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The Cyclosporin A-binding Immunophilin CyP-40 and the FK506-binding Immunophilin hsp56 Bind to a Common Site on hsp90 and Exist in Independent Cytosolic Heterocomplexes with the Untransformed Glucocorticoid Receptor*

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We have recently shown that hsp56, the FK506-binding immunophilin component of both the heat shock protein (hsp90·hsp70·hsp56) heterocomplex and the untransformed glucocorticoid receptor heterocomplex, is bound directly to hsp90 (Czar, M.J., Owens-Grillo, J. K., Dittmar, K. D., Hutchison, K. A., Zacharek, A. M., Leach, K. L., Deibel, M. R., and Pratt, W. B. (1994) *J. Biol. Chem.* 269, 11155–11161). In this work, we show that both untransformed glucocorticoid receptor and hsp90 heterocomplexes contain CyP-40, a 40-kDa immunophilin of the cyclosporin A-binding class. CyP-40 is present in both native glucocorticoid receptor heterocomplexes and receptor heterocomplexes reconstituted with rabbit reticulocyte lysate, and the presence of CyP-40 in the receptor heterocomplex is stabilized by molybdate. Immunoadsorption of hsp90 from cell lysate yields coimmunoadsorption of both hsp56 and CyP-40, showing that both immunophilins are in native heterocomplex with hsp90. However, immunoadsorption of hsp56 does not yield coimmunoadsorption of CyP-40; thus, the two immunophilins do not exist in the same heterocomplex with hsp90. Both purified CyP-40 and hsp56 bind directly to purified hsp90, and excess CyP-40 blocks the binding of hsp56, consistent with the presence of a common immunophilin binding site on hsp90. Our data also suggest that there are at least two types of untransformed glucocorticoid receptor-hsp90 heterocomplexes, one that contains hsp56 and another that contains CyP-40. The role played by the immunophilins in steroid receptor action is unknown, but it is clear that the peptidylprolyl isomerase activity of immunophilins is not required for glucocorticoid receptor-hsp90 heterocomplex assembly and proper folding of the hormone binding domain by the hsp90-associated protein folding system of reticulocyte lysate.

After cell rupture, untransformed steroid receptors are recovered in the cytosolic fraction of hormone-free cells in multiprotein complexes containing both heat shock protein (hsp)[1] and immunophilin chaperones (for review see Refs. 1 and 2). The immunophilins are ubiquitous and conserved proteins that bind immunosuppressant drugs, such as cyclosporin A, FK506, and rapamycin (Ref. 3 for review). All members of the immunophilin protein family have peptidylprolyl isomerase activity, and, like hsp70 and hsp90, they are thought to play major roles in protein folding and trafficking in the cell.

The first receptor-associated immunophilin was discovered when an antibody directed against the partially purified, untransformed progesterone receptor complex was found to react with a 59-kDa rabbit protein (4) that was shown to be associated with all untransformed steroid receptor heterocomplexes although its function in such complexes is unknown (5). The human protein, with an apparent M_r of 56,000 (6), was found to be both a heat shock protein (7) and a member of the FK506- and rapamycin-binding class of immunophilins (8, 9). Rabbit (10), human (11), and mouse (12) cDNAs for hsp56 were cloned, and because the human cDNA encodes an FK506-binding protein with a predicted molecular mass of 52,000 the protein is also called FKBP52 (11). Recently, the 50- and 54-kDa components of the untransformed chicken progesterone receptor heterocomplex (13) were found to have significant sequence identity with hsp56 (14) and to bind to an FK506 affinity resin (15). The 50-kDa FK506-binding protein is the avian homolog of hsp56, but the 54-kDa protein is the homolog of a novel 55-kDa human FKBP (15).

The immunophilins can be broadly divided into two classes according to their ability to bind either cyclosporin A (cyclophilins) or FK506 and rapamycin (FKBPs) to their peptidylprolyl isomerase site. In addition to the FKBPs noted above, a cyclophilin has been identified in the estrogen receptor heterocomplex. Purification of the bovine estrogen receptor using an estrogen-derivatized affinity resin yielded copurification of hsp90 and an ~40-kDa protein (16). When the cDNA for the 40-kDa protein was cloned (17), it was found to be the same as a 40-kDa cyclosporin A-binding protein that was previously purified from bovine brain (18) and cloned from a human library (19). This protein, known as cyclophilin-40 (CyP-40),

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[1] The abbreviations used are: hsp, heat shock protein; hsp56, hsp70, and hsp90, the 56-, 70-, and 90-kDa heat shock proteins, respectively; CyP-40, the 40-kDa cyclosporin A-binding protein; GR, glucocorticoid receptor; FKBP, FK506 binding protein; PAGE, polyacrylamide gel electrophoresis; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino}ethanesulfonic acid.

contains a C-terminal domain with significant sequence homology to an internal region of hsp56 (17, 19). To date, CyP-40 has not been reported in native hetercomplexes other than that of the bovine estrogen receptor, although it has been reported in progesterone receptor heterocomplexes reconstituted under cell-free conditions with rabbit reticulocyte lysate (20).

