P59 (FK506 Binding Protein 59) Interaction with Heat Shock Proteins Is Highly Conserved and May Involve Proteins Other Than Steroid Receptors†

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ABSTRACT: P59 [also known as FK506 binding protein 59 (FKBP59) or heat shock protein 56 (hsp56)] and heat shock proteins 90 and 70 (hsp90 and hsp70) associate with steroid receptors and are believed to maintain the receptors in an inactive state. Recently, we showed that p59 purified from human lymphocytes is an immunophilin (FKBP59) which binds both FK506 and rapamycin. It was also demonstrated that immunosuppressant–FKBP59 complexes associate with hsp90, hsp70, and the glucocorticoid receptor [Tai, P.-K. K., Albers, M. W., Chang, H., Faber, L. E., & Schreiber, S. L. (1992) *Science 256*, 1315–1318]. Here we provide evidence that rabbit uterine p59 also binds FK506 and rapamycin and that p59 or its homologue is associated with nontransformed progesterone receptors of rabbit uterus and chicken oviduct. This suggests that the immunophilin–heat shock protein–steroid receptor interaction is ubiquitous and not limited to immune systems. A FKBP59 homologue complexed with hsp90–hsp70 was also detected in yeast, which suggests that the immunophilin–heat shock protein association has been evolutionarily conserved. In addition, we found that the FKBP59–hsp complexes are more complicated than previously thought, involving other proteins such as actin and a 63-kDa protein, p63. The association of p63 to the p59 complex was inhibited by FK506 and rapamycin, suggesting that p63 could be a potential target for the immunosuppressive actions of these two drugs. Since both immunophilin and heat shock proteins have been suggested to be responsible for protein folding and assembly of new synthesized polypeptides, it is reasonable to propose that p59, in association with hsp70 and hsp90, forms the core structure of a universal molecular chaperone. This chaperone complex may be responsible for recognition, folding, assembly, and disassembly of steroid receptors or other regulatory proteins at or near the end of translation.

P59 was first described as a protein associated with progesterone receptors (PR)[1] isolated from rabbit uteri (Tai & Faber, 1985) and was later identified as a common component of nontransformed androgen, estrogen, and glucocorticoid receptors from a variety of sources (Tai et al., 1986; Renoir et al., 1990; Rexin et al., 1991). It complexes with hsp90 and hsp70 (Sanchez et al., 1990; Perdew & Whitelaw, 1991) and appears to be a heat shock protein (also called hsp56) (Sanchez, 1990). Recently, Matts et al. (1992) provided evidence that p59 (complexed with hsp90 and hsp70) is associated with heme-regulated protein kinase (HRI), a key enzyme in the regulation of protein synthesis [for reviews, see London et al. (1987) and Jackson (1991)]. The mechanism by which p59 (FKBP59) works in concert with two heat shock proteins to regulate steroid receptor and HRI activity is unknown. It has been proposed that the association of p59, hsp90, and hsp70 with steroid receptors and HRI maintains these two regulatory proteins in an inactive state, but the role of each component remains obscure.

Recently several laboratories established that p59 isolated from human lymphocytes and bovine thymus is an immunophilin, binding both FK506 and rapamycin (Tai et al., 1992; Yem et al., 1992). Immunophilins are proteins that bind immunosuppressive drugs and may be classified as either cyclophilins, which bind to cyclosporin A, or FKBPs, which bind FK506 and rapamycin [for review, see Schreiber (1991)]. Indeed, the deduced amino acid sequences from rabbit liver (Lebeau et al., 1991) and human p59 cDNAs (Peattie et al., 1992) revealed the existence of two domains with homology to FKBP12 (Standaert et al., 1990) and FKBP13 (Jin et al., 1991): the first domain is 50% and the second domain is 29% identical to FKBP12 (Tai et al., 1992).

In addition to demonstrating the ligand binding property of FKBP59, we showed that FKBP59, hsp90, hsp70, and glucocorticoid receptor (GR) exist as a heteromeric complex (Tai et al., 1992), thus linking two families of proteins thought to be involved in protein folding and assembly. The association of these two classes of highly conserved proteins in immune cells prompted us to investigate whether this complex is ubiquitous and has been conserved during evolution.

