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10974–10978
13. Pratt, W. B., and Welsh, M. J. (1993) J. Biol. Chem. 268, 10559–10568
14. Riezman, H., Hay, R., Jove, R., and Pratt, W. B. (1992) J. Biol. Chem. 267, 21285–21288
15. Riezman, H., Hay, R., Jove, R., and Pratt, W. B. (1992) J. Biol. Chem. 267, 21285–21288
16. Riezman, H., Hay, R., Jove, R., and Pratt, W. B. (1992) J. Biol. Chem. 267, 21285–21288
17. Riezman, H., Hay, R., Jove, R., and Pratt, W. B. (1992) J. Biol. Chem. 267, 21285–21288
18. Riezman, H., Hay, R., Jove, R., and Pratt, W. B. (1992) J. Biol. Chem. 267, 21285–21288
19. Riezman, H., Hay, R., Jove, R., and Pratt, W. B. (1992) J. Biol. Chem. 267, 21285–21288
20. Riezman, H., Hay, R., Jove, R., and Pratt, W. B. (1992) J. Biol. Chem. 267, 21285–21288
21. Riezman, H., Hay, R., Jove, R., and Pratt, W. B. (1992) J. Biol. Chem. 267, 21285–21288
22. Riezman, H., Hay, R., Jove, R., and Pratt, W. B. (1992) J. Biol. Chem. 267, 21285–21288
23. Riezman, H., Hay, R., Jove, R., and Pratt, W. B. (1992) J. Biol. Chem. 267, 21285–21288
24. Riezman, H., Hay, R., Jove, R., and Pratt, W. B. (1992) J. Biol. Chem. 267, 21285–21288
25. Riesz, M., Busch, W., and Gehring, U. (1991) J. Biol. Chem. 266, 24601–24605
26. Riesz, M., Busch, W., and Gehring, U. (1991) J. Biol. Chem. 266, 24601–24605
27. Riesz, M., Busch, W., and Gehring, U. (1991) J. Biol. Chem. 266, 24601–24605
28. Riesz, M., Busch, W., and Gehring, U. (1991) J. Biol. Chem. 266, 24601–24605
29. Riesz, M., Busch, W., and Gehring, U. (1991) J. Biol. Chem. 266, 24601–24605
20. Riesz, M., Busch, W., and Gehring, U. (1991) J. Biol. Chem. 266, 24601–24605
21. Riesz, M., Busch, W., and Gehring, U. (1991) J. Biol. Chem. 266, 24601–24605
22. Riesz, M., Busch, W., and Gehring, U. (1991) J. Biol. Chem. 266, 24601–24605
23. Riesz, M., Busch, W., and Gehring, U. (1991) J. Biol. Chem. 266, 24601–24605
24. Riesz, M., Busch, W., and Gehring, U. (1991) J. Biol. Chem. 266, 24601–24605
25. Riesz, M., Busch, W., and Gehring, U. (1991) J. Biol. Chem. 266, 24601–24605
26. Riesz, M., Busch, W., and Gehring, U. (1991) J. Biol. Chem. 266, 24601–24605
27. Riesz, M., Busch, W., and Gehring, U. (1991) J. Biol. Chem. 266, 24601–24605
28. Riesz, M., Busch, W., and Gehring, U. (1991) J. Biol. Chem. 266, 24601–24605
29. Riesz, M., Busch, W., and Gehring, U. (1991) J. Biol. Chem. 266, 24601–24605