Potentiation of Progesterone Receptor-Mediated Transcription by the Immunosuppressant FK506†

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ABSTRACT: The nontransformed steroid receptors contain several non-steroid binding proteins, such as hsp90, hsp70, and p59. Recently, we and others have shown that p59 (FKBP59) is an immunophilin which binds two potent immunosuppressants, FK506 and rapamycin. This raises the possibility that FK506 or rapamycin may modify the function of steroid receptors. To develop this line of inquiry, we chose a yeast model system in which the human progesterone receptor form B (hPR-B) was cotransformed with a reporter gene. The reporter contains two copies of a progesterone response element/glucocorticoid response element (PRE/GRE) upstream of the CYC1 promoter which are linked to the lacZ gene of *Escherichia coli*. We found that FK506 potentiated the ability of progesterone in activating transcription. To gain insight into the mechanism of FK506's regulation of PR action, we questioned whether calcineurin is involved, because it has been shown that FK506 is a specific inhibitor of calcineurin, a Ca^{2+}- and calmodulin-regulated phosphatase, through the formation of an FKBP12–FK506–calcineurin–calmodulin complex. We found that 15-*O*-desmethyl-FK520, an FK506 analogue which is an excellent ligand of FKBP12, but a poor inhibitor of calcineurin, failed to induce the same effect as FK506. We also found that calmidazolium, a calmodulin antagonist, mimicked FK506's action. Furthermore, immunoblot analysis showed that both FK506 and calmidazolium potentiated the effect of progesterone in decreasing the mobility of hPR-B upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). This suggests that FK506 and calmidazolium may cooperate with progesterone in increasing the level of hPR-B phosphorylation. Taken as a whole, we conclude that FK506 potentiates the transcriptional activity of PR, perhaps by protecting it from dephosphorylation. Thus, we suggest that calcineurin may play an important role in regulating PR function. Finally, our data eliminate FKBP12 as the mediator for the FK506 action observed in this study, because FK506 potentiation of progesterone receptor transactivation was preserved in FKBP12-deficient yeast mutant cells.

Steroids play important roles in the regulation of growth, differentiation, reproduction, and metabolism. The response to steroid hormones is mediated by specific intracellular receptors located in target cells. In the absence of steroids, the unliganded receptors are associated with several non-steroid binding proteins, including (hsp90),[1] hsp70, and p59 (Sanchez et al., 1985; Kost et al., 1989; Tai et al., 1986). Following steroid binding, receptors become phosphorylated, and subsequently bind to specific DNA sequences as dimers. Consequently, gene expression is regulated at the transcription level [for reviews, see Evans (1988), Beato (1990), and Carson-Jurica et al. (1990)]. A growing body of evidence suggests that the steroid-induced receptor phosphorylation is closely related to the modified receptor function [for reviews, see Auricchio (1989), Moudgil (1990), and Orti et al. (1992)]. However, the molecular details of the regulation of steroid receptor phosphorylation remain obscure, in part because the identities of the relevant kinases and phosphatases *in vivo* have not been clarified.

Recently, data from our laboratories as well as several others demonstrated that p59, one of the comon components of nontransformed steroid receptor complexes, is an immunophilin that binds the potent immunosuppressants FK506 and rapamycin (Tai et al., 1992; Lebeau et al., 1991; Yem et al., 1992; Peattie et al., 1992). Thus, p59 may be referred as FKBP59. The biological relevance of this finding is not clear; however, these two drugs may assist in the characterization and identification of the kinases and phosphatase which regulate steroid receptor function. This assumption is based on our current understanding of the mechanism of immunosuppressant action. FK506 and rapamycin, structurally related macrolides, share the same binding proteins (referred as FKBPs), but function as reciprocal antagonists; this has led to the suggestion that FK506–FKBP and rapamycin–FKBP may associate with or modulate different target proteins which are responsible for their distinct immunosuppressive activities (Schreiber, 1991; Schreiber & Crabtree, 1992; Sigal

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[1] Abbreviations: hsp90, heat shock protein 90; hsp70, heat shock protein 70; FKBP, FK506 binding protein; PR, progesterone receptor; GR, glucocorticoid receptor; PRE/GRE, progesterone response element/glucocorticoid response element; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ONPG, *o*-nitropenyl β-D-galactopyranoside; PMSF, phenylmethanesulfonyl fluoride; IgG, immunoglobulin G; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; RK, receptor kinase.