In the present study, we demonstrate that FKBP59, bound with immunosuppressant, may be isolated as a large complex with hsp90, hsp70, and PR from rabbit uterine and chick oviduct cytosols. Thus, the association of immunophilins, heat shock proteins, and steroid receptors is ubiquitous and not limited to the immune system. We also detected a yeast

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[1] Abbreviations: FKBP, FK506 binding protein; PR, progesterone receptor; GR, glucocorticoid receptor; HRI, heme-regulated protein kinase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; PBS, phosphate-buffered saline; EC1, monoclonal antibody KN382/EC1; Ig, immunoglobulin; IgG, immunoglobulin G; IgM, immunoglobulin M; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; hsp70, heat shock protein 70; hsp90, heat shock protein 90; N27, anti-hsp70 monoclonal antibody; AC88, anti-hsp90 monoclonal antibody; PR6 and PR22, anti-progesterone receptor antibodies; Ab-1, anti-actin antibody; CsA, cyclosporin A; PPIase, peptidylprolyl cis–trans isomerase.

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FKBP59 homologue complexed with hsp70 and hsp90, suggesting that the association between immunophilins and heat shock proteins has been highly conserved throughout evolution. We further explored the oligomeric composition of the FKBP59 and heat shock protein complex. It seems that actin and a new protein, p63, are also involved in this complex. This 63-kDa protein was only retained by the FKBP59–hsp90 complexes in the absence of FK506 or rapamycin. This suggests that the immunosuppressants may modify the state of assembly or disassembly of the FKBP59–heat shock protein complexes.

MATERIALS AND METHODS

Materials. HEPES, Tris, glycine, glycerol, PMSF, pepstatin A, RPMI-1640, L-glutamine, and antimycotic and antibiotic mixtures were purchased from Sigma. Affi-Gel 10, SDS, acrylamide, bisacrylamide, TEMED, ammonium persulfate, nitrocellulose paper, molecular weight standards, alkaline phosphatase-conjugated goat anti-mouse IgG, and color development reagents for the alkaline phosphatase assay were from Bio-Rad. Leupeptin and aprotinin were from Boehringer Mannheim. Fetal calf serum was purchased from Gibco. Yeast extract and trypticase peptone were from Becton Dickinson. [^{3}H]FK506 (58 Ci/mmol) was prepared according to Harding et al. (1989). ^{125}I-Protein A was from New England Nuclear.

Cell Culture. IM9 cells were grown in RPMI-1640 containing 10% fetal calf serum, 2 mM L-glutamine, and antimycotic and antibiotic mixtures in T-75 flasks. Cells were harvested when cell density reached 10^7 cells/mL. Yeast (a: X2180-1A) (kindly provided by Dr. R. Trumbly, Medical College of Ohio) was grown in YPD medium (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose) at room temperature. The cells were harvested when the OD_{600} reached 2.

Preparation of Cytosol. The entire procedure was carried out at 4 °C. A 10-g portion of frozen uteri purchased from Pel-freeze was homogenized in 30 mL of 10 mM potassium phosphate and 10% glycerol, pH 7.4 (PG), supplemented with 0.5 mM PMSF, aprotinin (20 μg/mL), leupeptin (100 μg/mL), and pepstatin A (2 μg/mL). The homogenate was centrifuged at 800*g* for 10 min. The pellet was discarded, and the supernatant was centrifuged at 100000*g* for 1 h to yield the cytosol. Chick oviduct cytosol was prepared in 10 mM PG supplemented with various protease inhibitors according to Toft et al. (1991). IM9 cells were washed with PBS and homogenized in 3 volumes of 10 mM HEPES and 10% glycerol, pH 7.4 (HG), with a Dounce homogenizer. Yeast cell homogenate was prepared by vigorously vortexing a mixture of cells, glass beads, and HG + protease inhibitors in a 1:1:2 ratio. The tissue or cell homogenates were further centrifuged at 100000*g* to generate a clear supernatant containing the cytosol.

Preparation of Affinity Matrix. Coupling of anti-FKBP59 monoclonal antibody (EC1) or nonimmune antibody to affigel 10 was performed according to Tai et al. (1986). FK506- and rapamycin-based affigel 10 were prepared according to Fretz et al. (1991).

