Protein Phosphatase 5 Is a Major Component of Glucocorticoid Receptor-hsp90 Complexes with Properties of an FK506-binding Immunophilin*

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Steroid receptors are recovered from hormone-free cells in multiprotein complexes containing hsp90, p23, an immunophilin, and often some hsp70. The immunophilin, which can be of the FK506- or cyclosporin A-binding class, binds to hsp90 via its tetratricopeptide repeat (TPR) domain, and different receptor heterocomplexes exist depending upon which immunophilin occupies the TPR-binding region of hsp90. We have recently reported that a protein serine/threonine phosphatase that is designated PP5 and contains four TPRs binds to hsp90 and is co-purified with the glucocorticoid receptor (GR) (Chen, M.-S., Silverstein, A. M., Pratt, W. B., and Chinkers, M. (1996) J. Biol. Chem. 271, 32315–32320). In this work, we show that PP5 is recovered with both GR that is nuclear and GR that is cytoplasmic in hormone-free cells. Approximately one-half of the GR-hsp90 heterocomplexes in L cell cytosol contains an immunophilin with high affinity FK506 binding activity, such as FKBP51 or FKBP52, and ~35% contains PP5. Only a small (but undetermined) fraction of the native GR-hsp90 heterocomplexes contain the cyclosporin A-binding immunophilin CyP-40. PP5, FKBP51, and CyP-40 exist in separate heterocomplexes with hsp90, and competition binding experiments with the PP5 TPR domain suggest that the three proteins occupy a common binding site on hsp90. A 55- residue connecting region between the N-terminal TPR domain of human PP5 and its C-terminal phosphatase domain has 50% amino acid homology and 22% identity with the central portion of the peptidylprolyl isomerase domain of human FKBP52. Of the 9 residues in this portion of FKBP52 involved in high affinity interactions with FK506, 3 residues are retained and 4 have homologous substitutions in PP5. Although immunoadsorbed PP5 did not bind [3H]FK506, we found that both rabbit PP5 in reticulocyte lysate and purified rat PP5 were specifically retained by an FK506 affinity matrix. Thus, we propose that PP5 possesses properties of an immunophilin with low affinity FK506 binding activity and that it determines a major portion of the native GR heterocomplexes in L cell cytosol.

In cytosols prepared from hormone-free cells, steroid receptors exist in multiprotein complexes that contain hsp901 and some hsp90-associated proteins, including p23 and some high molecular weight immunophilins (for review see Refs. 1 and 2). The immunophilins are ubiquitous and conserved proteins that bind immunosuppressant drugs, such as FK506 and cyclosporin A (for review see Ref. 3). All members of the immunophilins family have peptidylprolyl isomerase (PPIase) activity, and there are two classes: the FKBP's that bind compounds like FK506 and rapamycin and the cyclophilins (CyPs) that bind cyclosporin A. The drugs bind to the isomerase site on the immunophilin and inhibit cis-trans isomerization in vitro (4).

The low molecular weight immunophilins, such as FKBP12 and CyP-18, are thought to be the cellular components responsible for the immunosuppression and are the most studied. Three high molecular weight immunophilins, FKBP52 (5–9) (also known as p59 and hsp56), FKBP51 (10–12) (also known as avian p54), and CyP-40 (13–15), were discovered as components of steroid receptor heterocomplexes. In addition to the PPIase domains possessed by the low molecular weight immunophilins, these high molecular weight immunophilins possess three tetratricopeptide repeats (TPR) and a calmodulin-binding domain in their C-terminal half (14–16). TPRs are degenerate sequences of 34 amino acids that are often arranged in tandem repeats and are thought to be sites where intramolecular and intermolecular interactions occur (17).

The high molecular weight immunophilins FKBP52 and CyP-40 bind to a site on hsp90 that binds a variety of proteins containing TPR domains (18), and native immunophilin-hsp90 complexes exist in cytosols independent of the presence of steroid receptors. The existence of native immunophilin-hsp90 complexes is derived from the observation that immunoadsorption or affinity purification of FKBP52 or CyP-40 from cytosols yields co-retention of hsp90 (8, 18–20), and immunopurification of hsp90 yields co-purification of FKBP52 and CyP-40 (21, 22). Purified FKBP52 and CyP-40 bind directly to purified hsp90 (22, 23) at what is apparently a common binding site because they compete for the binding of each other (22, 24). Studies with mutant FKBP52 and CyP-40 showed that the region containing the three TPRs was required for binding to hsp90 (24–26), and the binding of both immunophilins was competed by a fragment of CyP-40 comprising its three tetratricopeptide repeats (18). Thus, TPR domains are critical for immunophilin interaction with hsp90.