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& Dumont, 1992). Now, it is clear that calcineurin, a Ca^{2+}- and calmodulin-dependent phosphatase, is involved in FK506 action (Liu et al., 1991). FK506 complexes with FKBP12 and induces the formation of an FKBP12–FK506–calcineurin A and B–calmodulin complex, resulting in the inhibition of the phosphatase activity of calcineurin. This inhibitory effect is specific for FK506, since rapamycin is unable to induce the formation of such a pentamer complex. Studies of FK506 analogues further demonstrated that there is a strong correlation between the ability of these analogues, when bound to FKBP12, to inhibit the phosphatase activity of calcineurin and their ability to inhibit transcriptional activation of NF–AT (Liu et al., 1992). The latter is a T-cell-specific transcriptional factor that regulates interleukin-2 expression. In contrast, recent studies from yeast suggest that a homologue of phosphatidylinositol 3-kinase is the target of the rapamycin–FKBP12 complex (Kunz et al., 1993). Similar results in mammalian cells are lacking. However, it has been reported that in T-cells, rapamycin inhibits the p70 S6 kinase activity stimulated by interleukin-2 (Chung et al., 1992; Price et al., 1992). In conclusion, FK506 and rapamycin provide powerful tools in establishing the involvement of calcineurin or certain kinases in signaling pathways.

In our present study, first we observed an FK506 effect in potentiating progesterone transactivation in yeast cells expressing hPR-B and a reporter gene. The effects of 15-*O*-desmethyl-FK520 and rapamycin on hPR-B are also compared. The former compound is an FK506 analogue, which has been considered as an avid ligand of FKBP12 but a poor inhibitor of calcineurin phosphatase (Liu et al., 1992). The specificity of FK506 would indicate a role of calcineurin in regulating the transcriptional activity of PR. The putative involvement of calcineurin was also tested by calmidazolium, a calmodulin antagonist, since calcineurin is a calmodulin-regulated phosphatase. Furthermore, if PR function is indeed modulated by a phosphatase, such as calcineurin, one would expect that hPR-B phosphorylation is affected by FK506 and calmidazolium. Results from these studies will provide molecular insight in understanding the mechanism of non-ligand regulation of PR in the heteromeric complex state. The roles of FKBP59 and calcineurin in the early events of progesterone signaling are thus suggested.

MATERIALS AND METHODS

Reagents. Yeast nitrogen base and trypticase peptone were purchased from Difco and Becton Dickinson, respectively. Glass beads (0.5 mm in diameter) were from Biospec Products. Calmidazolium, *o*-nitrophenyl β-D-galactopyranoside (ONPG), phenylmethanesulfonyl fluoride (PMSF), and pepstatin A were obtained from Sigma. R5020 was from New England Nuclear. AT414, a monoclonal antibody which recognizes huamn PR, was purchased from Affinity Bioreagents. Alkaline phosphatase-conjugated goat anti-mouse IgG, alkaline phosphate color development reagents, nitrocellulose paper, and all the reagents for SDS–PAGE were obtained from Bio-Rad.

Yeast Strains and Cell Culture. The yeast strain BJ3505 containing hPR-B (YEphPR-B) and the reporter (YRp PC3GS+) plasmids has been described by Vegeto et al. (1992). In this yeast system, hPR-B is expressed under the control of the copper-response CUP1 promoter. The reporter gene contains two copies of a PRE/GRE consensus sequence upstream of the CYC1 promoter which is linked to the lacZ gene of *Escherichia coli*. Cells were grown at 30 °C in medium containing 2% glucose, 0.5% trypticase peptone, and 6.7 g/L yeast nitrogen base. JHY2-1b, a rapamycin resistance strain which lacks FKB12, was produced by disruption of the FKBP12 gene (Heitman et al., 1991). This mutant strain was generously provided by M. Hall (University of Basel, Switzerland). Introduction of the hPR-B expression vector and the YRpPC3GS+ reporter into JHY2-1b was carried out following the lithium acetate transformation according to Ito et al. (1983). Mutant cells expressing the foreign proteins were selected by synthetic medium deficient in tryptophan and uracil according to Wickersham (1951) except that twice the recommended levels of the amino acids were used. The selective medium was also supplemented with 0.17% yeast nitrogen base and 0.25% trypticase peptone.