SDS–PAGE and Immunoblot Analysis. SDS–polyacrylamide gel electrophoresis was performed on 12.5% slab gels according to Laemmli (1970). Proteins separated on SDS gels were transferred to nitrocellulose paper for 6–16 h at 0.2 A with cooling. The blots were blocked with 1% BSA in 20 mM Tris and 0.15 M NaCl + 0.02% Tween-20, pH 7.4 (TBST + BSA) for 1 h and further incubated with the first antibody in TBST + BSA overnight. To detect FKBP59, the supernatant of EC1 hybridoma cells was used at 10× dilution. To detect hsp90 and hsp70, purified antibodies AC88 (Schuh et al., 1985) and N27 (Welch & Suhan, 1986) were diluted to 5 and 1 μg/mL, respectively (N27 was kindly provided by Dr. W. J. Welch, University of California, San Francisco). PR6 and PR22 (Sullivan et al., 1986), diluted to 2 μg/mL, were used to detect rabbit and chick oviduct progesterone receptors, respectively. The blots were further incubated with alkaline phosphatase-conjugated goat anti-mouse IgG at 1000× dilution for 2 h. Antigen was visualized by adding color development reagents according to the manufacturer's instructions. Anti-actin antibody (JLN-20) (Lin, 1982) was purchased from Oncogene Science. To detect actin, the antibody was diluted 500× with 3% milk in TBST. Because JLN-20 is an IgM, alkaline phosphatase-conjugated goat anti-total mouse Ig was used as the second antibody. Since AC88 does not recognize hsp90 in yeast, the identity of yeast hsp90 was verified by an anti-peptide antibody to yeast hsp90 (a generous gift from Dr. S. Lindquist, University of Chicago) (Picard et al., 1990). The immunoblot was blocked with 3% milk in TBST and incubated with the first antibody dilution at 500× in the same milk solution. The blots were further incubated with ^{125}I-protein A, followed by autoradiography.

[^{3}H]FK506 Binding Assay. Rabbit uterine cytosol (0.2 mL) in PG was incubated with 100 μL of either EC1–affigel 10 or nonimmune affigel 10 for 3 h at 4° C. Nonadsorbed proteins were removed with three washes of 10 mM HEPES, 10% glycerol, and 0.5 M KCl (pH 7.4) (1 mL/wash). Immunomatrices were resuspended in 100 μL of 10 mM HEPES (pH 7.4) that contained 34 nM [^{3}H]FK506 + 34 μM unlabeled FK506, rapamycin, or CsA; then, they were incubated for 2 h at 4° C. Free ^{3}H-ligand was eliminated by washing with 10 mM HEPES and 0.1 M KCl (pH 7.4). Bound [^{3}H]FK506 was eluted with 300 μL of 50 mM diethylamine in 10 mM HEPES (pH 10.5). Half of the eluate was counted with Scintiverse IL, and the second half was analyzed by SDS–PAGE followed by silver staining.

RESULTS AND DISCUSSION

Binding of [^{3}H]FK506 to Rabbit Uterine p59. Although p59 was first isolated from rabbit uterus, subsequent studies establishing it as the immunophilin FKBP59 employed only human lymphocytes and bovine thymus. Therefore, it was important to determine if rabbit uterine p59 also bound the immunosuppressants. Accordingly, [^{3}H]FK506 binding assays were performed. As shown in Figure 1A, substantial [^{3}H]FK506 was retained by p59–EC1–affigel 10 when compared to the nonimmune control matrix. The retained radioactivity was completely displaced by excess FK506 and rapamycin, whereas the same concentration of CsA had no effect. As shown in Figure 1B, only p59 was eluted from the resin. Previous work ruled out the possibility that EC1 interacts with [^{3}H]FK506 (Tai et al., 1992). Thus, we conclude that p59 isolated from rabbit uterus is an FK506- and rapamycin-binding protein.

Isolation of Nontransformed PR Complexes by FK506 and Rapamycin Affinity Matrices. Previous work showed that FKBP59 (p59), hsp90, hsp70, and GR copurified on an FK506- or rapamycin-based matrix. Since p59 was first identified as a nonsteroid binding subunit associated with PR in rabbit uterus (Tai & Faber, 1985), it was important to determine if the immunosuppressant–p59 interaction involved nontransformed PR. Rabbit uterine and chickn oviduct cytosol were incubated with affigel 10 conjugated with either FK506 or ethanolamine. Na_2MoO_4 (10 mM) was included in the

