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Molecular Cloning of Human FKBP51 and Comparisons of Immunophilin Interactions with Hsp90 and Progesterone Receptor

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A cDNA for human FKBP51 has been cloned and sequenced, and protein products have been expressed in both in vitro and bacterial systems. The deduced amino acid sequence for human FKBP51 is 90% identical to sequences of recently described murine proteins and is 55% identical to the sequence of human FKBP52. Human FKBP51 mRNA is expressed in a wide range of tissues, and the protein has peptidylprolyl isomerase activity that is inhibited by FK506 but not cyclosporine. FKBP51 is the same as a previously described progesterone receptor-associated immunophilin that, similar to FKBP52 and cyclophilin 40, is an Hsp90-binding protein and appears in functionally mature steroid receptor complexes along with Hsp90 and p23. Each of the three receptor-associated immunophilins displays interactions with progesterone receptor that are more dynamic than Hsp90-receptor interactions. Whereas FKBP52 and FKBP51 compete about equally well for binding to Hsp90 in a purified system, FKBP51 accumulates preferentially in progesterone receptor complexes assembled in a cell-free system. This observation provides a precedent for differential interactions between Hsp90-associated immunophilins and target proteins such as steroid receptors.

In the absence of hormone, progesterone receptor (PR) normally exists in hetero-oligomeric complexes with various components of the molecular chaperone machinery. The functionally mature progesterone receptor complex, which is competent for high-affinity progesterone binding, contains Hsp90, p23, and an immunophilin component; however, formation of the mature complex is preceded by PR interactions with chaperone components not present in the mature PR complex (33, 38). These earlier transient components include Hsp70, Hip, and Hop (p60). Hsp90 also appears at an intermediate assembly stage, but its interaction with the PR complex is biochemically distinct from its interaction in mature complexes (3).

Three immunophilins have been identified in steroid receptor complexes, and it appears that multiple immunophilins do not reside in the same receptor complex. Present evidence suggests that the immunophilins enter receptor complexes indirectly through their direct binding to Hsp90 (28, 31), perhaps in a ternary p23-Hsp90-immunophilin complex (11). The functional role for any of the receptor-associated immunophilins is unresolved.

The first receptor-associated immunophilin identified was initially called p59 (42) and later called Hsp56 (30) and p50 (36). After its subsequent recognition as a member of the FK506-binding immunophilin family (16, 41, 45), it has been variously termed FKBP59, FKBP56, FKBP52 (21), and Hsp90-binding immunophilin (17). The term FKBP52 is preferred here since this identifies the protein as an FKBP family member and all sequenced mammalian cDNAs for this protein encode a 52-kDa product.

Another steroid receptor-associated immunophilin, Cyp40, is a member of the cyclosporine-binding cyclophilin family and was first identified in estrogen receptor complexes (27). This protein has since been identified in progesterone (18) and glucocorticoid (20) receptor complexes as well.

The third receptor-associated immunophilin was initially identified in chicken PR complexes and termed p54 (36). Later, tryptic peptide sequences from chicken p54 were shown to have about 60% sequence identity with FKBP52 (35) and p54 was shown to bind FK506 (34); thus, it was termed FKBP54.

The FKBPs and cyclophilin family members have peptidylprolyl isomerase (PPIase) activity, but the catalytic domains, which are also the drug-binding sites, appear to have independently evolved in the two protein families (recently reviewed in reference 13). However, larger family members, such as FKBP52 and Cyp40, have some homology outside the catalytic domain in a region containing a tetratricopeptide repeat (TPR). TPR-like motifs, found in degenerate form in a number of proteins, are proposed to participate generally in protein-protein interactions (reviewed in reference 5), and in FKBP52 and Cyp40, the TPR region is required for Hsp90 binding (20, 25, 26). Interestingly, two nonimmunophilin, transient components of the PR assembly pathway also contain TPR motifs. The Hsp70-interacting protein Hip (6, 22), one of the earliest assembly components, contains a central TPR motif that is required for Hsp70 binding (23). Another intermediate component, a homolog of yeast Sti1 termed IEF SSP 3521 (7) or p60 (37), simultaneously binds Hsp70 and Hsp90 (3). Due to its Hsp-binding properties, the more descriptive name Hop, for Hsp70-Hsp90 organizing protein, has been proposed (3). An N-terminal TPR region of Hop is required for efficient Hsp70 binding, and a central TPR region is required for Hsp90 binding (3).

In this report, a cDNA sequence encoding the human homolog for FKBP54 is presented. Its deduced amino acid sequence is about 90% identical to the deduced sequence for mouse FKBP51, whose molecular cloning was recently reported by two laboratories (1, 44). Since the human cDNA also

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encodes a 51-kDa protein and given the high homology between the human and mouse sequences, it seems likely that the human clone and mouse FKBP51 are direct homologs. Therefore, we are adopting the term FKBP51 to denote the PR-associated protein previously termed p54 and FKBP54.

To begin addressing whether the immunophilin components in mature PR complexes behave distinctly or interchangeably, the following comparisons were made: (i) spatial patterns of mRNA expression for each immunophilin in 16 human tissues, (ii) levels of immunophilin protein in rabbit reticulocyte lysate (RL), the medium for cell-free assembly of PR complexes, (iii) FKBP51 and FKBP52 interactions with Hsp90, and (iv) interactions of each immunophilin with mature PR complexes.

MATERIALS AND METHODS

Cloning of cDNAs for human FKBPs. Expressed sequence tag (EST) entries in GenBank were searched for sequences showing 50 to 60% homology with FKBP52 and encoding any of seven tryptic peptide sequences derived from chicken p54 (35). One such EST (accession number F00724) was identified and used to design PCR primers for amplification from a HeLa cDNA phage library (UniZAP XR; Stratagene). The forward primer was 5′-AAGATAGTGTCCTG GTTAGAGATG, and the reverse primer was 5′-TCACCCCTCCTATACAAG. A radiolabeled hybridization probe was generated from the single 200-bp PCR product after isolation of the product on a 2% low-melting-point agarose gel. Labeled probe was prepared by using a High Prime DNA labeling kit (Boehringer Mannheim) and [α-^{32}P]dCTP (>3,000 Ci/mmol; ICN).

Plaque lifts were performed with nylon membrane filters (MagnaGraph; Micron Separations, Inc.) from plates containing bacteria transformed with the HeLa cDNA phage library. The filters were screened by hybridization (40) with a radiolabeled DNA probe. Prior to hybridization, filters were incubated 5 min in 0.5 M NaOH–1.5 M NaCl and then neutralized for 5 min in 0.5 M Tris-HCl (pH 8.0)–1.5 M NaCl. Filters were next rinsed for 1 min in 2× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate [pH 7.0]), blotted dry, and baked for 1 h at 80°C. Filters were prehybridized at 65°C for 1 h in 1 M NaCl–1% sodium dodecyl sulfate (SDS) and hybridized at 65°C overnight in 0.5 M sodium phosphate (pH 7.2)–1% bovine serum albumin–1 mM EDTA–7% SDS containing radiolabeled probe. After two 1-h washes in 0.25× SSC–0.1% SDS, filters were exposed on X-ray film.

Two positive clones were identified, and plasmids (pBluescript SK$^-$) were excised from the cloned phage according to Stratagene's instructions. Partial sequences were obtained for both clones by automated sequencing. Based on comparisons with the sequence for human FKBP52, neither of the original clones contained the 5′ end of an open reading frame (ORF). Clone 54-2 was sequenced entirely in both directions and found to contain 1,290 bases of ORF encoding an amino acid sequence approximately 55% identical to the FKBP52 sequence and with high homology with seven tryptic peptide sequences derived from chicken p54. From comparison with FKBP52, it appeared that approximately 80 bases were missing from the 5′ end of the cDNA ORF for human FKBP51.

In an attempt to obtain a full-length cDNA, an additional hybridization probe was prepared by PCR methods from the 5′ end of clone 54-2, and 21 additional positive clones were isolated from the HeLa cDNA library. While none of these clones contained a complete ORF, the 5′ sequence was extended an additional 58 bases with sequence from one of the clones (54-3). The novel sequence from 54-3 was used to search GenBank for an EST encoding this portion of FKBP51, and one EST (accession number Z17357) that had 100% identity with 112 bases from the 5′ end of 54-3 was found. The 5′ end of this EST also contained 20 additional bases encoding the presumed initiation codon for FKBP51.