It has been shown that a portion of the hsp90 and a portion of the hsp56 in cytosols exist together in a multiprotein complex independent of the presence of steroid receptors (6, 21–24). Both cross-linking (25) and purified protein binding studies (26) have established that hsp56 binds directly to hsp90 in this multiprotein complex. A 45-kDa yeast protein with N-terminal homology to mammalian CyP-40 has been isolated in a multiprotein complex with the yeast homolog of hsp90 (27), and incubation of a variety of tissue homogenates with a CyP-40/GST-fusion protein bound to glutathione-agarose resulted in retention of hsp90,[2] suggesting that CyP-40 also binds directly to hsp90. In this work, we show that both native and cell-free reconstituted GR heterocomplexes contain CyP-40. Like hsp56, a portion of the CyP-40 in cytosol exists in multiprotein, hsp90-containing heterocomplexes, but the FKBP and the cyclophilin do not exist simultaneously in the same complex. Purified CyP-40 binds to purified hsp90 and competes for the binding of purified hsp56, suggesting that there is a common immunophilin binding site on hsp90. Our data also suggest that there are at least two types of untransformed steroid receptor heterocomplexes, one that contains hsp56 and another that contains CyP-40.

EXPERIMENTAL PROCEDURES

Materials

Untreated rabbit reticulocyte lysate was from Green Hectares (Oregon, WI). [6,7-^{3}H]triamcinolone acetonide (49.5 Ci/mmol) and ^{125}I-conjugated goat anti-mouse and anti-rabbit IgGs were from DuPont NEN. Iron-supplemented bovine calf serum, powdered Dulbecco's modified Eagle's medium (high glucose), goat anti-mouse IgG-horseradish peroxidase conjugate, monoclonal nonimmune IgG and IgM, and nonimmune rabbit serum 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 AC88 monoclonal IgG against hsp90 and the N27F3–4 anti-72-/73-kDa heat shock protein monoclonal IgG (anti-hsp70) were from StressGen (Victoria, Canada). The BuGR2 IgG monoclonal antibody used to develop the GR on immunoblots and the anti-cyclophilin 40 (C-Terminal Peptide) antibody were from Affinity Bioreagents (Neshanic Station, NJ). The EC1 monoclonal antibody against hsp56 (28) was kindly provided by Lee Faber (Medical College of Ohio). The UPJ56 rabbit antiserum against hsp56 has been described previously (29). Hybridoma cells producing the 8D3 monoclonal anti-hsp90 IgM (30) were a gift from Gary Perdew (Purdue University), and hybridoma cells producing FiGR monoclonal IgG against the glucocorticoid receptor (31) were generously provided by Jack Bodwell (Dartmouth Medical School). Purified FiGR antibody was used to immunoadsorb the GR, and for this purpose it was covalently cross-linked to agarose using an Immunopure Ag/Ab Immobilization kit from Pierce. All of the monoclonal antibodies listed above were raised in mice.

Methods

Cytosol Preparation—The WCL2 line of Chinese hamster ovary cells overexpressing the mouse GR was established by Hirst *et al.* (32). Cells were grown in monolayer culture using Dulbecco's modified Eagle's medium plus 3 μM methotrexate, 40 μg/ml proline, and 10% iron-supplemented calf serum. Cells were harvested by scraping into Earle's balanced saline, suspended in 1.5 volumes of HE buffer (10 mM Hepes, 1 mM EDTA, pH 7.4), and ruptured by dounce homogenization. Cell homogenates were centrifuged for 1 h at 100,000 × *g*, and the supernatant from this centrifugation is referred to as "cytosol."

Immunoadsorption—hsp90 was immunoadsorbed from rabbit reticulocyte lysate or WCL2 cytosol with 10 or 20 μl, respectively, of 8D3 antibody (ascites) prebound to 14 μl of protein A-agarose. Antibody was preadsorbed by suspending protein A-agarose pellets in 300 μl of HE buffer and 50 μl of goat anti-mouse IgM for 1 h. Pellets were centrifuged, aspirated, and resuspended for 1 h in 300 μl of HE buffer with 10 μl of 8D3 (IgM) ascites fluid and then centrifuged and aspirated prior to immunoprecipitation. The 8D3-preadsorbed protein A-agarose pellet was rotated with 400 μl of rabbit reticulocyte lysate or WCL2 cytosol to prepare the immune pellet. The immunoadsorbed pellets were washed three times by suspension in 1 ml of Hepes buffer (10 mM Hepes, pH 7.4) and centrifugation. hsp56 was immunoadsorbed from 200 μl of rabbit reticulocyte lysate by incubating for 2 h at 4 °C with 10 μl of protein A-agarose preadsorbed with UPJ56 antiserum. After immunoadsorption, the immune pellet was washed three times by suspension in 1 ml of HE buffer and centrifugation. The GR heterocomplex was immunoadsorbed from 300-μl aliquots of WCL2 cytosol by rotation for 2 h at 4 °C with 5 μl of covalently coupled FiGR-agarose. Some samples were incubated with FiGR-agarose that had been preincubated for 1 h at 4 °C with 50 μg of epitope peptide to block the antibody-combining site and prevent GR immunoadsorption. Immune pellets were washed three times by suspension in 1 ml of HE buffer containing 20 mM sodium molybdate (HEM) or in the experiment of Fig. 1 with TEG buffer (10 mM TES, 50 mM NaCl, 4 mM EDTA, 10% glycerol, pH 7.4). In experiments where purified hsp56 and CyP-40 were bound to purified hsp90, the purified hsp90 was immunoadsorbed to a 5-μl pellet of Actigel ALD precoupled with 50 μl of 8D3 ascites.