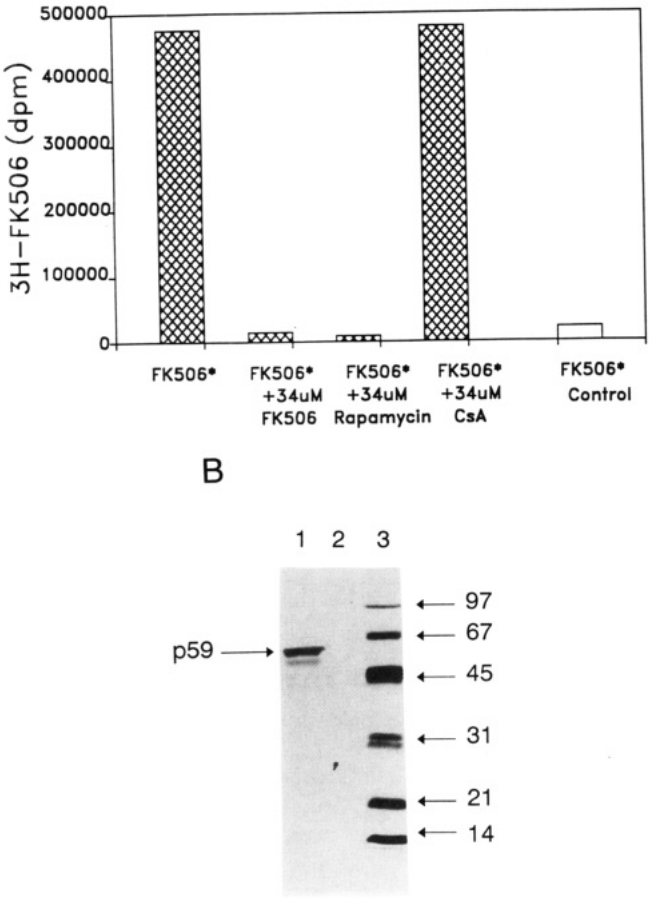

FIGURE 1: Binding of [^{3}H]FK506 to rabbit uterine p59. Panel A Binding specificity. Rabbit uterine cytosol was incubated either with EC1–Affi-Gel 10 (crosshatched bars) or nonimmune Affi-Gel 10 (open bars). P59 was purified and an [^{3}H]FK506 binding assay was performed as described. Binding specificity was determined by including a 1000-fold excess of unlabeled FK506, rapamycin, or CsA. Panel B Silver-stained SDS gels. Lane 1, sample eluted from EC1–Affi-Gel 10; lane 2, blank; lane 3, molecular weight standards.

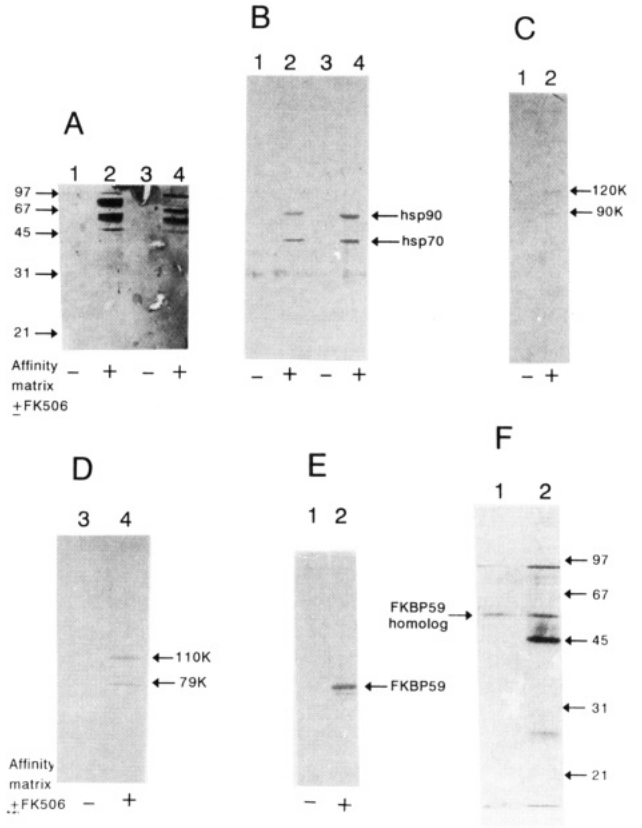

FIGURE 2: Affinity purification of nontransformed PR. Panels A–E Isolation of the components of nontransformed PR by FK506 matrix. Aliquots (0.5 mL) of rabbit uterine cytosol (lanes 1 and 2) and chick oviduct cytosol (lanes 3 and 4) were incubated with 40 μL of Affi-Gel 10 conjugated with (lanes 2 and 4) or without (lanes 1 and 3) FK506. The matrices were extensively washed with 100 mM PG + 10 mM Na_2MoO_4, and the proteins were eluted with 1 mM FK506 and subjected to SDS–PAGE followed by immunoblot analysis. Panel A, silver stain; panels B–E, immunoblot analysis. The blots were probed with AC88 + N27 (panel B), PR6 (panel C), PR22 (panel D), and EC1 (panel E). Panel F Identification of chicken FKBP59 homologue by rapamycin affinity purification. A 0.5-mL aliquot of chicken oviduct cytosol was incubated with 40 μL of rapamycin–Affi-Gel 10. The matrix was washed either with buffer containing 0.6 M KCl + 0.02% Triton X-100 (lane 1) or with 100 mM PG (lane 2). The adsorbed proteins were eluted with 1 mM rapamycin and analyzed by SDS–PAGE and silver staining.