A cDNA containing the complete putative ORF for human FKBP51 was constructed by PCR methods using the 54-2 sequence subcloned into the pET28c bacterial expression plasmid (Novagen) as the template. The reverse primer (5′-ACTTTCAAATAAATCCTCTCC) was complementary to the 5′ end of 54-2 downstream from a unique *Sac*I restriction site; the forward primer (5′-AAGA CCATGGAGACTGATGAAGGTGCCAAGAACAATGAAGAAAGCCCC) contained novel sequences derived from the second EST with an *Nco*I restriction site at the presumed initiation codon. The 440-bp PCR product was digested with *Nco*I and *Sac*I and used to replace the 5′ end in the original 54-2/pET28c. The sequence of the PCR-generated 5′ end was verified by automated sequencing.

A cDNA for human FKBP52 was cloned from the HeLa cDNA library by using a PCR-generated hybridization probe. Primer sequences were derived from the published sequence for human FKBP52 (21): forward primer 5′-TGG ACATCAGCCCCAAACAG and reverse 5′-GCGGTAGAAGGAGAAGGC AG. Using the HeLa cDNA library as a template, a 378-bp product was generated and radiolabeled probe was prepared. Seven positive clones were obtained, and plasmids were excised. Partial sequencing of a clone with a 2.2-kb insert confirmed that it contained the complete ORF for FKBP52.

Bacterial expression of human FKBP51 and FKBP52. In addition to the construct encoding the complete putative sequence for human FKBP51, a construct encoding a His-tagged FKBP51 was made by subcloning the complete ORF into pET30a (Novagen); expression of this cDNA produces an FKBP51 product with an N-terminal fusion of 44 amino acids including six histidines at positions 2 to 7. Another expression vector was constructed with pET28 and the full ORF of cloned human FKBP52 cDNA; the resulting plasmid encodes a full-length FKBP52 protein with an N-terminal fusion of 20 amino acids including six histidines at positions 5 to 10.

Escherichia coli was transformed with either of the two expression plasmids and grown at 37°C to an optical density at 600 nm of between 0.6 and 1.0. Protein production was induced with 0.4 mM isopropylthiogalactopyranoside (IPTG) at room temperature for 3 h. Cells were pelleted, washed once with cold phosphate-buffered saline, and lysed by sonication in ice-cold phosphate-buffered saline or, for lysates containing His-tagged products, in nickel resin binding buffer (Novagen). Disrupted cells were microcentrifuged at 17,000 × *g* for 30 min, and the clear supernatants were collected and stored at −80°C.

Production of monoclonal antibodies Hi51 and Hi52. Recombinant protein products served as antigens for monoclonal antibody production. FKBP52 antigen was full-length FKBP52 with an N-terminal polyhistidine tag. The FKBP51 antigen was generated by subcloning the partial cDNA 54-2 into pET28 in frame with plasmid sequences encoding an N-terminal His tag. Protein was expressed in bacteria as described above and purified by elution from a Ni^{2+} affinity resin (Novagen). Monoclonal antibody was generated as previously described (22). Briefly, BALB/c mice were injected intraperitoneally with 50 μg of antigen on days 0, 21, 30, and 45. On day 48, mice with the highest serum titers against each antigen were euthanized. Splenocytes from these two mice were isolated and fused with MOPC-21 NS-1 murine myeloma cells (4:1 ratio) to prepare hybridoma cell lines. Hybridoma supernatants were screened by enzyme-linked immunosorbent assay for recognition of partially purified recombinant proteins. Positive cell lines were further screened for the ability of antibody to immunoprecipitate full-length, native FKBP51 or FKBP52. We cloned one hybridoma cell line that produces an immunoglobulin G (IgG) antibody, termed Hi51, that specifically recognizes FKBP51. We cloned three hybridoma cell lines that produce IgG antibodies, termed Hi52a, Hi52b, and Hi52c, that each specifically recognize FKBP52. These four cell lines were used for ascites production.

Northern blot analyses for immunophilin expression. Radiolabeled probes for Northern blot analysis of immunophilin mRNA expression were prepared from PCR-generated fragments of cDNAs for each human immunophilin. The template cDNA, product size and range, and primers for each PCR were as follows: human FKBP51, 200-bp product, nucleotides (nt) 854 to 1036 in accession number U42031, primers same as those used to generate original hybridization probe for cloning cDNA; human FKBP52, 378-bp product, nt 167 to 544 in accession number M88279, forward 5′-TGGACATCAGCCCCAAACAG, reverse 5′-CGCCATCTTCCTCTTCCGTC; and human Cyp40, 111-bp product, nt 562 to 672 from accession number L11667, forward 5′-GGAATAGGAGTGGC AAGG, reverse 5′-TTCCCCCGTCATCTCC.

Radiolabeled probes were prepared from each gel-purified PCR product and a human β-actin cDNA (Clontech), using [α-^{32}P]dCTP and a High Prime DNA labeling kit. Blots containing, per lane, 2 μg of poly(A)$^+$ RNA from each of 16 human tissues were obtained commercially (Multiple Tissue Northerns; Clontech). In successive hybridizations, blots were prehybridized (2 h) and hybridized (overnight) in Expresshyb solution (Clontech) at 65°C. Following hybridization with one of the radiolabeled probes, blots were rinsed five times and washed for 45 min at room temperature in 2× SSC–0.5% SDS. Blots were further washed twice for 40 min each time at 50°C in 0.1× SSC–0.1% SDS. Wet blots were exposed to Kodak X-AR film with two intensifying screens at −80°C. Between successive probings, membranes were stripped by first incubating for twice 15 min each time at 98°C in diethyl pyrocarbonate-treated water–0.75% SDS and next incubating in 0.5% SDS at 98°C with 20 min of cooling. The loss of hybridized probe was verified by reexposure to film.

Enzymatic activity of recombinant FKBP51. PPIase activity of recombinant FKBP51 was measured as described for mouse FKBP51 (44), with some minor modifications. Purified His-tagged FKBP51 (130 nM, final concentration) was premixed with 250 μg of α-chymotrypsin *N*α-*p*-tosyl-L-lysine chloromethyl ketone [TLCK] treated; Sigma) per ml and various concentrations of FK506 or cyclosporine in 32.5 mM HEPES (pH 7.9)–0.014% Triton X-100. The substrate for reactions was *N*-succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide (BACHEM) at a final concentration of 56.4 μM. Reactions in a 1.5-ml final volume were performed at 10°C and were initiated by addition of substrate to the FKBP51 mixture. Release of *p*-nitroanilide in the presence or absence of His-FKBP51 was monitored at 410 nm with a Cary model 1E spectrophotometer.

Coprecipitation patterns of FKBPs and Hsp90. Monoclonal antibodies to PR, FKBP51, and FKBP52 were used separately to compare the compositions of complexes containing each protein. Approximately 3 μg of PR was adsorbed to PR22-protein G-Sepharose and used for in vitro assembly of PR complexes, as previously described (38), in 1.5 ml of RL. FKBP51- or FKBP52-containing complexes were immunoprecipitated from RL in the absence of added PR. First, immunoaffinity resins were prepared by preadsorbing antibody Hi51 or Hi52c to protein G-Sepharose (15-μl bed volume). The resins were then incubated with RL for 30 min at 30°C and washed four times with 1 ml of wash buffer (WB; 20 mM Tris [pH 7.4], 50 mM NaCl, 1% Tween 20). Proteins were extracted from all resins with urea sample buffer and separated by two-dimensional (2-D) polyacrylamide gel electrophoresis (PAGE) as described previously (38).

Some factors affecting the coprecipitation of Hsp90 and p23 with FKBP51 were examined as follows. Hi51 resin was added to each of three 500-μl aliquots of RL that contained one of the following: 500 mM NaCl, an ATP-regenerating system (ATPRS; 2.5 mg of phosphocreatine and 4 U of creatine phosphokinase), or 5 U of apyrase. After a 30-min incubation at 30°C, resins were washed with WB and extracted with SDS sample buffer for gel separations.