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† The abbreviations used are: hsp, heat shock protein; PPIase, peptidylprolyl isomerase; GR, glucocorticoid receptor; TPR, tetratricopeptide repeat; PR, progesterone receptor; PP5, protein phosphatase 5; FKBP, FK506-binding protein; CyP, cyclosporin A-binding protein; PAGE, polyacrylamide gel electrophoresis; TES, 2-(2-hydroxyethyl)-1-(2-hydroxyethyl)aminomethanesulfonic acid.
Because hsp90 exists as a dimer, with dimerization being required for its function in vivo (27), it is conceivable that two different immunophilins could exist in the same hsp90 complex. However, under conditions where isolation of FKBP52 or CyP-40 from cytosol co-isolation of hsp90, there is no co-isolation of the other immunophilin (18, 20, 22, 28). This is important because it suggests that there are different steroid receptor-hsp90 heterocomplexes depending upon the immunophilin that is bound. This prediction has been confirmed by direct studies that showed independent heterocomplexes of GR-hsp90-FKBP52 and GR-hsp90-CyP-40 (20, 22).

The binding of high molecular weight immunophilins to hsp90 via their TPR domains is conserved in plants as well as in the animal kingdom, suggesting that this may be a basic protein interaction that is critical to immunophilin function (29). However, the biological function(s) of the high molecular weight immunophilins and the role they play as components of steroid receptor heterocomplexes has not been defined. It has been suggested that FKBP52 is involved in targeting the cytoplasmic nuclear trafficking of receptors (18, 30), and there is some evidence in support of this notion for the GR (31). Some TPR proteins are components of the mitochondrial and peroxisomal import receptor complexes (32), which may suggest a general usefulness for TPR domains in other aspects of protein trafficking.

In 1994, three laboratories isolated cDNA clones encoding a protein serine/threonine phosphatase designated PP5 (protein phosphatase 5), which is distinctive in that it contains four TPRs in an N-terminal domain (33–35). We have recently shown that immunoadsorption of PP5 from cytosols yields co-immunoadsorption of hsp90 and that the expressed TPR domain of PP5 is sufficient for binding to hsp90 (36). Like FKBP52 and CyP-40, PP5 was co-adsorbed with the glucocorticoid receptor from L cell cytosol, suggesting that it is also a component of receptor heterocomplexes (36). Because the TPR domain of PP5 is related to those of the hsp90-binding immunophilins (35), we predicted that it might utilize the same binding site on hsp90 (36). That TPR domains per se are important for GR action is suggested by the fact that expression of the PP5 TPR domain in CV-1 cells has a dominant negative effect on GR-mediated activation of transcription from a reporter plasmid (36).

It is not known whether PP5 is a major component or a trace component of the GR heterocomplex profile or whether PP5 is related in any way to the immunophilins, other than by the possession of TPR domains. Previously, it has been determined that about half the GR heterocomplexes immunoadsorbed from chick oviduct cytosol (37) or GR heterocomplexes immunoadsorbed from L cell cytosol (38) contains FKBP5s. Also, we have previously reported that only a few GR-hsp90 complexes contain CyP-40, which binds rather weakly to hsp90 (18, 22). In this work, we provide evidence that PP5 binds to the same site on hsp90 as the immunophilins and that complexes containing PP5 account for 35% of the GR heterocomplexes in L cell cytosol. We also show that a region in the center of PP5 has some homology with the FK506 binding site of FKBP52 and that PP5 binds in a specific manner to an FK506 affinity matrix. This leads us to suggest that PP5 has properties of an immunophilin with a low affinity FK506 binding activity and that it accounts for one of every three native GR-hsp90 heterocomplexes in L cell cytosol.