incubation to stabilize the receptor complex. Nonadsorbed material was removed by washing with 100 mM PG and 10 mM Na_2MoO_4, and the adsorbed proteins were eluted with 1 mM FK506 or rapamycin. As shown in Figure 2A, silver staining the mini SDS gel revealed three major proteins (59, 70, and 90 kDa) adsorbed by the FK506 matrix. No protein was detected in material eluted from the control affinity matrix (ethanolamine).

Immunoblotting with antibodies N27 and AC88 revealed that the 70- and 90-kDa proteins were hsp70 and hsp90, respectively (Figure 2B). The presence of PR was verified by anti-PR antibodies (Sullivan et al., 1986). In eluates of rabbit uterine cytosol, two proteins of 120 and 92 kDa were recognized by antibody PR6 (Figure 2C). This antibody recognizes only the B form (120 kDa) of mammalian PR, and the 92-kDa protein probably represents a proteolytic product of PR. These results are consistent with our previous studies showing that rabbit uterine PR is extremely susceptible to proteolysis (Tai & Faber, 1985). PR22 recognized the A (110 kDa) and B (79 kDa) forms of chick oviduct PR upon immunoblotting (Figure 2D). An association of the avian PR with an FKBP59 homologue had not been observed previously (Toft et al., 1991). Whether this is a predominant form for this receptor remains unknown. The presence of p59 (FKBP59) in rabbit uterus was verified by EC1 antibody (Figure 2E). The existence of an FKBP59-like protein in chick oviduct has not been established because none of the anti-FKBP59 antibodies interact with nonmammalian proteins. To establish the identity of FKBP59 in chicken, rapamycin affinity purification was performed. As shown in Figure 2F, a single 56-kDa protein was detected following stringent washing with 0.6 M KCl and 0.02% Triton X-100 (lane 1). Several other proteins, including hsp90, hsp70, and a 45-kDa protein, were visualized after a milder wash with 100 mM PG (lane 2). These results suggest that the 56-kDa protein is an FKBP, directly interacting with rapamycin. This may be the chicken FKBP59 homologue.

Interaction of Immunophilins and Heat Shock Proteins May Involve Actin and Other Proteins. Rapamycin and FK506 affinity chromatography of rabbit uterine and chick oviduct cytosol using relatively mild washing conditions allowed us to detect proteins associated with immunophilins. As shown in Figure 2, panels A and F, we consistently observed a 45-kDa protein and several other proteins copurifying with the FKBP59–heat shock protein complexes. The two major proteins, FKBP59 and a 45-kDa protein, were retained by the rapamycin affinity matrix under stringent washing conditions including 0.6 M KCl and 0.02% Triton X-100 in the washing buffer (Figure 3, panels A and B, lane 1). With milder washing