Competition between FKBPs for Hsp90 binding. An Hsp90 immunoaffinity resin was prepared by cross-linking monoclonal antibody D7α to protein A-Sepharose (Pharmacia); dimethyl pimelimidate (Sigma) was used as cross-linker as described elsewhere (32). This resin was added to chick oviduct cytosol containing 0.5 M NaCl, and the slurry was rocked at 4°C for 1 h. Resin with bound chicken Hsp90 was washed two times in WB containing 0.5 M NaCl and once in WB alone.

Competition for Hsp90 binding by FKBP51 and FKBP52 was examined as follows. Hsp90-resin (approximately 7 μg of chicken Hsp90 per sample) was incubated 30 min at room temperature with 6 μg of purified FKBP52 and a range of FKBP51 amounts (0 to 27 μg) in a final volume of 100 μl of incubation buffer (20 mM Tris [pH 7.4], 50 mM NaCl, 5 mM $MgCl_2$, 5 mM ATP, 1% Tween 20). Resin pellets were washed two times with incubation buffer and extracted into SDS sample buffer for gel separations. A small amount of D7α heavy chain is extracted from the Hsp90 immunoaffinity resin and migrates close to FKBP51 and FKBP52. Since quantitation of Coomassie blue-stained FKBP bands would be complicated by the contaminating heavy chain, samples were screened instead for associated FKBPs by Western immunostaining with Hi51 and Hi52c.

Quantitation of immunophilins in RL. His-tagged, recombinant forms of FKBP51, FKBP52, and Cyp40 were prepared and purified. Aliquots of each purified protein were separated by SDS-PAGE and Coomassie blue stained. Protein amounts were quantitated by laser densitometry (Molecular Dynamics Personal Densitometer) of stained bands in comparison with a known quantity of SDS-PAGE size standards. Western immunostaining was used to estimate the concentration of each immunophilin in RL. A range of amounts for each purified recombinant protein was electrophoresed alongside a given volume of RL, and the gel lanes were transferred to a polyvinylidene difluoride membrane and immunostained with the appropriate monoclonal or polyclonal antibody: FKBP51 with Hi51, FKBP52 with rabbit antiserum UPJ56 (29), and Cyp40 with rabbit antiserum PA3-022 (Affinity Bioreagents, Golden, Colo.). Optical densities of immunostained bands from RL were compared with densities for recombinant protein standards to estimate the amount of each immunophilin in a given volume of RL.

Interactions of immunophilins with PR. Radiolabeled immunophilins were prepared by expressing cDNAs for each in a combined transcription-translation system (TnT Lysate; Promega) containing [^{35}S]methionine. Approximately equal amounts of incorporated radioactivity for each of the three immunophilins were added to RL to support in vitro assembly of PR complexes. PR was immunoaffinity purified from chick oviduct cytosol by using monoclonal antibody PR22 adsorbed to protein G-Sepharose (Pharmacia). Components in each final gel sample were PR from 1 ml of oviduct cytosol (~1 μg of PR) bound to 10 μg of PR22 on a 10-μl resin pellet. Steady-state assembly of PR complexes was established by incubating PR resin for 45 min at 30°C in 1 ml of RL containing an ATPRS and radiolabeled immunophilins. PR complexes on resin were then washed in WB and transferred to fresh, prewarmed RL containing an ATPRS but lacking radiolabeled immunophilins. Aliquots (100 μl) were removed from this assembly mixture at various times from 0 to 15 min and rapidly quenched by addition to 1 ml of ice-cold WB. Resin complexes were washed in WB, extracted into SDS sample buffer, and separated by SDS-PAGE. Gels were Coomassie blue stained for total protein levels and then autoradiographed for levels of each radiolabeled immunophilin associated with PR complexes. Protein bands were quantitated by laser densitometry.

In a separate comparison of immunophilin interactions with PR complexes, radiolabeled immunophilins were added to RL at relative molar levels equivalent to the molar ratios determined for endogenous immunophilins. Approximately 0.5 μg of PR was assembled in vitro with 600 μl of RL, a volume determined to give saturated binding of all three immunophilins. One sample was assembled in RL containing 10 μg of the Hsp90-binding drug geldanamycin (GA) (43), and three replicate samples were assembled in RL lacking GA. Incubations were at 30°C for 45 min, followed by washing of resin complexes and extraction into SDS sample buffer.

Complexes of p23-Hsp90-immunophilin in the absence of PR were also isolated from RL containing radiolabeled immunophilins. For the isolation of p23-containing complexes, monoclonal antibody JJ3 (9) adsorbed to protein G-Sepharose was added to 100 μl of RL containing an ATPRS and radiolabeled immunophilins. Similar to conditions for the PR assembly, one sample contained GA and three replicate samples lacked the drug; again, the resin mixtures were incubated at 30°C for 45 min.

RESULTS

Cloning of human FKBP51 cDNA and tissue distribution of immunophilin expression. Previously (35), partial amino acid sequences were obtained from tryptic peptides of PR-associated chicken p54. These sequences suggested an approximately 60% overall identity between p54 and FKBP52. Unsuccessful attempts were made to clone a cDNA for p54 by either PCR procedures using degenerate oligonucleotides derived from the peptide sequences or expression screening using monoclonal antibody FF1, which recognizes p54 by Western immunostaining but not by immunoprecipitation (35). As a third alternative, DNA sequences from ESTs in the GenBank database were searched for similarity with FKBP sequences. We identified one EST (accession number F00724) whose sequence did not correspond to any known FKBP family member, was approximately 60% identical to FKBP52, and encoded one of the avian p54 tryptic peptides. The sequence for this human EST was used to design PCR primers for creating a hybridization probe; HeLa cell poly(A)$^+$ RNA was used as the template. As detailed in Materials and Methods, we obtained a cDNA sequence (accession number U42031) that encodes most of the human FKBP51 ORF; the remaining DNA sequence from the 5′ end of the ORF was derived from an additional human EST (accession number Z17357) that has 100% identity with 112 bases from the 5′ end of the cloned FKBP51 cDNA.

In Fig. 1, the deduced amino acid sequence for human

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hFKBP51 MTTDEGAKNN EESPTATVAE QGEDITSKKD RGVL....KI VKRVGNGEET 46
mFKBP51 MTTDEGTSNN GENPAATMTE QGEDITTKKD RGVL....KI VKRVGTSDEA 46
hFKBP52 MTADEMKATE SGAQSAPLPM EGVDISPKQD EGVL....KV IKREGTGTEM 46
 hCyp40 MSHPSPQAKP SNPSNPRVFF DVDIGGERVG RIVLELFADI VPKTAENFRA 50

hFKBP51 PMIGDKVYVH YKGKLSNGKK FDSSHDRNEP FVFSLGKGQV IKAWDIGVAT 96
mFKBP51 PMPGDKVYVH YKGMLSDGKK FDSSHDRKKP FAFSLGQGQV IKAWDIGVST 96
hFKBP52 PMIGDRVFVH YTGWLLDGTK FDSSLDRKDK FSFDLGKGEV IKAWDIAIAT 96
 hCyp40 LCTGEKGIGH TTGKPLHFK. .GCFFHR... .........I IKKFMIQGG. 85

hFKBP51 MKKGEICHLL CKPEYAYGSA GSLPKIPSNA TLFFEIELLD FKGEDLF..E 144
mFKBP51 MKKGEICHLL CKPEYAYGSA GHLQKIPSNA TLFFEIELLD FKGEDLF..E 144
hFKBP52 MKVGEVCHIT CKPEYAYGSA GSPPKIPPNA TLVFEVELFE FKGEDLTEEE 146
 hCyp40 .......... ...DPSNQNG TGGESIYGEK ...FEDENFH YK......ED 113

hFKBP51 DGGIIRRTKR KGEGYSNPNE GATVEIHLEG RCGGRMFDCR DVAFTVGEGE 194
mFKBP51 DSGVIRRIKR KGEGYSNPNE GATVKVHLEG CCGGRTFDCR DVVFVVGEGE 194
hFKBP52 DGGIIRRIQT RGEGVAKPNE GAIVEVALEG YYKDKLFDQR ELRFEIGEGE 196
 hCyp40 REGLL..... ...SMANAGR NTNGSQFFIT TVPTPHLDGK HVVF.....G 150