Transcription Assay. Cells were harvested for the transcription assay when the cell density reached an OD_{600} of 0.15–0.2. Two milliliters of cells was preincubated with or without 10 μM drugs as designated, for 90 min. Then, 100 μM $CuSO_4$ was added to initiate the expression of hPR-B. At the same time, 0–1000 nM R5020 was added to the cells to initiate β-galactosidase expression. After 2 h of incubation, cells were washed with H_2O, and cell extracts were prepared by vigorously vortexing the cells with 100 mg of glass beads in 250 μL of extraction buffer containing 10 mM Na_2HPO_4 and 1 mM PMSF (pH 7.5) followed by centrifugation. The β-galactosidase assay was initiated by adding ONPG to cell extracts and further incubated for 5 min at 37 °C. The assay mixture contained 5–9 μg of cell extracts, 0.88 mg/mL ONPG, 1 mM $MgCl_2$, and 45 mM β-mercaptoethanol in 0.1 M Na_2HPO_4, pH 7.5, in a total volume of 300 μL. The reaction was stopped by adding 500 μL of 1 M Na_2CO_3. Readings at OD_{420} were recorded and normalized for protein. The transcriptional activity of each sample was expressed as a percentage of the maximal response.

Immunoblot Analysis. Twenty milliliters of cells (BJ3505) was incubated with effectors, and cell extracts were prepared as described above; 50 mM NaF, 10 mM Na_2MoO_4, and 10 mM EDTA were included in the extraction buffer to prevent protein dephosphorylation. Pepstatin A (2 μg/mL), leupeptin (100 μg/mL), and aprotinin (100 μg/mL) were added to prevent proteolysis. Fifty micrograms of cell extracts was applied into each well of a 10% polyacrylamide SDS-gel. Following electrophoresis, the proteins were transferred onto nitrocellulose paper at 0.2 A overnight with cooling. The nitrocellulose was incubated with the anti-hPR monoclonal antibody AT414 at 5 μg/mL in Tris-buffered saline containing 1% BSA and 0.05% Tween-20 overnight. The immunogen of AT414 is a 15-residue peptide which was synthesized corresponding to the amino acid sequence residues of 533–547 of hPR-B, found near the DNA binding domain (Traish & Wotiz, 1990). Following incubation with the second antibody, alkaline phosphatase-conjugated goat anti-mouse IgG, hPR-B was detected by adding color development reagents according to the manufacturer's protocol.

RESULTS AND DISCUSSION

Potentiation of PR-Dependent Transactivation by FK506. To determine whether FK506 affects PR-mediated transactivation, we tested the effect of FK506 on hPR-B transactivation in a reconstituted transcription system in yeast strain BJ3505 (Vegeto et al., 1992). As shown in Figure 1, β-galactosidase activity was augmented following addition of increasing concentrations of R5020, the progesterone agonist. The response saturated between 100 and 1000 mM R5020. A 90 min preincubation of the cells with 10 μM FK506 alone did not induce β-galactosidase activity. However, at sub-

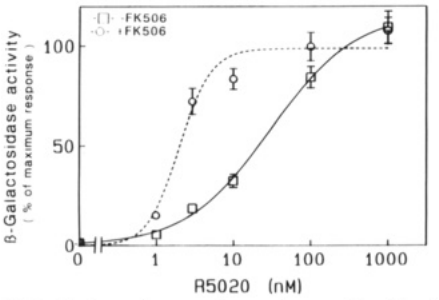

FIGURE 1: Potentiation of progesterone transactivation by FK506. Two milliliters of BJ3505 expressing hPR-B and containing a PR-responsive reporter gene was preincubated with (O---O) or without (□—□) 10 μM FK506 at 30 °C for 90 min; 100 μM $CuSO_4$ and increasing concentrations of R5020 was added to initiate the expression of hPR-B and in subsequent induction of β-galactosidase, respectively. After 2 h, cell extracts were prepared, and the β-galactosidase activity in each sample was measured. The transcriptional activity of hPR-B is expressed as a percentage of the maximal response. Means and standard errors for nine separate experiments are shown.