### EXPERIMENTAL PROCEDURES

#### Materials

Uptreated rabbit reticulocyte lysate was from Green Hectares (Oregon, WI). [6,7-3H]Triamcinolone acetonide (35.4 Ci/mmol), [propyl-3H]FK506 (85 Ci/mmol), and 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). Actigel ALD (activated aldehyde agarose) affinity support for protein immobilization was purchased from Stergene Biochemicals (San Gabriel, CA). Powdered Dubecco’s modified Eagle’s medium (high glucose), goat anti-mouse IgG-horseradish peroxidase conjugate, monoclonal antibody to IgG and IgM, nonimmune rabbit serum, and protein A-Sepharose were from Sigma. Donkey anti-rabbit IgG-horseradish peroxidase conjugate was from Pierce. Amphotericin B was from Bio-Rad. Immodulon-P was from Millipore (Bedford, MA). Cyclosporin A was provided by Sandoz Research Institute (East Hanover, NJ), and FK506 was from Alexis Biochemicals (San Diego, CA). The AC88 monoclonal IgG against hsp90 was from StressGen (Victoria, British Columbia). The BuGR2 IgG monoclonal antibody against the GR, the 3G3 monoclonal IgG was from IBI (New Haven, CT). The DS14F5 monoclonal antibody against p60 (39) and Escherichia coli expressing human p60 were kindly provided by Dr. David Smith (University of Nebraska, Omaha, NE). The EC1 monoclonal antibody against hsp56 (40) was kindly provided by Dr. Lee Faber (Medical College of Ohio). The UPJ56 rabbit antiserum against hsp56 (41) was a kind gift from Dr. Karen Leach (The Upjohn Co., Kalamazoo, MI). Hybridoma cells producing FgGR monoclonal IgG against the GR (42) were generously provided by Dr. James Kelly (Dartmouth Medical School, Lebanon, NH). Rabbit anti-serum to PP5, purified FLAG-PP5, and the FLAG-tagged TPR domain of rat PP5 were prepared as described previously (36).

#### Methods

### Cell Culture and Cytosol Preparation

The WCL2 line of Chinese hamster ovary cells overexpressing the mouse GR was established by Hirst et al. (43). WCL2 cells and L929 mouse fibroblasts (L cells) were grown in monolayer culture in Dulbecco’s modified Eagle’s medium supplemented with 10% iron-supplemented calf serum. For WCL2 cells, this medium was supplemented with 3 μM methotrexate and 40 μg/ml proline. Cells were harvested by scraping into Earle’s balanced saline, washed once, suspended in 1.5 volumes of HE buffer (10 mM Hepes, 1 mM EDTA) or HEM (HE plus 50 μM sodium molybdate), and ruptured by Dounce homogenization. Homogenates were centrifuged for 1 h at 100,000 × g, with the supernatant from this step being the “cytosol” from which the GR was immunoadsorbed.

### Immunoadsorption

The GR heterocomplex was immunoadsorbed from 400-μl aliquots of L cell cytosol or 300-μl aliquots of WCL2 cytosol by rotation for 2 h at 4°C with 15% volume of covalently coupled FgGR-Actigel. The immune pellets were washed three times with 1 ml of TEGM buffer (10 mM TES, 50 mM NaCl, 4 mM EDTA, 10% (w/v) glycerol, 20 mM sodium molybdate, pH 7.6), and proteins were resolved by SDS-polyacrylamide gel electrophoresis. For immunoadsorption of hsp90 or immunophilins, aliquots (100 μl) of rabbit reticulocyte lysate were immunoadsorbed for 2 h at 4°C to 7.5-μl pellets of Actigel-ALD preconjugated with nonimmunoglobulin mouse ascites or 20–80 μl of protein A-agarose prebound with the EC1 monoclonal IgG against FKBP52 (10%), anti-CyP-40 (5%), anti-PP5 serum (10%), non-immune mouse IgG, or nonimmune rabbit serum. Immunopellets were washed twice by suspension in 1 ml of HEG buffer (10 mM Hepes, pH 7.4, 1 mM EDTA, 10% glycerol) and centrifugation prior to gel electrophoresis.

### Gel Electrophoresis and Western Blotting

Immunopellets were boiled in SDS sample buffer, and proteins were resolved on 10 or 12% SDS-polyacrylamide gels. For two-dimensional gel electrophoresis, immunopellets were dissolved by incubating them in 9.5 M urea, 2% (w/v) Nonidet P-40, 5% ampholytes (pH range 5–8), and 5% β-mercaptoethanol. Proteins were transferred to Immobilon membranes and probed with 1 μg/ml BuGR2 antibody for the GR, 1 μg/ml AC88 for hsp90, 0.1% UPJ56 or 1 μg/ml EC1 for hsp56, 0.1% PP5 antiserum for PP5, 1 μg/ml M2 monoclonal for the FLAG-PP5 and FLAG-TPR, 0.1% DS14F5 for p60, or 0.1% anti-cyclophilin 40 for CyP-40. The immunoblots were then incubated a second time with the appropriate 125I-conjugated antibody to visualize the immunoreactive bands.