hFKBP51 DHDIPIGIDK ALEKMQREEQ CILYLGPRYG FGEAGKPKFG IEPNAELIYE 244
mFKBP51 DHDIPIGIDK ALVKMQREEQ CILYLGPRYG FGEAGKPKFG IDPNAELMYE 244
hFKBP52 NLDLPYGLER AIQRMEKGEH SIVYLKPSYA FGSVGKEKFQ IPPNAELKYE 246
 hCyp40 QVIKGIGVAR ILENVEVKGE KPAKL...CV IAECGELKEG .DDGGIPPKD 196

hFKBP51 VTLKSFEKAK ESWEMDTKE. ..KLEQAAIV KEKGTVYFKG GKYMQAVIQY 291
mFKBP51 VTLKSFEKAK ESWEMDTKE. ..KLTQAAIV KEKGTVYFKG GKYTQAVIQY 291
hFKBP52 LHLKSFEKAK ESWEMNSEE. ..KLEQSTIV KERGTVYFKE GKYKQALLQY 293
 hCyp40 GSGQDSHPDFP EDADTDLKDV DKTLLITFDL ENIGNTFFKS QNWEMAIKKY 246

hFKBP51 GKIVSWLEME YGLSEKESKA S.ESFLLAAF LNLAMCYLKL REYTKAVECC 340
mFKBP51 RKIVSWLEME YGLSEKESKA S.ESFLLAAF LNLAMCYLKL REYNKAVECC 340
hFKBP52 KKIVSWLEYE SSFSNEEAQK A.QALRLASH LNLAMCHLKL QAFSAAIESC 342
 hCyp40 AEVLRYVDSS KAVIETADRA KLQPIALSCV LNIGACKLKM SNWQGAIDSC 296

hFKBP51 DKALGLDSAN EKGLYRRGEA QLLMNEFESA KGDFEKVLEV NPQNKAARLQ 390
mFKBP51 DKALGLDSAN EKGLYRRGEA QLLMNDFESA KGDFEKVLAV NPQNRAARLQ 390
hFKBP52 KKALELDSNN EKGLFRRGEA HLAVNDFELA RADFQKVLQL YPNNKAAKTQ 392
 hCyp40 LRALELDPSN TKALYRRAQG WQGLKEYDQA LADLKKAQGI APEDKAIQAE 346

hFKBP51 ISMCQKKAKE HNERDRRIYA NMFKKFAEQD AKEEANKAMG KKTSEGVTNE 440
mFKBP51 ISMCQRKAKE HNERDRRVYA NMFKKFAERD AKEEASKAGS KKAVEG.AAG 439
hFKBP52 LAVCQQRIRR QLAREKKLYA NMFERLAEEE NKAKAEASSG DHPTDTEMKE 442
 hCyp40 LLKVKQKIKA QKDKEKAVYA KMFA*..... .......... .......... 370

hFKBP51 KGTDSQAMEE EKPEGHV* 457
mFKBP51 KQHESQAMEE CKAKGHV* 456
hFKBP52 EQKSNTAGSQ SQVETSA* 459
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FIG. 1. Immunophilin amino acid sequence comparisons. Amino acid sequences deduced from cDNAs for human FKBP51 (*hFKBP51*; accession number U42031 and additional 5′ sequence described herein), mouse FKBP51 (*mFKBP51*; accession numbers U16959 and U36220), human FKBP52 (*hFKBP52*; accession number M88279), and human cyclophilin 40 (*hCyp40*; accession number L11667) were aligned by using the Gap program from the Genetics Computer Group package. Amino acids identical to the corresponding residue in hFKBP51 are in boldface type. Dots represent gaps introduced during sequence alignments; asterisks represent termination codons.

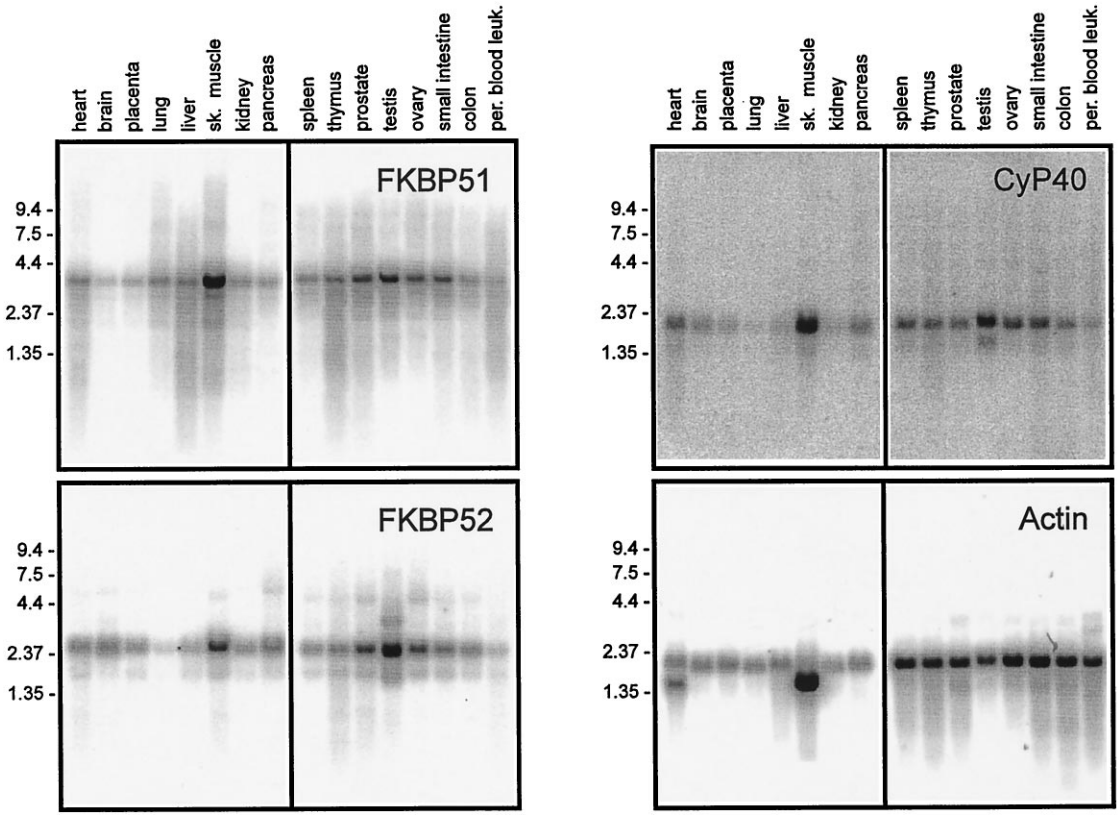

FIG. 2. Northern blot analyses for immunophilin expression. Multiple-tissue Northern blots were hybridized successively with radioactively labeled probes for FKBP51, FKBP52, Cyp40, and actin. Each lane represents approximately 2 μg of poly(A)$^+$ RNA. The migration positions for RNA size markers are indicated in kilodaltons on the left, and the tissue source for RNA is indicated above each lane. Abbreviations for tissues: sk. muscle, skeletal muscle; per. blood leuk., peripheral blood leukocytes.

FKBP51 is compared with the sequences for mouse FKBP51 (1, 44), human FKBP52 (21), and human Cyp40 (14). Similar to cDNAs for mouse FKBP51, the human cDNA encodes a protein with a predicted molecular mass of 51,212 Da. The extents of identity (similarity) for sequences compared with human FKBP51 are as follows: mouse FKBP51, 87% (92%); human FKBP52, 55% (74%); and human Cyp40, 21% (47%). As noted for mouse FKBP51, human FKBP51 contains an apparent PPIase- and FK506-binding domain within its N-terminal 200 amino acids and contains a TPR region between amino acids 270 and 390. Cyp40, as found in comparisons with other FKBPs, has little homology with the FKBP PPIase/FK506-binding domain, but there is some similarity in the TPR regions of FKBPs and Cyp40.

To compare the tissue distributions of FKBP51, FKBP52, and Cyp40 mRNA expression, specific hybridization probes were prepared for each and used to probe multiple-tissue Northern blots representing poly(A)$^+$ RNA from 16 different human tissues (Fig. 2). Each of the immunophilin mRNAs displays a broad pattern of tissue expression; note that there are detectable message levels for each immunophilin in all 16 tissues examined. Some relative differences in mRNA levels are seen among the tissues, such as the relatively lower level of FKBP51 message in brain and higher level in lung compared to messages for FKBP52 and Cyp40. Upon normalization for actin mRNA levels, expression of all three immunophilin mRNAs is enriched in testis relative to other tissues. Overall, however, there are no striking differences in the distribution of mRNAs for FKBP51, FKBP52, and Cyp40.