Reconstitution of GR Heterocomplexes—Reconstitutions were performed essentially as described previously (33, 34). Immunoadsorbed GR was stripped of associated hsp90 by incubating the immunopellet for 2 h at 4 °C with 0.5 M NaCl followed by two washes with 1 ml of Hepes buffer. Salt-stripped immune pellets were then incubated for 20 min at 30 °C with 100 μl of rabbit reticulocyte lysate plus 10 μl of an ATP-regenerating system (50 mM ATP, 250 mM creatinine phosphate, 20 mM $MgCl_2$, and 100 units/ml creatinine phosphokinase). The mixtures were resuspended every 5 min during the incubation. After the incubation, the pellets were washed two times with 1 ml of HEM, and replicate washed pellets were assayed for steroid binding activity or GR-associated proteins. For assay of steroid binding activity, pellets were incubated with 50 nM [^{3}H]triamcinolone acetonide in HEM overnight on ice, washed twice with HEM buffer, and counted for radioactivity in a scintillation counter. Pellets from duplicate incubations were analyzed by SDS-PAGE and Western blotting for GR, hsp90, hsp70, hsp56, and CyP-40.

Gel Electrophoresis and Western Blotting—Immunoadsorbed protein A-agarose pellets 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 BuGR2 antibody for the GR, 1 μg/ml AC88 for hsp90, 1 μg/ml N27F3–4 for hsp70, 0.1% anti-cyclophilin 40 for CyP-40, and either 0.1% UPJ56 or 1 μg/ml EC1 for hsp56. The immunoblots were then incubated a second time with the appropriate ^{125}I-conjugated or horseradish peroxidase-conjugated counterantibody to visualize the immunoreactive bands.

Protein Purification—Calf hsp56 was purified essentially as described by Yem *et al.* (8). Briefly, calf thymus cytosol prepared in 50 mM Tris-HCl, pH 7.5, with a protease inhibitor mixture was passed through 0.5 ml of FK506-Affi-Gel-10 matrix for 2 h at 4 °C. After washing the matrix with 50 ml of 50 mM Tris HCl, pH 7.5, followed by 50 ml of the Tris buffer containing 0.5 M NaCl, followed by 50 ml of water, the bound proteins were eluted with 3 ml of 5% acetic acid, and Tris base was added to bring the pH to 7.5. The eluant was concentrated to 0.3 ml by filtration through an Amicon YM30 membrane to yield a concentration of 0.02 μg of hsp56/μl. For the experiment of Fig. 6*A*, hsp56 was partially purified from rabbit brain by chromatography of cytosol on DE52. Fractions containing hsp56 and hsp90 were identified by immunoblotting, and fractions with hsp56 but free of hsp90 were combined and contracted to original volume. Rabbit hsp90 was purified from the brain cytosol by chromatrography of the pooled DE52 fractions on hydroxylapatite followed by chromatrography over ATP-agarose exactly as described in Hutchison *et al.* (35).

The purified human CyP-40 used in this work is a mutant in which His-141 is replaced by Trp. The preparation of the cDNA and its expression in *E. coli* are described elsewhere.[2] The protein was purified on a cyclosporin A affinity matrix as described by Kieffer *et al.* (18). After thorough washing, the protein was eluted with pH 3.0 phosphate buffer (50 mM) and immediately neutralized with pH 7.8 Tris buffer (200 mM). The eluates were concentrated by ultrafiltration (Centricon 30) to 2 mg/ml, and the purified CyP-40 was stored at 4 °C.

[2] K. Hoffmann and R. E. Handschumacher, submitted for publication.