### Building of Proteins to Purified hsp90

Rabbit hsp90 was purified from brain cytosol by sequential chromatography over DE52, hydroxyapatite, and ATP-agarose exactly as described by Hutchison et al. (44). Aliquots (30 μl) of purified rabbit hsp90 (1 mg/ml) were immunoadsorbed to 7.5-μl pellets of Actigel precoupled with 75 μl of 3G3 ascites. Pellets were washed once with 1 ml of HE buffer and suspended in Hepes buffer, pH 7.4, plus 0.1% Nonidet P-40 in a final volume of 130 μl, including 30 μl of the hsp90-free DE52 fraction of rabbit brain cytosol.
containing p60, PP5, FKBP52, and CyP-40. This is DE52 fraction pool A as described by Dittmar et al. (45). In experiments where binding of proteins to hsp90 was competed with the PP5 TPR domain, 7.5 μg of purified FLAG-tagged PP5 TPR in 30 μl of 20 mM Hepes, 1 mM dithiothreitol, 150 mM NaCl were added, maintaining the same final incubation volume of 150 μl. Samples were incubated on ice with occasional manual resuspension for 40 min, washed three times with 1 ml of HEG, and proteins were resolved by SDS-PAGE and Western blotting.

Reconstitution of GR Heterocomplexes—Reconstitution of GR-hsp90 heterocomplexes was performed essentially as described previously (44, 45). Immunoabsorbed GR was stripped of associated hsp90 by incubating the immunopellet for 2 h at 4 °C with 0.5 x 10^6 CFU followed by two washes with 1 ml of HEG buffer. Salt-stripped immune pellets were then incubated for 20 min at 30 °C with 50 μl of rabbit reticulocyte lysate plus 5 μl of an ATP-regenerating system (50 mM ATP, 250 mM creatine phosphate, 20 mM MgCl₂, and 100 units/ml creatine phosphokinase). The mixtures were resuspended every 5 min during the incubation. After the incubation, the pellets were washed three times with 1 ml of TEGM, 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 [³²H]triamcinolone acetonide in TEGM overnight on ice, washed twice with HEM buffer, and counted for radioactivity in a scintillation counter. Pellets from replicate incubations were analyzed by SDS-PAGE and Western blotting with anti-hsp90, PP5, FKBP52, and CyP-40.

Expression of p60—For bacterial lysates containing p60, cDNA for the 60-kDa human protein (IFP SSP 3521) cloned by Honôri et al. (46), which is the homolog of the rabbit p60 (39), was subcloned into a pET23c vector (Novagen) using the EcoRI and NotI sites. This construct was used to transform E. coli strain BL21 (DE3), which harbors an integrated T7 polymerase gene. Control E. coli and bacteria expressing p60 were grown to an A₆₀₀ of 0.6, induced with isopropyl-β-D-thiogalactopyranoside for 3 h at 25 °C, and harvested. Bacterial lysates were prepared by sonication in phosphate-buffered saline, and aliquots were flash frozen and stored at −70 °C.

Binding to FK506 Matrix—The preparation of FK506 immobilized onto an Affi-Gel 10 matrix was described by Yen et al. (47), and the affinity matrix used here is the remainder of that original batch prepared at the Upjohn Co. Rabbit reticulocyte lysate (100 μl) or purified PP5 (100 μl, 10 μg/ml) was rotated with 100 μl of affinity resin at 4 °C for 2 h. The resin was then washed six times with 1 ml of HE buffer by rotating at 4 °C for 15 min on each wash. After washing, the resin was rotated at 4 °C for 15 min with 100 μl of vehicle (10 or 4% ethanol, 0.02% Triton X-100), cyclosporin A, or FK506 as indicated. The matrix was then stripped by incubating it with 100 μl of 0.1% trifluoroacetic acid for 1 h at 4 °C. Proteins eluted from 1-ml washes were contracted to 100 μl in a rapid vacuum apparatus. The proteins eluted from affinity resin were analyzed by SDS-PAGE followed by Western blotting with anti-hsp90 antibody.

Protein Phosphatase Assay—³²P-Labeled casein was prepared according to Dobrowolsky et al. (47) and contained ~7 nmol of ³²P/pg of protein. PP5 (100 μg) was incubated for 15 min at 37 °C in 15 μl of 50 mM Tris-HCl, pH 7.0, 0.1 mM EGTA, 1 mM MnCl₂, 0.1% β-mercaptoethanol, 0.005% bovine serum albumin, and various concentrations of FK506 or ethanol vehicle. Reactions were initiated by addition of 5 μl of ³²P-casein in the same buffer, and they were terminated by trichloroacetic acid precipitation. Phosphate release was determined by the method of McGoWan and Cohen (48).