FKBP51 protein expression, enzymatic activity, and FK506 inhibition. For studies of human FKBP51 protein, several expression plasmids were prepared. The original FKBP51 cDNA clone in pBluescript is under transcriptional control of a T3 promoter; this cDNA, lacking the 5′ end of the ORF, encodes a partial FKBP51 protein beginning at the internal Met^{48} codon. This cDNA was subcloned into pET28c to produce a polyhistidine-tagged antigen for production of an anti-FKBP51 monoclonal antibody.

As detailed in Materials and Methods, a bacterial expression plasmid encoding the putative sequence for full-length FKBP51 was created, and another expression plasmid encoding this FKBP51 with an N-terminal polyhistidine fusion was also created. Both wild-type and His-tagged FKBP51 proteins are expressed at high levels in *E. coli* and are recovered in the soluble supernatant from sonicated cells (results not shown).

To verify that recombinant human FKBP51 has PPIase activity and binds FK506, affinity-purified His-FKBP51 was added to an assay system for PPIase activity. As shown in Fig. 3, His-FKBP51 greatly enhanced basal conversion of *cis* to *trans* peptidyl proline, and this activity was partially inhibited by 0.1 to 1 μM FK506 but not by 1 μM cyclosporine.

Immunoisolation of FKBP-containing complexes and interactions with Hsp90. The compositions of PR complexes assembled in RL and complexes containing FKBP51 and FKBP52 were compared by 2-D PAGE separations and Coomassie staining (Fig. 4A). Each of the three panels shows the gel region containing Hsp90 and the immunophilins. Note that PR complexes (top panel) contain all three immunophilins—

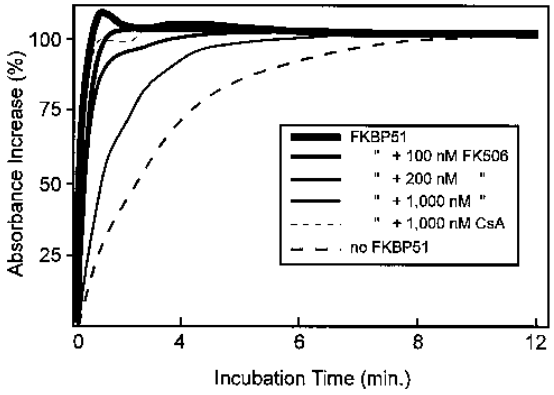

FIG. 3. FK506 inhibition of FKBP51 enzymatic activity. Recombinant human FKBP51 with an N-terminal polyhistidine fusion was expressed in bacteria and purified on a Ni^{2+}-agarose affinity resin. The ability of His-FKBP51 to enhance chymotryptic cleavage of the substrate N-succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide was measured by changes in absorbance at 410 nm. Reactions were performed at the indicated concentrations of FK506 or cyclosporine (CsA).

FKBP52, FKBP51, and Cyp40—along with Hsp90 and Hsp70. In the absence of PR, Hsp90 and some Hsp70 coprecipitate with FKBP51, but no FKBP52 or Cyp40 is detected (middle panel). Likewise, Hsp90 and Hsp70 coprecipitate with FKBP52, but neither FKBP51 nor Cyp40 is detected (bottom panel). In addition to Hsp90 and Hsp70, p23 is present in each of the three complexes examined, but it migrates outside the gel region depicted.

The effects of ionic strength and ATP on the coprecipitation of Hsp90, Hsp70, and p23 with FKBP51 are shown in Fig. 4B. FKBP51 is present in lanes 2 and 3 but is poorly resolved from the Hi51 heavy chain. At elevated ionic strength (lane 2), no proteins that specifically coprecipitate with FKBP51 were detected. The Hsp70 band in lane 2 appears to represent nonspecific binding of Hsp70 to antibody resin. When precipitations from RL were performed at 30°C in the presence of an ATPRS (lane 3), three coprecipitating proteins, Hsp90, Hsp70, and p23, were identified. Each of these has been previously identified as associating directly or indirectly with large immunophilins (4, 11, 28, 31). In the presence of an ATPase (lane 4), coprecipitation of Hsp90 was unaffected, but Hsp70 was reduced to background binding levels and p23 was quantitatively lost. Thus, it appears that Hsp90, Hsp70, and p23 bind directly or indirectly to FKBP51 in a salt-sensitive manner and that at 30°C, recovery of Hsp70 and p23 with FKBP51 complexes is ATP dependent. The band appearing in lanes 3 and 4 immediately below the band marked HC, as noted previously (3), is an RL protein that binds nonspecifically to antibody resins in a salt-sensitive manner. The relevance of other proteins appearing in the samples has not been determined.

The ability of FKBP51 and FKBP52 to compete for Hsp90 binding was examined (Fig. 5). Hsp90 was immunoisolated from chicken oviduct cytosol and incubated with FKBP52 plus a range of FKBP51 concentrations. Shown in Fig. 5 are Western immunostains of the Hsp90 samples probing with anti-FKBP51 and anti-FKBP52 antibodies. Note the corresponding decrease in recovery of FKBP52 (upper panel) as the recovery of FKBP51 (lower panel) increases. While this limited analysis does not provide affinity measurements for Hsp90 interactions with either FKBP, it does suggest that the two FKBPs interact with Hsp90 in a competitive manner, with neither FKBP binding Hsp90 in a dominant fashion. Similar competition for

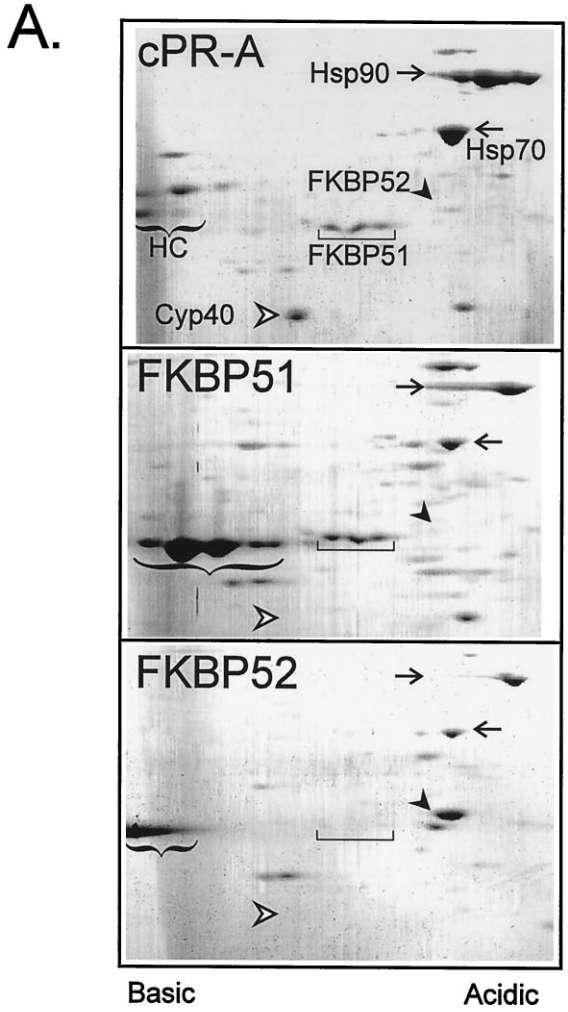

FIG. 4. (A) Two-dimensional gel separations of complexes targeted by antibodies to PR, FKBP51, or FKBP52. For the sample shown in the upper panel, recombinant chicken PR-A (cPR-A) was expressed in *Spodoptera frugiperda* Sf9 cells and purified from cell extracts by immunoprecipitation with PR22 adsorbed to protein G-Sepharose. PR on resin was incubated with RL to promote assembly of PR complexes. For the lower two panels, monoclonal antibodies recognizing FKBP51 and FKBP52 were also used to immunoprecipitate native immunophilin complexes from RL. Each of the three preparations were separated by 2-D PAGE and Coomassie stained. The migration positions for several proteins, as labeled in the upper panel, are indicated by corresponding markers in all three panels. Antibody heavy chains (HC), which differ for each panel, are indicated by braces. (B) Ionic strength and ATP effects on FKBP51 interactions with Hsp90 and p23. Mouse monoclonal IgG Hi51 was adsorbed to protein G-Sepharose (lane 1) and used to immunoprecipitate FKBP51 from RL containing 0.4 M NaCl (lane 2), an ATPRS (lane 3), or the ATPase apyrase (lane 4). After washing, resin samples were extracted with SDS sample buffer and proteins were separated by SDS-PAGE. Proteins were visualized by Coomassie staining (upper panel) or by Western immunostaining (lower panel) with an anti-p23 monoclonal antibody. FKBP51 in lanes 2 to 4 is not resolved from Hi51 heavy chain (HC).