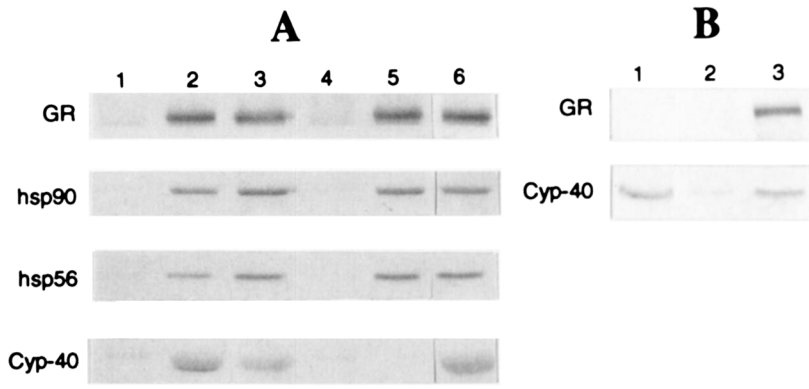

FIG. 1. **CyP-40 is a component of the native mouse GR heterocomplex.** *A*, GR was immunoadsorbed from aliquots of WCL2 cytosol (200 μl) with FiGR-agarose in the presence or absence of competing epitope peptide. The immunopellets were washed three times with 1 ml of buffer, and the proteins were resolved by SDS-PAGE followed by Western blotting. *Lanes 1* and *2*, immunopellets prepared in the presence (*lane 1*) or absence (*lane 2*) of competing FiGR epitope peptide and washed with HE buffer; *lane 3*, immunopellet prepared in the absence of competing epitope peptide and washed with HE buffer containing 20 mM molybdate; *lanes 4* and *5*, immunopellets prepared in the presence (*lane 4*) or absence (*lane 5*) of competing epitope peptide and washed with TEG buffer; *lane 6*, immunopellet prepared in the absence of competing epitope peptide and washed with TEG buffer containing 20 mM molybdate. *B*, GR was immunoadsorbed from aliquots (500 μl) of L cell cytosol as above. *Lane 1*, 40 μl of whole cytosol; *lanes 2* and *3*, immunopellets prepared in the presence (*lane 2*) or absence (*lane 3*) of competing epitope peptide.

RESULTS

GR Heterocomplex Contains Some CyP-40—To determine whether CyP-40 is present in native GR heterocomplexes, receptors were immunoadsorbed from WCL2 cytosol and both the GR and coimmunoadsorbed proteins were assayed by Western blotting. As shown in Fig. 1*A*, GR heterocomplexes that are immunoadsorbed and washed in buffer without salt contain CyP-40 (*lane 2*). We have noted previously (26) that, in contrast to hsp90, hsp56 is a relatively loosely associated component of the GR heterocomplex. CyP-40 appears to be a less stable component of the GR heterocomplex than hsp56. As shown in *lane 5* of Fig. 1*A*, most of the CyP-40 was eliminated when the immunopellet was washed with a common immune complex lavage buffer containing 50 mM NaCl, but the presence of molybdate in the washing buffer inhibited the loss of CyP-40 (*lane 6*). It is clearly the salt that causes the dissociation by the TEG lavage buffer, because CyP-40 dissociates if we simply add 50 mM NaCl to the HE buffer used to wash the immune pellets in *lanes 1–3* (data not shown). Fig. 1*B* shows that CyP-40 is also present in the native GR heterocomplex immunoadsorbed from mouse L cell cytosol.

Unliganded receptors that have been stripped free of hsp90 are reassociated with hsp90 when immunopellet-bound GR is incubated with rabbit reticulocyte lysate (33). Formation of the GR-hsp90 complex in reticulocyte lysate is due to an ATP/Mg^{2+}-dependent and K^+-dependent (34) protein folding mechanism that requires hsp70 (35). When the GR-hsp90 complex is formed by reticulocyte lysate, hsp56 is also present in the newly formed heterocomplex (35). As shown in Fig. 2, when the immunoadsorbed GR (*lane 2*) is stripped of receptor-associated proteins (*lane 3*) and incubated with reticulocyte lysate (*lane 5*), the mouse receptor enters a heterocomplex (or heterocomplexes) containing some rabbit CyP-40 in addition to hsp90 and hsp56. This complex is functional in the sense that the GR has been returned to the steroid binding conformation (compare *lanes 3* and *5* in the *bar graph* in Fig. 2).

CyP-40 Is a Component of the hsp Heterocomplex—To determine whether CyP-40 is a component of the hsp heterocomplex, hsp90 was immunoadsorbed from rabbit reticulocyte lysate (Fig. 3*A*) and WCL2 cytosol (Fig. 3*B*) with the 8D3 monoclonal antibody, and the immunocomplexes were assayed for associated proteins. As shown in Fig. 3, immunoadsorption of hsp90 is accompanied by coimmunoadsorption of CyP-40 as well as hsp70 and hsp56. By cutting out and counting the ^{125}I-counter antibody-labeled bands from 8D3 immunoadsorptions, such as