RESULTS

PP5 Is a Component of Native and Reconstituted GR Heterocomplexes—Fig. 1 shows an immunoblot of three TPR domain proteins co-adsorbed with the untransformed mouse GR from L cell cytosol or from WCL2 cell cytosol. In hormone-free L cells, the GR is cytoplasmic (31), whereas the mouse receptor overexpressed in Chinese hamster ovary cells (WCL2 cell line) is nuclear (49). There is a difference in the two heterocomplexes in that the L cell GR is not associated with hsp70, whereas the WCL2 cell GR heterocomplex contains hsp70 (49). As shown in Fig. 1, immunoadsorption of both the cytoplasmic L cell (Fig. 1A) and nuclear WCL2 cell (Fig. 1B) GR is accompanied by immunospecific co-adsorption of PP5 as well as of the immunophilins FKBP52 and CyP-40 (cf. lanes 2 and 3).

When hormone-free receptors that have been stripped of hsp90 are incubated with rabbit reticulocyte lysate, they are reassembled into complexes that contain rabbit hsp90 and either FKBP52 or CyP-40 (22). The immunophilin is co-adsorbed with the GR only when the receptor is complexed with hsp90 (23), and because hsp70 is required for assembly of the GR-hsp90 complex, hsp70 is also required for the presence of the immunophilin (44). As shown in Fig. 2, when the immunoabsorbed GR (lane 2) is stripped of its associated proteins (lane 4) and incubated with reticulocyte lysate (lane 6), the mouse receptor is assembled into heterocomplexes containing rabbit PP5 as well as heterocomplexes containing the rabbit immunophilins FKBP52 and CyP-40. The bar graph in Fig. 2 shows that about half the receptors are restored to the steroid binding conformation upon cell-free heterocomplex assembly.

PP5 and the Immunophilins Are in Different Heterocomplexes with hsp90—As shown in Fig. 3A, rabbit PP5 (lane 3) and FKBP52 (lane 2) migrate very close to each other in a 10% polyacrylamide gel. Lane 4 of Fig. 3A was sliced down the middle, and the left portion was immunoblotted for FKBP52 and the right portion was immunoblotted for PP5, to clearly demonstrate the small but distinct difference in their migration. Importantly, the antibody against PP5 does not react against FKBP52, and the UP56 antibody against FKBP52 does not recognize PP5. Thus, despite their close migration, the two proteins can be individually identified by dividing samples and immunoblotting proteins independently. This is the way the samples of Figs. 1 and 2 were assayed.

As shown in Fig. 3B, immunoadsorption of hsp90 from rabbit reticulocyte lysate yields immune specific co-adsorption of PP5, FKBP52, and CyP-40. However, immunoadsorption of FKBP52 or CyP-40 does not yield co-immunoadsorption of PP5 or the other immunophilin, although hsp90 is co-immunoadsorbed. This suggests that the three TPR domain proteins exist in independent complexes with hsp90. Unfortunately, the antiserum against PP5 does not immunoadsorb rabbit PP5, but the failure of the anti-immunophilin antibodies to yield co-adsorption of PP5 is strong evidence that PP5 is not present in native hsp90 complexes that contain either FKBP52 or CyP-40.

Competitive Binding of the PP5 TPR—We previously showed that immunoadsorption of the FLAG-tagged TPR domain of PP5 expressed in COS-7 cells yielded co-immunoadsorption of monkey hsp90 and that the TPR domain alone bound more tightly to hsp90 than the intact PP5 (36). In Fig. 4, we show that the PP5 TPR domain blocks the binding of intact PP5 to hsp90. For this experiment, the proteins in rabbit brain cytosol were first fractionated by chromatography on DE52 and fractions containing proteins eluting before hsp90 were pooled to yield the hsp90-free fraction pool A described by

![Fig. 1. PP5 is a component of both nuclear and cytoplasmic GR heterocomplexes.](Image)
Fig. 2. Rabbit reticulocyte lysate reconstitutes GR-hsp90 complexes containing PP5. GR was immunoadsorbed from replicate aliquots of L cell cytosol with nonimmune IgG or BuGR2, and the immunopellets were stripped of hsp90, PP5, FKBP52, and CyP-40 with 0.5 M NaCl. The salt-stripped immunopellets were incubated with rabbit reticulocyte lysate and an ATP-generating system. GR, hsp90, PP5, FKBP52, and CyP-40 were assayed in each sample by SDS-PAGE and Western blotting. Identical immune 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 nonimmune pellet; lane 4, stripped immune pellet; lane 5, stripped nonimmune pellet incubated with reticulocyte lysate; lane 6, stripped immune pellet incubated with reticulocyte lysate.