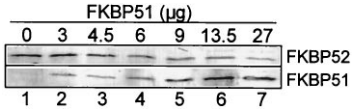

FIG. 5. Competitive binding of FKBPs to Hsp90. Hsp90 was purified from chicken oviduct cytosol by using monoclonal antibody D7α covalently cross-linked to protein A-Sepharose. After stringent washing to remove Hsp90-associated proteins, resin containing approximately 7 μg of Hsp90 was incubated for 30 min at room temperature with 6 μg of purified, recombinant His-FKBP52 plus His-FKBP51 in the amount indicated above each lane. Resins were washed three times, and adsorbed proteins were extracted into SDS sample buffer. The PAGE-separated samples were transferred to membrane and Western immunostained with either anti-FKBP52 or anti-FKBP51 antibody, as indicated on the right.

Hsp90 binding between FKBP52 and Cyp40 has previously been demonstrated (20, 26).

Immunophilin interactions with PR complexes. Consistent with the apparent competition among immunophilins for Hsp90 binding, each large immunophilin appears to interact separately with steroid receptors (20). The interactions of the immunophilins with PR complexes were compared by using in vitro assembly in RL. First, the level of each immunophilin in RL was estimated (Fig. 6). Recombinant His-tagged immunophilins were prepared, purified, and quantitated to serve as standards for semiquantitative Western immunostaining. From densitometric analysis of the immunostained bands in Fig. 6, it is estimated that the approximate molar ratio for FKBP51, FKBP52, and Cyp40 in RL is 1:5:10, respectively. The total concentration of the three immunophilins in RL is approximately 300 nM, compared to Hsp90's typical concentration of approximately 1 to 2 μM.

Comparative interactions of the immunophilins during PR assembly were first examined as shown in Fig. 7. Each immunophilin was synthesized and radioactively labeled by cell-free expression in the presence of [^{35}S]methionine, and approximately equivalent amounts of incorporated radioactivity for each were collectively added to normal RL containing an ATPRS.

In an earlier study (33), it was shown that Hsp90 in mature PR complexes exchanges with a half-life of 5 min during steady-state cell-free assembly reactions. To compare immunophilin exchanges in mature PR complexes, the RL mixture supplemented with radioactive immunophilins was used to support in vitro assembly of PR complexes for 30 min to reach steady-state assembly conditions. PR-resin complexes were rapidly washed and transferred to fresh RL lacking radioactive components, where assembly reactions were allowed to proceed. At various time points, aliquots of the assembly mix were removed, resin was rapidly washed in cold buffer, and bound proteins were separated by SDS-PAGE. Proteins recovered with PR resin were visualized by Coomassie staining (Fig. 7A, upper panel) and autoradiography of the dried gel (lower panel).

Unlabeled, endogenous FKBPs are not evident in the stained gel due to their comigration with contaminating immunoglobulin heavy chain. For reference, FKBP51 (detected by autoradiography in the lower panel) comigrates with the darker heavy-chain band seen in the stained gel image. The lower region of the stained gel containing endogenous Cyp40 and p23 has been cropped from the image.

In lane 1 (lower panel only), an aliquot of the initial assembly mix is shown to illustrate the relative levels of radioactive immunophilins added. In lane 2 (upper and lower panels), a negative assembly control in which mock assembly was performed with immunoaffinity resin lacking PR is shown. Lanes 3 to 11 show PR samples taken over the exchange time course; the 0-min time point (lane 3) is an aliquot of the assembly mix removed before resin transfer to nonradioactive RL. Note from the stained gel patterns that the overall status of PR complexes is unchanged over the entire time course, indicating achievement and maintenance of steady-state PR assembly. Based on the autoradiograph (lower panel), it might appear that the level of FKBP51 in PR complexes is higher than the levels of FKBP52 and Cyp40. However, in this experiment, the amount of radioactive FKBP51 present was approximately equal to that for FKBP52 and Cyp40 whereas the relative amounts of endogenous FKBP51 in RL were 5- and 10-fold lower, respectively (Fig. 6). In other words, the specific activity of the FKBP51 pool in this experiment is higher than the specific activities for FKBP52 and Cyp40, but a later experiment does in fact suggest that FKBP51 interacts preferentially with PR complexes.

Densitometric data from the autoradiograph (lower panel) are summarized in graphical form in Fig. 7B. The rapid reduction of all three labeled immunophilins from PR complexes suggests a highly dynamic interaction by each of these components with mature PR complexes. A 50% reduction between the maximum and new steady-state level for each radioactive immunophilin was observed in less than 1 min. Based on the 5-min period observed for Hsp90 exchange in PR complexes (33), it appears that the immunophilins must interact dynamically with Hsp90 while Hsp90 remains associated with PR.

For FKBP51, the new steady-state level is exactly 50% of the maximal level before the chase period. This could be explained if FKBP51 from the initial assembly reaction is quantitatively associated with PR complexes; thus, transferring the PR complexes to an equivalent volume of RL would double the pool of FKBP51 in the chase reaction. If this is true, for the volume of RL and amount of PR used in this experiment (approximately 1 μg of PR in 100 μl of RL for each sample shown in Fig. 7), the level of FKBP51, but not the levels of other immunophilins, appears to be limiting. This was borne out by additional assembly reactions in which a fixed amount of PR was assembled in the presence of increasing volumes of RL. The relative ratio of FKBP51 to other immunophilins recovered in PR complexes reached a maximal, constant value at approximately 1 μg of PR in 400 μl of RL (results not shown).

The next experiment (Fig. 8) was designed to more carefully examine whether any of the three immunophilins display a preferential association with PR complexes. First, immunophilins labeled with [^{35}S]methionine (Fig. 8A, lanes 1 to 3) were added to RL at approximately the same molar ratio as en-

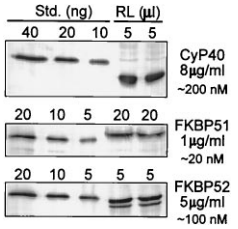

FIG. 6. Estimation of immunophilin levels in RL. Amounts of Cyp40, FKBP51, and FKBP52 present in RL were estimated by quantitative comparisons of Western immunostain intensity for each protein in RL samples (duplicates in the final two lanes) with various amounts of purified recombinant protein standard (Std.). Mobility differences between endogenous RL proteins and standards are due to His tag fusions (Cyp40 and FKBP52) or a combination of His tag fusion and N-terminal truncation (FKBP51) of the standard proteins. Optical densities for immunostained bands were measured by laser scanning densitometry. Estimated protein and molar concentrations of each immunophilin in RL are shown on the right.