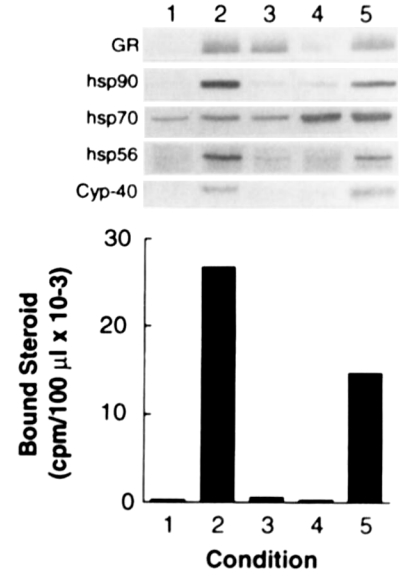

FIG. 2. **Rabbit reticulocyte lysate mediates reconstitution of GR complexes with both CyP-40 and hsp56.** GR was immunoadsorbed to 10% FiGR-agarose from replicate aliquots of WCL2 cell cytosol in the presence (nonimmune) or absence (immune) of the epitope peptide, and the immunopellets were stripped of hsp90, hsp56, and CyP-40 with 0.5 M NaCl. The salt-stripped immunopellets were incubated with rabbit reticulocyte lysate and an ATP-generating system. Receptor, hsp90, hsp70, hsp56, and CyP-40 were assayed in each sample by SDS-PAGE and Western blotting. Duplicate pellets were incubated with 50 nM [^3H]triamcinolone acetonide to determine steroid binding activity (*bar graph*). *Lane 1*, untreated nonimmune pellet; *lane 2*, untreated immune pellet; *lane 3*, salt-stripped immune pellet; *lane 4*, stripped nonimmune pellet incubated with reticulocyte lysate; *lane 5*, stripped immune pellet incubated with reticulocyte lysate.

that of Fig. 3*A*, as well as from aliquots of whole lysate resolved on the same immunoblots, we can estimate that 10–15% of the total CyP-40 in lysate is bound to hsp90. However, as the CyP-40 is likely dissociating from the complex during its isolation, this estimate may be low.

CyP-40 Is in Different Heterocomplexes from hsp56—Although we have referred to the hsp heterocomplex as if it were a single multiprotein unit, there may be multiple complexes, and the two immunophilins may not exist together in the same complex. Thus, we asked whether complexes that contain hsp56 also contain CyP-40. In the experiment of Fig. 4, hsp56 was immunoadsorbed from reticulocyte lysate with the UPJ56

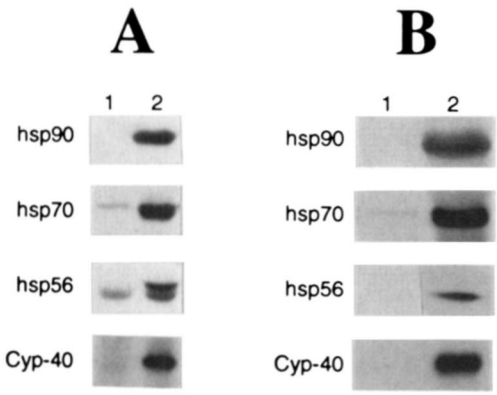

FIG. 3. **CyP-40 is coimmunoadsorbed with hsp90.** hsp90 was immunoadsorbed from aliquots (400 μl) of rabbit reticulocyte lysate (*A*) or WCL2 cytosol (*B*) with the 8D3 monoclonal antibody or nonimmune mouse IgM. After washing the immunopellets, the proteins were resolved by SDS-PAGE and Western blotting with AC88 for hsp90, anti-hsp 72/73 for hsp70, anti-CyP-40, or antibody against hsp56. The rabbit hsp56 (*dark band above* the IgG heavy chain in *lane 2* of *panel A*) was probed with the EC1 antibody, and the hamster hsp56 in *panel B* was resolved by two-dimensional gel analysis of replicate immunopellets prior to probing with the UPJ56 antiserum. *Lane 1*, nonimmune pellet; *lane 2*, immune pellet.

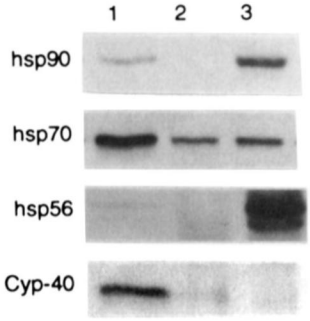

FIG. 4. **CyP-40 is not in the same hsp90 heterocomplex as hsp56.** Aliquots of rabbit reticulocyte lysate (200 μl) were immunoadsorbed to protein A-agarose with 2.5% UPJ56 antiserum or with preimmune rabbit serum. The immunopellets were washed in HE buffer and resolved by SDS-PAGE followed by Western blotting. *Lane 1*, 10 μl of whole lysate; *lane 2*, preimmune pellet; *lane 3*, immune pellet.

antiserum, yielding coadsorption of hsp90 but not CyP-40 (compare *lanes 2* and *3*), indicating that the two immunophilins exist in different heterocomplexes with hsp90. The reverse experiment cannot be done because the antibody against CyP-40 can only be used for Western blotting. As we have noted before (26), the hsp56·hsp90 complex from reticulocyte lysate contains little if any hsp70 (Fig. 4). This stands in contrast to IM-9 lymphoblasts (6) and some other cells where considerable hsp70 is coimmunoadsorbed with hsp56.

If CyP-40 is in a different hsp90 heterocomplex than hsp56, then there may be two classes of untransformed GR-hsp90 heterocomplexes as well, one containing the FKBP hsp56 and another containing the cyclophilin CyP-40. This possibility was tested in the experiment of Fig. 5. In this experiment WCL2 cytosol was immunoadsorbed with sufficient UPJ56 antiserum to remove all of the hsp56 (compare preimmune supernatant in *lane 3* with UPJ56 supernatant in *lane 4*). As expected, some GR but no CyP-40 is coimmunoadsorbed with the hsp56 (*lane 2*). When the now hsp56-free supernatant from UPJ56 immunoadsorption (*lane 4*) was immunoadsorbed with the BuGR antibody against the GR, hsp90 and CyP-40 were coadsorbed (*lane 6*). These observations are consistent with the notion that there are at least two different GR heterocomplexes, depending upon the immunophilin that is bound. GR in the UPJ56 immunopellet condition of *lane 2* binds steroid (9,400 cpm [^{3}H]triamcinolone acetonide *versus* 146,700 cpm for the condition of *lane 6*).