Dittmar et al. (45). This DE52 pool was then incubated on ice with Actigel pellets bound with 3G3 antibody alone (lane 1) or 3G3 antibody prebound with purified hsp90 (lanes 2 and 3). When hsp90 is present, the PP5 in the DE52 pool binds to the Actigel pellet (cf. lanes 1 and 2), but PP5 binding is blocked if the purified PP5 TPR domain is present (lane 3). In lanes 4 and 5 of Fig. 4A, 3G3-Actigel (lane 4) or 3G3-Actigel prebound with purified hsp90 (lane 5) was incubated with purified PP5 to demonstrate that the complex is due to a direct interaction between the two proteins.

We have previously shown that p60, which is a required component of the receptor-hsp90 heterocomplex assembly machinery (45) and contains six to eight TPRs (46), binds very tightly to hsp90 and blocks the binding of immunophilins (18). By mutational analysis, Chen et al. (50) have shown that a central region of p60 containing TPRs is required for binding to hsp90. As shown in Fig. 4B, p60 inhibits PP5 binding to hsp90. In this experiment 3G3-Actigel alone (lane 1) or 3G3-Actigel prebound with hsp90 (lanes 2–4) was incubated with the hsp90-free DE52 protein pool. When hsp90 is present, PP5 is bound (lane 2), and PP5 binding is prevented in the presence of lysate from bacteria expressing p60 (lane 4) but not in the presence of control bacterial lysate (lane 3).

CyP-40 contains three TPRs (14), and a C-terminal fragment of CyP-40 containing the three TPRs binds to hsp90 (26). Previously, we have shown that this fragment competes for the binding of the immunophilins, which are relatively weakly bound to hsp90, but we were unable to affect the binding of p60 at the highest achievable concentration of the CyP-40 TPR fragment (18). The FLAG-tagged TPR domain of PP5 binds more avidly to hsp90, and it competes for the binding of p60 in DE52 pool A to hsp90 (Fig. 4C). In the experiment of Fig. 4C, the p60 bands, which were probed with 125I-labeled counterantibody, were excised and counted to determine the extent of the inhibition. In the presence of the PP5 TPR domain (lane 3), the binding of p60 was reduced by 90% with respect to the control binding in the absence of the TPR (lane 2). The PP5 TPR domain competes for p60 binding to hsp90 at about one-tenth the concentration at which the CyP-40 TPR fragment was previously ineffective (18).

Stoichiometry of PP5 and FKBP5s in GR Heterocomplexes—To determine the stoichiometry of PP5 relative to receptor in native GR heterocomplexes, L cells were ruptured in molybdate-containing buffer, GR was immunoadsorbed, and after washing the immune pellet, the proteins were resolved on 12% SDS-polyacrylamide gels. After transfer to an Immobilon membrane, the PP5 was quantitated by reacting blots containing both immunoadsorbed complexes and standard amounts of purified FLAG-FP5 with 125I-anti-rabbit IgG and directly counting the excised PP5 bands. Fig. 5 shows a typical immune pellet and standard curve. Because only GR that is bound to hsp90 binds steroid (44, 45, 51), the number of GR-hsp90 complexes was assayed by incubating replicate immune pellets with a saturating level of [3H]tixamcinolone acetonide. Similarly, GR-associated immunophilins possessing high affinity FK506 binding activity were assayed by incubating replicate immune pellets with [3H]FK506 as described previously (38). Fig. 5 summarizes stoichiometry data from five experiments. Thirty-five percent of the GR-hsp90 heterocomplexes immunoadsorbed from L cell cytosol contain PP5 and about 50% contain an FKBP. We found that large amounts of [3H]cyclosporin A bound to immune pellets in a nonspecific manner; thus, we were not able to determine the percentage of receptor hetero-
PP5 binds directly to hsp90 via its TPR domain. A, the PP5 TPR domain competes for binding of PP5 to hsp90. 3G3-Actigel or 3G3-Actigel prebound with purified hsp90 was incubated with the rabbit brain DE52 hsp90-free protein pool in the presence or absence of 7.5 μg of the purified, FLAG-tagged TPR domain of PP5 as described under “Methods.” After washing, the pellet-bound proteins were resolved by SDS-PAGE and Western blotting. Lane 1, 3G3-Actigel pellet without hsp90 but with DE52 pool; lane 2, hsp90-bound 3G3-Actigel and DE52 pool; lane 3, hsp90-bound 3G3-Actigel incubated with DE52 pool and TPR domain. For lanes 4 and 5, 3G3-Actigel (lane 4) or hsp90-bound 3G3-Actigel (lane 5) was incubated with 0.3 μg of purified FLAG-PP5. B, bacterially expressed p60 competes for binding of PP5 to hsp90. 3G3-Actigel or 3G3-Actigel prebound with purified hsp90 was preincubated at 4 °C in the presence of 0.3 μg of purified FLAG-PP5. C, incubated with lysate from bacteria expressing p60 and DE52 pool. Lane 1, tagged TPR domain of PP5 and treated as described in “Methods.” After washing, the pellet-bound proteins were resolved by SDS-PAGE and Western blotting.