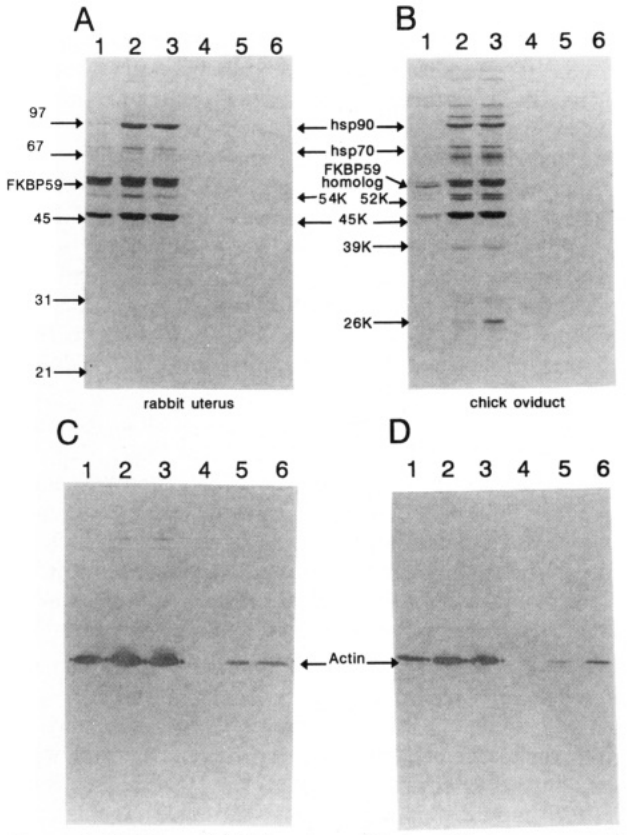

FIGURE 3: Isolation of FKBPs and associated proteins by a rapamycin-based matrix. Aliquots (0.5 mL) of rabbit uterine cytosol (panels A and C) or chick oviduct cytosol (panels B and D) were incubated with 40 μL of rapamycin–Affi-Gel 10 (lanes 1–3) or Affi-Gel 10 only (lanes 4–6). The affinity matrix was either stringently washed with 10 mM PG containing 0.6 M KCl and 0.02% Triton X-100 (lanes 1 and 4) or mildly washed with 100 mM PG in the absence (lanes 2 and 5) or presence (lanes 3 and 6) of 10 mM Na_2MoO_4. Adsorbed proteins were eluted with 1 mM rapamycin and subjected to SDS–PAGE. Panels A and B, silver stain; panels C and D, immunoblot for actin.

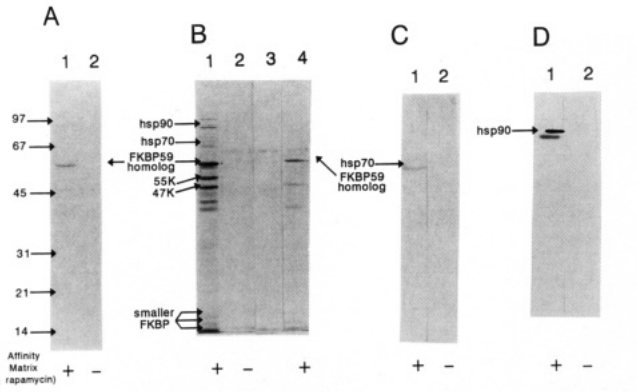

FIGURE 4: Affinity purification of yeast immunophilin heat shock protein complexes. Yeast cytosol in HG (1 mL) was incubated with rapamycin–Affi-Gel 10 (lane 1) or Affi-Gel 10 only (lane 2). The matrix was washed with 100 mM PG and the adsorbed proteins were eluted and subjected to SDS–PAGE. Panel A, Coomassie Blue stain; panel B, silver stain; lane 3 of panel B, blank; lane 4 of panel B, material eluted from rapamycin matrix by stringent washing with 0.6 M KCl + 0.02% Triton X-100; panel C, immunoblot with N27; panel D, immunoblot with anti-yeast hsp90 antibody.

(100 mMPG), hsp90, hsp70, a 54-kDa protein in rabbit or a 52-kDa protein in chicken, and other proteins of lower molecular mass were observed (lane 2). Molybdate had no effect on the intensity of the bands for the majority of proteins with the exception of a chick oviduct 26-kDa protein (Figure 2F; Figure 3, panels A and B, lanes 2 and 3).

Koyasu et al. (1986) reported that hsp90 interacts with actin, and we suspected that the 45-kDa protein might be actin. As shown in Figure 3, panels C and D, immunoblotting with anti-actin antibody revealed that actin copurified with FKBP59 from the rapamycin matrix (lanes 1–3). Although small amounts of actin were eluted from the control matrix (lanes 5 and 6), two observations suggest that actin was selectively adsorbed by the rapamycin matrix. First, as shown in Figure 3, panels C and D, the intensity of the actin band is significantly greater in material isolated from the rapamycin matrix than from the control matrix. Second, adsorption of actin to the control matrix was eliminated by stringent washing with 0.6 M KCl and 0.02% Triton X-100. On the other hand, FKBP59 and actin copurified from the rapamycin matrix even after vigorous washing, suggesting that actin either is a rapamycin-binding protein or is bound to FKBP59. The former does not seem likely, because the sequence of actin does not resemble any of the FKBPs (McHugh & Lessard, 1988). Recently, Miyata and Yahara (1991) showed that the inactive GR (8S form) binds actin filaments through hsp90. Earlier, data from the same laboratory (Nishida et al., 1986) suggested that the hsp90 actin binding was inhibited by calmodulin and Ca^{2+}. Callebaut et al. (1992) and Massol et al. (1992) suggested that the C-terminus of p59 bears a calmodulin binding consensus sequence. From the data of our study, we suggest that p59 directly interacts with actin. Thus, it is quite possible that the hsp90–actin complex, described by Nishida et al. (1986), involves p59. Our present conclusion is consistent with recent observations of Massol et al. (1992). They found that purified rabbit p59 is retained by a calmodulin affinity column in the presence of Ca^{2+}, suggesting p59 (FKBP59) is a calmodulin-binding protein. It is also reasonable to speculate that calmodulin may interact with the hsp90–p59–actin complex in a Ca^{2+}-dependent manner, which provides a potential control mechanism for transporting steroid receptors or other regulatory proteins to their sites of action.