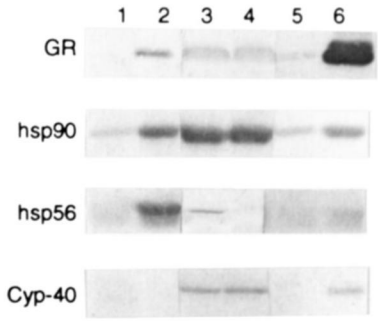

FIG. 5. **CyP-40 and hsp56 are in different GR heterocomplexes.** Aliquots (200 μl) of WCL2 cytosol were immunoadsorbed with UPJ56 antiserum or preimmune serum. An aliquot of each immunoadsorption supernatant was retained for Western blotting, and the remainder was immunoadsorbed with FiGR-agarose to isolate the GR heterocomplex. *Lane 1*, preimmune immunopellet; *lane 2*, UPJ56 immunopellet; *lane 3*, 50 μl of preimmune supernatant; *lane 4*, UPJ56 supernatant; *lane 5*, UPJ56 supernatant immunoadsorbed with FiGR in the presence of competing epitope peptide; *lane 6*, UPJ56 supernatant immunoadsorbed with FiGR without peptide. To blot hsp56, which runs with the heavy chain of the immunoadsorbing antibody, three different development procedures were used. *Lanes 1* and *2* were probed with the EC1 monoclonal antibody, which does not react with the rabbit UPJ56 heavy chain. *Lanes 3* and *4* were probed with UPJ56 and developed with horseradish peroxidase-conjugated counterantibody. *Lanes 5* and *6* were probed with UPJ56 and developed with ^{125}I-conjugated goat anti-rabbit counterantibody, which does not react with the FiGR monoclonal antibody heavy chain.

CyP-40 Competes for Binding of hsp56 to hsp90—Because CyP-40 and hsp56 share a region of homology (19) and are present in independent heterocomplexes with hsp90, it is possible that they share the same binding site on hsp90. To determine if CyP-40 can compete for the binding of hsp56, we performed the experiment shown in Fig. 6*A*. Because only a very limited quantity of highly purified human hsp56 was available to us, we used a DE52 fraction of rabbit brain cytosol as a source of hsp56 for working out the competition assay conditions. As shown in *lane 1*, this hsp56 preparation does not contain hsp90, but it does contain a small amount of CyP-40 that can be seen on the blot but is not visible in the photograph. When the hsp56 is incubated with purified rabbit brain hsp90 that was preimmunoadsorbed to 8D3-Actigel pellets, hsp56 is bound (*lane 5*) in a manner that is specific for the presence of hsp90 (compare *lanes 4* and *5*). When purified human CyP-40 is added to the incubation, the binding of rabbit hsp56 to hsp90 is inhibited (*lanes 6* and *7*). It is important to note that 0.1% Nonidet P-40 is required to keep the purified CyP-40 from aggregating under the conditions of the experiment, and the detergent is present in all samples.

To demonstrate that no other proteins are required for CyP-40 and hsp56 binding to hsp90, we performed the experiment shown in Fig. 6*B* with human CyP-40, calf hsp56, and rabbit hsp90, all purified to near homogeneity. CyP-40 (compare *lanes 2 and 3*) binds directly to hsp90, and an excess of CyP-40 competes for the binding of hsp56 (compare *lanes 4* and *5*), an observation that is consistent with the two immunophilins binding to the same site on hsp90. Because of the very limited amount of purified hsp56 available, we were not able to test whether excess hsp56 would compete for binding of CyP-40. The binding of hsp56 and CyP-40 to hsp90 is not affected by the presence of 1 μM FK506 or cyclosporin A (data not shown).

The Immunophilins and Receptor Folding—Because the immunophilins have a peptidylprolyl isomerase site, they are thought to be involved in protein folding in the cell (3). We have previously shown that inhibition of prolyl isomerase activity