PP5 possesses an FK506-binding type domain—Because the other TPR proteins identified in native steroid receptor heterocomplexes (FKBP51, FKBP52, CyP-40) are all immunophilins, it seems possible that immunophilin structure could be in some way important for the function(s) of proteins binding to GR-hsp90 complexes at this common TPR acceptor site. Thus, the sequence of human PP5 (34) was scanned for a best fit alignment with both the FKBP consensus sequence of Peattie et al. (9) and the FK506 binding site of human FKBP52 (3). Accession number X89416. In the middle rank, the top sequence is that of human FKBP52 (9) (accession number M88279), and the bottom is that of hPP5. The sequence of hPP5 was scanned for the best fit with the PPlase domain of hFKBP52 as defined by the domain analysis of Callebaut et al. (16). The alignment was made using the GCG program BESTFIT (with a gap weight of 3.0). [sequence identities; †, conserved replacements; ‡, partially conserved replacements. This sequence contains 9 of the 12 residues (vertical arrows) involved in high affinity interactions between hFKBP52 and FK506 (9), based on analysis of the structure of the FKBP12-FK506 complex by Van Duyn et al. (52). The domain diagrams at the bottom locate the above sequences (stippled areas), the tetratricopeptide repeats (hatched bars), and a calmodulin-binding domain (solid bar) in FKBP52 and hPP5. PPlase, the peptidylprolyl isomerase domain of FKBP52.

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immunoadsorbed FLAG-PP5 with [3H]FK506. Under conditions where we could readily demonstrate specific binding of [3H]FK506 to FKBP52 immunoadsorbed from rabbit reticulocyte lysate, we could not demonstrate binding to immunoadsorbed PP5. However, weak binding that cannot be detected by this assay can sometimes be detected by affinity chromatography. We had available a small amount of Affi-Gel-linked FK506 affinity matrix, and we used it to adsorb reticulocyte lysate. As shown in Fig. 7A, after the matrix was washed multiple times, PP5 was eluted with 1 mm FK506 (lane 4, middle row). In the next to the bottom row of Fig. 7A, lane 4 was cut in half, and lanes 1–3 and the left half of lane 4 were rebotted for FKBP52 to show that it was also eluted by FK506. The top row of Fig. 7A
show the immunoblot of the same fractions for hsp90, which co-elutes with FK506-bound immunophilins, as reported previously by Tai et al. (8). Neither CyP-40 nor p60, both of which bind to hsp90 via TPR domains in the same manner as FKBP52, were retained by the FK506 affinity matrix (Fig. 7A), and they serve as negative controls.

Because it is possible that the PP5 eluted with FK506 is somehow co-eluted with other FK506-binding immunophilins rather than having FK506 binding activity of its own, purified FLAG-PP5 was incubated with the affinity matrix. As shown in Fig. 7B, purified PP5 bound to the affinity matrix and was eluted by FK506. It appears that PP5 is a weak FK506 binding activity that allows it to be retained by an FK506 affinity matrix and eluted in a stereospecific manner. We have not detected any effect of FK506 on PP5 function. As we previously reported for FKBP52 (38), FK506 does not affect the binding of PP5 to hsp90, nor does it stabilize or dissociate the GR-hsp90 complex (data not shown). At concentrations up to 1 μM, FK506 does not affect the ability of PP5 to release phosphate from [γ-32P]labeled casein (data not shown). At this time, it is not known whether PP5 in the GR-hsp90 heterocomplex affects the phosphorylation state of the GR protein itself.

**DISCUSSION**

Gehring and co-workers (53–55) have cross-linked molybdate-stabilized glucocorticoid (53), progesterin (54), and estrogen (55) receptor heterocomplexes and isolated 300–350-kDa heterocomplexes that contain 1 molecule of receptor, 2 molecules of hsp90, and 1 molecule of FKBP52. This has led to a static model in which it is assumed that there is a common heterotetrameric receptor structure (see Ref. 56 for review). Now that is realized that there are different receptor heterocomplexes depending upon the immunophilin that is bound to the TPR-binding site on hsp90 (18, 20, 22, 24), the static model with uniform composition derived from cross-linking studies is not adequate. Rather, we should think of these heterocomplexes as dynamic structures in which the hsp90 component is capable of binding multiple proteins, including FKBP51, FKBP52, CyP-40, PP5 and possibly unidentified proteins, in equilibria that depend upon the local abundance of the protein, its affinity for the TPR acceptor region of hsp90, and likely the affinity of a second direct interaction with the receptor protein itself (30, 31).