Interaction of Immunophilins and Heat Shock Proteins in Yeast. Both immunophilins and heat shock proteins are highly conserved. The finding that these two classes of protein complex with each other in mammalian cells prompted us to investigate whether a similar situation exists in yeast. Yeast cytosol was incubated with rapamycin affinity matrix. The matrix was extensively washed with 100 mM PG, and the adsorbed proteins were eluted with 1 mM rapamycin. As shown in Figure 4, panels A and B, a major protein of 62 kDa and several other proteins were retained by the rapamycin matrix (lanes 1 and 2). Immunoblot analyses using N27 and an anti-peptide antibody to yeast hsp90 revealed that hsp70 and hsp90 were in the rapamycin eluant. These data suggest that these two heat shock proteins associate with immunophilins in yeast. To identify the FKBP59 homologue in yeast, affinity purification was performed with stringent washing (0.6 M KCl and 0.02% Triton X-100). Three proteins of 62, 47, and 37 kDa (Figure 4B, lane 4) were detected. The 62-kDa protein is the major band and the 47- and 37-kDa proteins are the minor bands, suggesting taht the 62-kDa protein is a yeast FKBP59 homologue. At the present time, we do not know if the 47-kDa protein is actin since the anti-actin antibody described above does not recognize yeast actin. The 47- and 37-kDa proteins may be immunophilins or proteins associated with yeast FKBP59.

Identification of a 63-kDa Protein Whose Binding to FKBP59 Is under Immunosuppressant Control. The ubiquity of immunophilin binding both FK506 and heat shock proteins

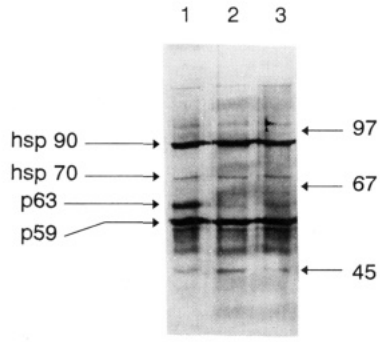

FIGURE 5: Identification of an FK506- and rapamycin-sensitive protein (p63) associated with FKBP59. IM9 cytosol (1 mL) in 10 mM Hepes, 1 mM $CaCl_2$, 2 mM $MgCl_2$ and 10% glycerol (pH 7.4) was incubated with buffer alone (lane 1), 20 μM FK506 (lane 2), or 20 μM rapamycin (lane 3) overnight and further incubated with EC1–Affi-Gel 10 for 3 h at 4 °C. The immunomatrix was washed five times with 1 mL of 50 mM Tris-HCl and 0.1 M NaCl, pH 7.4. The retained proteins were eluted with 50 mM diethylamine (pH 10.4) and analyzed by SDS–PAGE and silver staining.

leads us to inquire about the effects of FK506 and rapamycin on the subunit composition of the oligomeric complex. To address this issue, we immunopurified FKBP59 and its associated proteins in the absence and presence of the drug. Cytosol (1 mL) isolated from IM9 lymphocytes was incubated with EC1 affinity matrix either in the absence or in the presence of 20 μM FK506 or rapamycin. After mild washing (50 mM Tris + 100 mM NaCl, pH = 7.4), the adsorbed proteins were eluted with 50 mM diethylamine (pH = 10.4) and analyzed by SDS-PAGE. As shown in Figure 5, silver staining revealed that FKBP59 and hsp90 were the two major proteins and hsp70 was a minor protein adsorbed by the EC1 antibody. The intensities of these three protein bands were not affected by preincubation with FK506 or rapamycin. We also noted a 63-kDa protein (p63) adsorbed by the EC1 immunoaffinity matrix. Interestingly, the intensity of the p63 band was drastically reduced by preincubation of the cells with FK506 or rapamycin. These results suggest that binding of the immunosuppressants to FKBP59 does not affect its interaction with hsp90, hsp70, or the EC1 antibody. Preincubation of the drugs does affect the association with p63, implying that p63 interacts with FKBP59 via the immunosuppressant-binding site. However, indirect effects of the ligand in inducing structural modifications cannot be ruled out. Perdew and Whitelaw (1991) reported a 63-kDa protein that copurified with hsp90, hsp70, FKBP59, and p50 by anti-hsp90 antibody. Subsequently, p50 was identified as the protein associated with the hsp90–pp60^{src} complex (Whitelaw et al., 1991). Smith et al. (1992) also reported a 60-kDa protein which reassociated with hsp90 and hsp70 during reconstitution of chick oviduct progesterone receptor complexes in rabbit reticulocyte lysates. Immunoblotting using the anti-60-kDa antibody F5 (provided by D. Smith) revealed that F5 did recognize a 60-kDa protein immunoprecipitated by the EC1 antibody. However, the intensity of the immunodetected p60 was not affected by FK506 or rapamycin (data not shown). This suggests that our p63 is not the p60 identified by Smith et al. (1992). At present, we do not know if p63 of this study is the same protein observed by Perdew and Whitelaw (1991). Nevertheless, all three proteins are closely associated with heat shock proteins, suggesting that they may be structurally or functionally related.