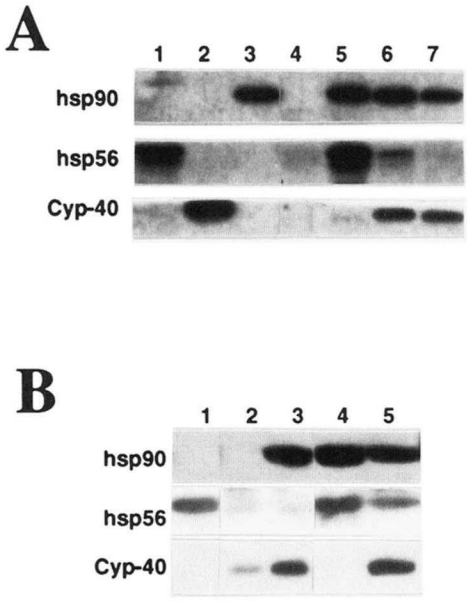

FIG. 6. **CyP-40 competition of hsp56 binding to hsp90.** *A*, CyP-40 competes for binding of partially purified rabbit brain hsp56 to purified hsp90. Rabbit brain cytosol was chromatographed on a column of DE52, and fractions containing hsp56 were pooled as described under "Methods." Aliquots (25 μl) of purified rabbit hsp90 (1 mg/ml) were immunoadsorbed to 5-μl pellets of Actigel precoupled with 8D3 antibody. Pellets were washed twice with 1 ml of Hepes, pH 7.35, and suspended in Hepes plus 50 mM KCl and 0.1% Nonidet P-40 in a final volume of 100 μl including 20 μl of DE52-purified hsp56 and 20 or 40 μg of purified human CyP-40 as noted. Incubations were rotated for 1 h at 4 °C and washed twice with 1 ml of Hepes, and proteins were resolved by SDS-PAGE and Western blotting. *Lane 1*, 2 μl of DE52-purified hsp56; *lane 2*, 4 μg of purified CyP-40; *lane 3*, 2 μg of hsp90; *lane 4*, Actigel-8D3 pellet without hsp90 incubated with hsp56; *lane 5*, Actigel-8D3 pellet with hsp90 incubated with hsp56; *lanes 6* and *7*, hsp90-bound Actigel-8D3 pellets incubated with hsp56 in the presence of 20 μg (*lane 6*) or 40 μg (*lane 7*) of purified CyP-40. *B*, purified human CyP-40 competes for binding of purified calf hsp56 to rabbit hsp90. Aliquots of purified hsp90 were immunoadsorbed to 8D3-precoupled Actigel pellets, and washed pellets were suspended in Hepes plus 25 mM KCl and 0.02% Nonidet P-40 (0.1% Nonidet P-40 in the incubations of *lanes 2* and *3*) in a final volume of 50 μl including 0.4 μg of purified calf hsp56 plus or minus 20 μg purified human CyP-40. Incubations were rotated 1 h at 4 °C, and pellet-associated proteins were resolved by Western blotting. *Lane 1*, 0.1 μg of purified calf hsp56; *lane 2* Actigel-8D3 pellet without hsp90 plus 20 μg of CyP-40; *lane 3*, Actigel-8D3 pellet with hsp90 plus 20 μg of CyP-40; *lane 4*, 8D3 pellet with hsp90 plus hsp56; *lane 5*, 8D3 pellet with hsp90 plus hsp56 plus 20 μg of purified CyP-40.

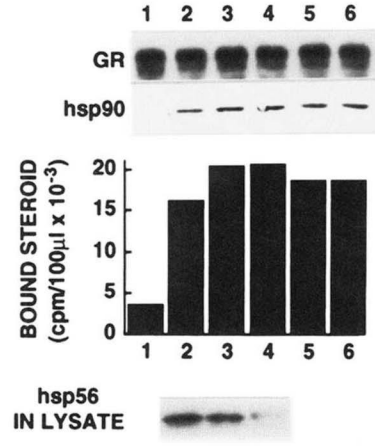

FIG. 7. **Evidence that prolylisomerase activity of immunophilins is not required for reconstitution of GR heterocomplexes with hsp90.** GR immunoadsorbed to 10% FiGR-agarose was salt-stripped of associated proteins, and the immune pellets were incubated with either whole rabbit reticulocyte lysate or lysate that was preabsorbed with a protein A-agarose-preimmune serum pellet or preadsorbed with a protein A-agarose-UPJ56 pellet to deplete hsp56. All samples contained an ATP-generating system, and receptor and hsp90 in each sample were resolved by SDS-PAGE and Western blotting. One half of each pellet was incubated with [^{3}H]triamcinolone acetonide to determine steroid binding activity (*bar graph*). *Lane 1*, stripped receptor; *lane 2*, stripped receptor plus whole lysate; *lane 3*, stripped receptor plus lysate extracted with preimmune serum; *lane 4*, stripped receptor plus UPJ56-extracted lysate; *lane 5*, stripped receptor plus UPJ56-extracted lysate and 1 μM cyclosporin A; *lane 6*, stripped receptor plus whole lysate and both cyclosporin A and FK506 at 1 μM. The Western blot *under* the graph shows the hsp56 in 5 μl of whole lysate (*lane 2*), preimmune extracted lysate (*lane 3*), and UPJ56-extracted lysate (*lane 4*).

with FK506 does not affect the ability of reticulocyte lysate to form a receptor heterocomplex with hsp90 and fold the hormone binding domain of the GR into a high affinity steroid binding conformation (36). It is entirely possible, however, that any requirement for peptidylprolyl isomerase activity in GR folding could be performed by a cyclophilin when the activity of FKBPs is blocked by FK506. The experiment of Fig. 7 argues strongly for the conclusion that hsp56 is not required to return the GR to the steroid-binding conformation. In this experiment, reticulocyte lysate was preadsorbed with UPJ56 to eliminate hsp56. As shown in *lane 4*, GR incubated with hsp56-depleted lysate is bound to hsp90 and is reactivated to the steroid binding state as well as GR incubated with whole lysate (*lane 2*). Addition of 1 μM cyclosporin A to the hsp56-depleted lysate has no effect on receptor folding (*lane 5*); nor does the addition of both cyclosporin A and FK506 to whole lysate (*lane 6*).