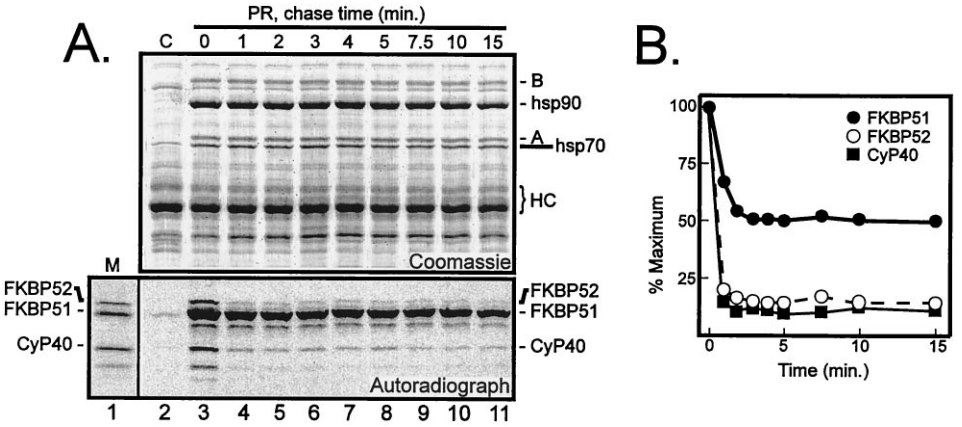

FIG. 7. Dynamics of immunophilin interactions with PR complexes. (A) cDNAs encoding each immunophilin were expressed in vitro by combined transcription and translation in the presence of [^{35}S]methionine. Aliquots of each synthesis mix containing equivalent amounts of incorporated radioactivity were combined and added to RL. PR was purified from chicken oviduct cytosol on PR22-protein G-Sepharose; the PR-resin was added to RL for in vitro assembly of receptor complexes. PR assembly in the presence of labeled immunophilins proceeded for 30 min to establish steady-state assembly. As a control for nonspecific binding to antibody resin, an aliquot of PR22-protein G-Sepharose lacking bound PR was incubated with RL containing radioactive components (lane 2). Following the initial assembly, an aliquot of the PR mixture was removed (lane 3), and the remainder was rapidly washed and transferred to fresh RL lacking radioactive components. Aliquots were removed over a 15-min time period (chase time). Total proteins associated with resins were separated by gel electrophoresis and visualized by Coomassie staining (upper panel). Bands identified on the right are the A and B forms of PR, Hsp90, Hsp70, and PR22 heavy chain (HC). Immunophilins are not evident in the stained gel due to their comigration with HC. Radioactive immunophilins in the samples were detected by drying the stained gel and exposing it to X-ray film (lower panel). (B) Changes over time in the levels of radioactive immunophilins were estimated by laser densitometry of bands in the autoradiograph. Values are plotted as a percent of radioactivity found in PR complexes at the 0-min time point (lane 3).

dogenous immunophilins (lane 4). To do this, the number of methionines in each immunophilin—14 in FKBP51, 11 in FKBP52, and 7 in Cyp40—had to be considered. Also, as illustrated in Fig. 8A, the FKBP52 synthesis product consists of two protein forms that are resolved by SDS-PAGE (lane 3). The smaller, minor form, which may initiate from a methionine codon downstream from the authentic initiation codon, represents approximately one-third of the total radioactivity incorporated into FKBP52 products and comigrates with the FKBP51 product (lane 2). PR assembly reactions with radioactive FKBP52 alone showed that the two FKBP52 forms associate equally well with PR complexes (not shown). Thus, in PR assembly mixtures containing both FKBP52 and FKBP51, adjustments must be made in quantitating the amounts of radioactive FKBP52 and FKBP51 recovered with PR.

The following lanes contain samples of PR complexes (lanes 5 to 8) assembled in an excess of this mixture (500 μl of RL per 0.5 μg of PR) and p23-containing complexes (lanes 9 to 12) isolated from 100 μl of RL lacking PR. Three replicate samples were prepared for both PR and p23 complexes to demonstrate reproducibility. The final sample in each set (lanes 8 and 12) is a negative control. GA has been shown to disrupt p23 binding to Hsp90 (12, 38) and to efficiently block assembly of mature PR complexes containing Hsp90, p23, and immunophilins (38). Thus, any immunophilins recovered in PR or p23 complexes in the presence of GA is likely nonspecifically adsorbed to affinity resins. However, note that very little radiolabeled immunophilin is recovered with PR or p23 complexes in the presence of GA (lanes 8 and 12).

In Fig. 8B, data from Fig. 6 relating to the relative levels of immunophilins in total RL and data from Fig. 8A showing the relative immunophilin levels in PR and p23 complexes are plotted. For the latter data sets, individual autoradiographic bands (Fig. 8A, lanes 5 to 7 and 9 to 11) were quantitated by laser densitometry. To arrive at molar equivalents, the densitometric values were adjusted for methionine number and the comigration of FKBP51 with FKBP52's minor form. If the immunophilins interact indiscriminately with p23 and PR complexes, then their percentages should reflect that observed in RL as a whole. In fact, FKBP51 associates with PR complexes at a much higher percentage (over 70% of the total immunophilins) than would be suggested by its relatively low level in RL (less than 10%) or by its recovery in p23 complexes (just over 20%).

DISCUSSION

We have cloned and sequenced a cDNA from a HeLa cell cDNA library for FKBP51, a protein previously identified as a component of mature PR complexes. In earlier reports, this protein was first termed p54 (36) to reflect its apparent size in kilodaltons by SDS-PAGE; later it was concluded from peptide sequence similarities (35) and the protein's ability to bind an FK506 affinity matrix (34) that the protein is a member of the FK506-binding family of immunophilins and was thus termed FKBP54. Now, however, based on its predicted size deduced from the human cDNA sequence and its high homology with the recently identified mouse FKBP51 (1, 44), it seems appropriate to agree that "FKBP51" will designate all vertebrate forms of this protein.

Northern blot analyses suggest that FKBP51 and two other PR-associated immunophilins, FKBP52 and Cyp40, are expressed in a wide range of tissues. FKBP51, FKBP52, and Cyp40 are similar in having PPIase activity and in their abilities to competitively bind Hsp90. The three immunophilins have highly dynamic interactions with PR complexes, but FKBP51 associates with PR complexes preferentially over FKBP52 or Cyp40 (Fig. 8).

Yeh et al. (44) identified the mouse FKBP51 mRNA due to its higher expression level during the early, clonal expansion phase of 3T3-L1 cell differentiation to adipocytes. They proposed that FKBP51 may be preferentially expressed in mitotically active cells, but no function for FKBP51 in the differentiation process was identified. Baughman et al. (1) independently cloned a cDNA for mouse FKBP51 and reported that it is a T-cell-specific immunophilin. The respective cloned

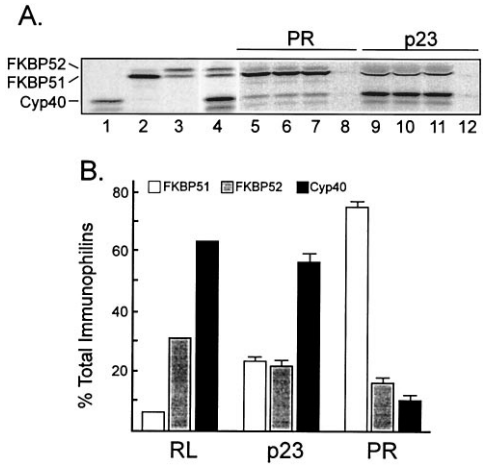

FIG. 8. Relative binding of immunophilins to PR and p23 complexes. (A) Two microliters of each in vitro-synthesized, radioactively labeled immunophilin product was separated by SDS-PAGE and autoradiographed to measure the incorporation of [^{35}S]methionine into protein products (lanes 1 to 3). Radiolabeled FKBP52, FKBP51, and Cyp40 synthesis mixtures were combined at a molar ratio (5:1:10, respectively) matching the ratio estimated for immunophilins endogenous to RL. An aliquot of this mixture is shown in lane 4; the remaining mixture was added to RL containing an ATPRS from which PR complexes were assembled (lanes 5 to 8) or p23 complexes were isolated (lanes 9 to 12). In each of three replicate reactions (lanes 5 to 7), approximately 0.5 μg of PR-A was assembled in a total volume of 500 μl of RL; a fourth reaction (lane 8) was supplemented with GA (20 μg per ml of RL mixture). Anti-p23 antibody was used to isolate p23-containing complexes from additional 100-μl aliquots of the RL mixture either lacking (lanes 9 to 11) or containing (lane 12) GA. Resin samples were washed, separated by SDS-PAGE, and autoradiographed to visualize radioactive immunophilins associated with the samples. (B) The bands shown in lanes 5 to 7 and 9 to 11 were quantitated by laser densitometry, and the values were adjusted to reflect the number of methionines in each product. The values for FKBP52 and FKBP51 were further adjusted to account for the minor FKBP52 product that comigrates with FKBP51. Finally, values for the three replicate PR or p23 samples were averaged and the standard errors were calculated. These values are plotted as a percentage of the total radiolabeled immunophilin in each sample, and error bars are shown. For comparison, the relative amounts of endogenous immunophilins in RL (as determined from the data in Fig. 6) are plotted on the left.