Because p63 is displaced by immunosuppressants, p63 becomes a potential FK506 and rapamycin target protein. The dependency of the association of p63 with FKBP59 upon the presence or absence of drugs leads us to specualte that two distinct mechanisms may possibly be in operation. First, the binding of FK506 or rapamycin may change the conformation of p59, thus disrupting the interaction of p59 with the 63-kDa protein. On the other hand, there may be one or several natural ligands that are structurally related to FK506 and rapamycin. Under normal physiological conditions, p63 and p59 would bind to different domains of the natural ligand which serves as the molecular glue holding p63, p59, hsp70 and hsp90 together in a large heteromeric complex. In crude cell extracts, the addition of exogenous FK506 and rapamycin drives the dissociation of p63 from the p59 complexes. In support of this concept, Freidman and Weissman (1991), using the cyclophilin C fusion protein as an affinity ligand, identified a 77-kDa protein associated with cyclophilin C in the absence of CsA. They also speculated on the existence of a CsA-related naturally occurring ligand *in vivo*. On the other hand, in the presence of the drugs, Liu et al. (1991) identified calcineurin as the common target of FKBP12 and cyclophilin. Thus, p63 and p77, detected in the absence of the immunosuppressant(s), may be responsible for modulating the native functions of immunophilins, whereas calcineurin, identified in the presence of FK506 and rapamycin, is involved in the signal transduction events blocked by CsA and FK506.

Speculation on the Oligomeric Composition and Function of p59 Complexes. Although the binding of hsp90, hsp70, and p59 to steroid hormone receptors has been known for years, the biochemical processes involved in the formation of this complex remain obscure. Our finding that the association between immunophilins and heat shock proteins is ubiquitous and highly conserved may provide some clues in studying the assembly of receptor complexes. A viable approach might involve the physiological functions of these two families of proteins. Using synthetic peptides as substrates, most of the immunophilins were identified as peptidylprolyl cis–trans isomerases (PPIases or rotamases) (Harding et al., 1991). Although the physiological relevance of this activity is unclear, it was proposed that proline cis–trans isomerization may be essential for protein folding *in vivo*. This is based on the observation that polypeptides generated from mRNA translation contained prolines all in the trans configuration, and cis-proline bonds are occasionally found in mature, globular proteins (Fisher & Schmid, 1990; Jaenicke, 1991). Heat shock proteins, by definition, play important roles in allowing cells to cope with environmental abuses such as heat shock and chemical poisoning (Lindquist & Craig, 1988). Recently, the concept of "molecular chaperones" has been advanced (Pelham, 1986; Ellis, 1987). It suggests that heat shock proteins serve to recognize and stabilize partially folded proteins during polypeptide maturation, assembly, and transport to sites of action. To summarize the proposed functions of immunophilins and heat shock proteins, it is reasonable to speculate that FKBP59, in association with hsp90 and hsp70, forms a core structure of a molecular chaperone entity responsible for recognizing, folding, processing, assembling, and trafficking target proteins at or near the end of translation. Currently, we know of only two potential targets for this molecular chaperone: steroid receptors and HRI. These two important regulatory proteins share no structural and functional similarities. They exist in different cells and tissues (Chen et al., 1991), suggesting the specificity of the chaperone target resides in the state of differentiation. Future work should focus on searching for target proteins in different tissues and species and their various physiological states. For example, one should look for proteins associated with the p59–hsp90–hsp70 complex in activated T lymphocytes during proliferation, which is known to be inhibited by FK506 and rapamycin. The

identity of these proteins may shed some light on the mechanism of FK506 and rapamycin-mediated immunosuppression.

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