Although the cross-linking method yields an appropriate estimate of the size of a heterotetrameric complex detected with a steroid affinity label, the technique does not differentiate between receptor complexes that contain FKBP52 and receptor complexes that contain other molecules of a similar size instead of FKBP52. The presence of PP5 would be missed because of its nearly identical migration with FKBP52 on denaturing gels (Fig. 3A). Yet, one gets the rough impression from immunoblots that PP5 may be about as abundant as FKBP52 in native GR heterocomplexes (Fig. 1). When the stoichiometry of immuno-purified PR heterocomplexes was determined from scanning stained gels, a ratio of one molecule of PR to 0.56 molecule of FKBP52 (avian p50) was obtained (37). By ligand binding, a ratio of one steroid binding site to 0.55 [3H]FK506 binding sites was determined for immunoadsorbed GR heterocomplexes (38). The stoichiometry of 0.52 [3H]FK506 binding sites per molecule of GR determined in the experiments of Fig. 5 is in close agreement with these data. The majority of the remaining half of the receptor complexes, or approximately 1 in 3 of the total complexes, contain PP5 (Fig. 5). By calculation, this leaves only a few of the native GR-hsp90 heterocomplexes containing CyP-40, which agrees with the rough impression obtained from the immunoblots shown in Fig. 1. In contrast to native complexes isolated from L cell cytosol, we consistently find that GR-hsp90 heterocomplexes reconstituted by reticulo-cyte lysate contain higher relative amounts of CyP-40 and lower relative amounts of PP5 (Fig. 2). This suggests that stoichiometry estimates made from receptor heterocomplexes reconstituted in vitro may not reflect the stoichiometry of native receptor heterocomplexes recovered from cells.

Although PP5 is not a member of the very conserved FKBP family of immunophilins, it contains TPRs that are related to those of the high molecular weight immunophilins (36), and it has a weak FK506 binding activity (Fig. 7). The FK506 binding site appears to be in the center of the molecule between the TPR and phosphatase domains (Fig. 6) in a region we are calling an FK506-binding type domain.

Because the immunophilins are protein chaperones (4, 57), it has been thought that they might be required for assembly of receptor-hsp90 heterocomplexes. However, it is clear that cell-free heterocomplex assembly is not affected by FK506 or cyclosporin A (22, 38), and GR-hsp90 complexes have been assembled in a reconstituted system that does not contain either PP5 or immunophilins (45). We have previously suggested that the immunophilin components of steroid receptor heterocomplexes may play a role in their cytoplasmic-nuclear trafficking (18, 30, 31). The majority of both PP5 (34) and FKBP52 (58) is localized to the nucleus where both proteins are distributed in a mottled, nonrandom manner, while being excluded from nucleoli. Interestingly, the GR receptor heterocomplexes of WCL2 cells (Fig. 1B) are localized to the nucleus where the proteins are distributed in a mottled, nonrandom fashion and are excluded from nucleoli.
mutations in the gene for the CyP-40 homolog is unknown. It is a gene in yeast (61). However, the specific step in the pathway of optimal GR-mediated transcriptional activation of a reporter their having a role in any other receptor function. Genetic experiments suggest that a CyP-40 homolog is required for optimal GR-mediated transcriptional activation of a reporter gene in yeast (61). However, the specific step in the pathway of transcriptional activation that is adversely affected by null mutations in the gene for the CyP-40 homolog is unknown. It is interesting that, compared with FKBP52 and PP5, CyP-40 in mammalian cells is more weakly bound to the GR-hsp90 complex (22), is found in only a small number of native GR hetero- complexes (see above), and is localized primarily to nucleoli (18), a localization that has no obvious relevance to the recep- tors. It should be noted that none of the immunophi- lins examined to date appear essential for the viability of haploid yeast cells (57). This lack of essentiality may reflect a level of redundancy that covers the loss of one or more of these proteins, and this could seriously complicate defining the role of immunophi- lins in receptor action. Given their similar cellular localizations, their similar abundance in native GR heterocomplexes, as well as their common TPR interaction with hsp90 and ability to bind FK506, it is entirely possible that the functions of FKBP52 and PP5 are largely redundant. However, the posses- sion of phosphatase activity on the part of PP5 (33–35) and calmodulin binding activity on the part of FKBP52 (62) sug- gests that the proteins must have functions independent of one another as well.

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