FKBP51 cDNAs are 100% identical over 2.2 kb of overlapping sequence, differing only in the extent of the 5′ and 3′ untranslated sequences obtained by each group. Given published data (44) in which FKBP51 mRNA expression was noted in each of eight mouse tissues, the highly restricted expression of FKBP51 in mouse T cells (1) seems doubtful. We propose that human FKBP51 is the direct species homolog of the mouse protein based on an 87% deduced amino acid sequence identity, but we cannot presently exclude the possibility that there are other, highly homologous FKBP51-like genes. For comparison, FKBP52 sequences share approximately 60% amino acid sequence identity with FKBP51.

FKBP51 interactions with Hsp90 and PR complexes. Similar to FKBP52 and Cyp40, FKBP51 is an Hsp90-binding protein. Hsp90 coprecipitates with FKBP51 from RL (Fig. 4), and purified FKBP51 directly binds purified, recombinant FKBP51 (Fig. 5). As has been shown for FKBP52 and Cyp40 (20, 26), FKBP51 and FKBP52 compete for binding to purified Hsp90 in roughly equivalent manners (Fig. 5). Hsp90 can independently bind an immunophilin and p23, and Hsp90 will bind both associated proteins simultaneously. However, binding of p23 to Hsp90 is ATP dependent (10, 12) but binding of immunophilins to Hsp90 does not strictly require ATP (Fig. 4B and references 7 and 31). The p23-Hsp90-immunophilin complex is potentially important since functionally mature PR complexes are associated with Hsp90, p23, and any one of the immunophilins (33, 38).

As estimated from the data in Fig. 6, the three immunophilins are present in RL at approximately a 1:5:10 (FKBP51:FKBP52:Cyp40) molar ratio. Furthermore, all three immunophilins enter and leave PR complexes in a highly dynamic manner (Fig. 7). One might expect to recover the three immunophilins in p23 complexes and in mature PR complexes at a 1:5:10 ratio, but this was not observed (Fig. 8). p23 complexes isolated from RL contained approximately equal amounts of the two FKBPs and a twofold greater amount of Cyp40. More strikingly, PR complexes assembled in RL contained roughly four times more FKBP51 than either FKBP52 or Cyp40. The preferred interaction of FKBP51 with PR complexes was not appreciated in our previous assembly studies since it was not realized that FKBP51 is present in RL at a limiting concentration. For the results in Fig. 8, the volume of RL was increased fivefold from the typical proportion used in earlier studies (200 μl of RL per μg of PR), to 1 ml of RL per μg of PR.

In an earlier study (36) on the composition of native PR complexes isolated from oviduct cytosol, the avian FKBP51 homolog (previously termed p54) was approximately 1.5-fold more abundant than the FKBP52 homolog (termed p50). In that analysis of native PR complexes, Cyp40 was not observed, probably because phosphate buffer was used and Cyp40 recovery is now known to be sensitive to phosphate (33a). The apparently small enhancement of FKBP51 in native complexes differs from the maximal enhancement observed in Fig. 8; however, the molar ratio of the two FKBPs and Cyp40 in oviduct cytosol would presumably affect the relative abundance of FKBP51 in native PR complexes. The endogenous levels of the three large immunophilins in oviduct cytosol have not been determined.

Earlier examinations of FKBP51 and FKBP52 interactions in native chicken PR complexes also revealed a marked difference in their sensitivities to progesterone (35, 36). Using in vitro conditions (4°C and 10 mM molybdate) that otherwise stabilize PR complexes against hormone-dependent dissociation, FKBP51 recovery in purified PR complexes was greatly reduced from cytosol supplemented with progesterone compared to cytosol lacking hormone. In the same samples, recovery of FKBP52, Hsp90, and p23 in PR complexes was unaffected by progesterone. The exclusive, hormone-dependent dissociation of FKBP51 from PR complexes implied that FKBP51 may have more direct interactions with the hormone-binding domain of PR than FKBP52. Therefore, despite the overall sequence and biochemical similarities between FKBP51 and FKBP52, there are potentially important differences in how the two FKBPs interact with PR complexes.

Functions of the large immunophilins. To date, the best-characterized interactions involving the high-molecular-weight immunophilins are their assembly into steroid receptor complexes. The PPIase activity of these proteins was an obvious first focus for functional studies in receptor complexes, but it remains unclear whether the N-terminal PPIase domain has a functional importance in receptor complexes. FK506 and cyclosporine bind in the respective PPIase active sites and inhibit enzymatic activity, but in vitro studies have failed to show a drug effect on receptor function or structure even though binding of drug to the immunophilin-receptor complex can be readily demonstrated. Several in vivo studies have described effects of either FK506 or cyclosporine on steroid hormone signalling, but a direct connection between drug-inhibited PPIase activity in one or more of the three receptor-associated immunophilins has not been clearly demonstrated. There are multiple cyclophilin and FKBP family members in any given

cell that may indirectly mediate drug actions on steroid signalling. One example is inhibition of the phosphatase calcineurin by FK506- and cyclosporine-immunophilin complexes (reviewed in reference 13). In fact, it has been demonstrated that FKBP51-FK506 complexes can inhibit calcineurin in vitro (1). Another possibility is that immunosuppressant drugs inhibit the function of membrane transporters that can alter intracellular steroid concentrations (15).

It has been proposed that FKBP52 may serve as a nuclear shuttle protein to cross-link nucleus-targeted proteins to cytoskeletal elements that direct and facilitate nuclear transport (24). However, since experimental data directly supporting this model are limited, and several observations, such as the diverse and dynamic nature of immunophilin-receptor interactions suggested from in vitro studies (Fig. 7 and references 33 and 38), tend to be conflicting, this model remains controversial.

In the most comprehensive comparison made to date, we have presented evidence that the multichaperone PR assembly pathway (PRAP) acts on proteins other than steroid receptors (19). In addition to progesterone and estrogen receptors, other potential targets examined for interactions with PRAP components were the arylhydrocarbon receptor, human heat shock transcription factor 1, and the cytoplasmic tyrosine kinase Fes. Each of these proteins was selected for its reported binding to Hsp90. In each case, treatment of the target in RL, using the same conditions that produce functional PR complexes, achieved Hsp90 binding and revealed related interactions with other PRAP components, including FKBP51, FKBP52, and Cyp40. Other, less comprehensive analyses have shown that Src (8) and Raf (39) kinases also interact with chaperones manner similarly to steroid receptors. (One distinction between protein kinases and other targets, and one also observed by us with Fes, is the additional presence of p50, a 50-kDa Hsp90-binding protein, in kinase complexes [reviewed in reference 2].)

Given the apparently promiscuous nature of this multichaperone pathway, it appears likely that it may serve a more or less general role in cellular protein folding. Participation of most PRAP components in nascent chain folding has not been observed, but the pathway's actions may be restricted to end-stage folding processes that could occur after dissociation of the peptide chain from the ribosome. Also, participation by this putative folding pathway is probably not required for every cytoplasmic protein.

We have proposed (19, 33, 38) that a number of proteins are evolutionarily adapted to remain in this end-stage chaperone pathway as a regulatory mechanism for repression, proteolytic stabilization, or other purposes. If this is the case, then for some target proteins, there may be no active, continuing role played by immunophilin-containing complexes on the target other than their passive association. Alternatively, the immunophilins may actively participate, perhaps through modulating the properties of Hsp90, to maintain dynamic interactions between the target and chaperone components or to facilitate progression of the target through the chaperone pathway.

In conclusion, despite investigation from several laboratories over several years, a satisfying definition for the function of large immunophilins relating to steroid receptor complexes, in particular, or the cellular chaperone machinery, in general, remains elusive. Steroid receptors continue to be an attractive model for reaching this understanding. Perhaps with the identification of multiple immunophilins directly participating in steroid receptor complexes and the development of a complete set of molecular and immunological reagents specific for each immunophilin, advances in understanding the functions of the large immunophilins, collectively and individually, will be forthcoming.

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The first two authors contributed equally to this work.

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