DISCUSSION

CyP-40, which was first found to copurify with the native bovine estrogen receptor-hsp90 complex (16, 17), has now been identified in progesterone receptor heterocomplexes reconstituted by reticulocyte lysate (20) and in both native (Fig. 1) and reconstituted (Fig. 2) GR heterocomplexes. CyP-40 is also present in native GR heterocomplexes immunoadsorbed from L cell cytosol (Fig. 1*B*); thus, the association is not unique to the overexpressed receptor. The stoichiometry of CyP-40 with respect to the steroid receptor in these complexes is unknown. Molybdate stabilizes the presence of hsp56 in steroid receptor heterocomplexes (26) and it stabilizes the presence of p50 in pp60^{src} (36) and Raf (23) heterocomplexes with hsp90. Given the likelihood that hsp56 and CyP-40 may bind in the same manner to a common site on hsp90, it is perhaps not surprising that molybdate inhibits salt-mediated dissociation of CyP-40 from the GR heterocomplex (Fig. 1). Molybdate is thought to bind directly to the hsp90 component of these heterocomplexes (37), and the metal oxyanion has been shown to induce a conformational change in purified hsp90 (38) that may account for its stabilizing effect.

A portion of the hsp56 is known to exist in a native cytosolic heterocomplex with hsp90 independent of the association of hsp90 with steroid receptors or protein kinases. This has been shown by copurification of the two proteins with antibodies directed against either hsp56 (6) or hsp90 (21) and by copurification of hsp90 with hsp56 on an FK506 affinity matrix (9). In that purified hsp56 and purified hsp90 bind to each other (26), the association of the two hsps reflects a direct protein-protein interaction. Because passage of cytosol through a matrix with immobilized CyP-40 yields coretention of hsp90,[2] a direct interaction between the cyclophilin and hsp90 is also likely. The coimmunoadsorption of CyP-40 with the 8D3 monoclonal antibody against hsp90 shown in Fig. 3 confirms the presence of CyP-40 in native cytosolic hsp heterocomplexes.

While this work was in progress, Johnson and Toft (24) reported that immunoadsorption of rabbit reticulocyte lysate with the EC1 monoclonal antibody against hsp56 did not yield

coimmunoadsorption of CyP-40, suggesting that the immunophilins exist in different complexes with hsp90. The experiment of Fig. 4 supports the conclusion that the two immunophilins are in different native heterocomplexes with hsp90, and the data of Fig. 5 are consistent with the conclusion that there are different GR-hsp90 heterocomplexes depending upon the immunophilin that is bound to hsp90.

The existence of a direct protein-protein interaction between CyP-40 and hsp90 is demonstrated by the complex formation with the purified proteins in Fig. 6*B*. The fact that purified CyP-40 competes for the binding of hsp56 to hsp90 (Fig. 6, *A* and *B*), is consistent with the existence of a common immunophilin binding site on hsp90. The C-terminal 150 amino acids of CyP-40 share 30.7% identify with an internal region of hsp56 (19). This region of homology contains three repetitive sequence motifs of 34 amino acids called tetratricopeptide repeat domains or TPR domains (17), and it is known that the TPR domains of hsp56 are required for its binding to hsp90 (39). The N-terminal and C-terminal halves of CyP-40 have been expressed independently, and the hsp90 binding site was localized to the C-terminal half containing the TPR domains.[2] TPR domains are thought to be involved in protein-protein interactions (40–42), and it is likely that they are responsible for immunophilin binding to hsp90.

The role of hsp56 and CyP-40 in steroid receptor function is unknown. It has been suggested that the immunophilins are required for proper receptor folding and heterocomplex assembly with hsp90 (9, 10, 17, 24). The data of Fig. 7 suggest that this is not the case. It is possible that, when hsp56 is removed from reticulocyte lysate, any role it plays in receptor folding and heterocomplex assembly with hsp90 is subsumed by CyP-40 and/or other immunophilins. However, the failure of high concentrations of FK506 and cyclosporin A to affect GR folding to the high affinity steroid-binding state and receptor association with hsp90 (Fig. 7) argues strongly against any requirement for a cyclosporin A or FK506-inhibited peptidyl-prolyl isomerase activity in the process. It is possible that an unknown third class of isomerase exists in reticulocyte lysate that could substitute for the activity of the cyclosporin A- and FK506-binding classes of isomerase.

It has also been suggested that hsp56 may play a role in nuclear localization of the steroid receptors (43), and the great majority of hsp56 in the cell is located in the nucleus where it is found by confocal imaging to colocalize in the same nonrandom, mottled pattern as the GR (44). Curiously, both the GR and hsp56 are excluded from nucleoli (44), but CyP-40 is localized to nucleoli by indirect immunofluorescence,[3] a localization that makes any potential role for CyP-40 in steroid receptor function even more cryptic.

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