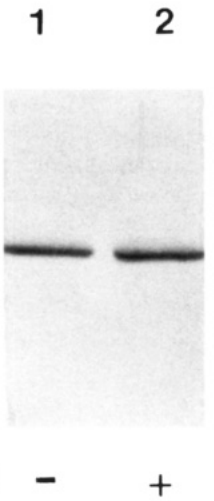

FIGURE 2: FK506 does not change the level of hPR-B expressed in yeast. Twenty milliliters of yeast cells was grown in the absence (lane 1) and presence of 10 μM FK506 (lane 2) and 3.5 h; 100 μM $CuSO_4$ was added at the beginning of the culture to initiate the expression of hPR-B. Fifty micrograms of cell extract was applied to each well of a 10% polyacrylamide SDS gel and subjected to electrophoresis, followed by immunoblot analysis. hPR-B was detected by anti-hPR monoclonal antiody AT414.

maximal concentrations of hormone (1–10 nM), FK506 significantly increased the efficiency of R5020 as an hPR-B agonist. It appears that FK506 increases the potency of R5020 by shifting the dose–response curve to the left without affecting the maximal response. The ED_{50} for R5020 action was estimated to be 30 ± 0.08 nM (n = 9) in the absence of FK506 and to be 2 ± 0.12 nM (n = 9) in the presence of FK506. Since FK506 did not affect the maximal response of R5020 in stimulating transactivation, the possibility that FK506 may increase the expression of hPR-B seems unlikely. Data from Western blot analysis using an anti-hPR monoclonal antibody, AT414, further confirmed this conclusion. AT414 is specific to hPR, since it does not recognize any protein in cells untreated with $CuSO_4$, which is required for the induction of hPR-B expression (data not shown). As shown in Figure 2, in the presence of $CuSO_4$, hPR-B was recognized by AT414 as a 114 kDa protein, and the intensity of hPR-B was not changed by FK506, suggesting that the expression of hPR-B was not affected by addition of FK506.

Involvement of Calcineurin in PR-Dependent Transactivation. To gain insight into the molecular mechanism of the

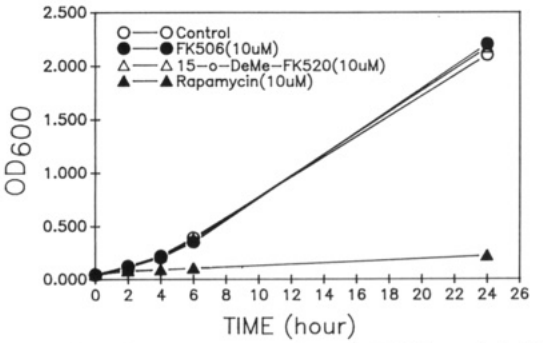

FIGURE 3: Effect of FK506, rapamycin, and 15-*O*-desmethyl-FK520 (DeFK520) on the growth of the yeast strain BJ3505. Cells were incubated with or without 10 μM FK506, rapamycin, or 15-*O*-desmethyl-FK520 for 24 h. Cell density was monitored at 0, 2, 4, 6, and 24 h.

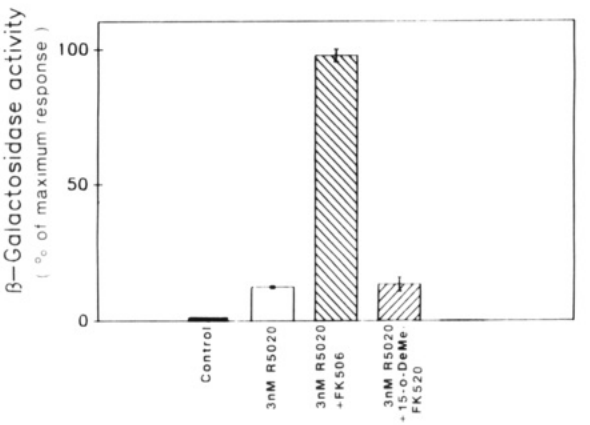

FIGURE 4: Differential effect of FK506 and 15-*O*-desmethyl-FK520 on potentiation of progesterone transactivation. Two millilters of BJ3505 was preincubated with or without 10 mM FK506 or 15-*O*-desmethyl-FK520 for 90 min; 100 μM $CuSO_4$ and 3 nM R5020 were added to initiate the expression of hPR-B and β-galactosidase, respectively. After 2 h, cell extracts were prepared, and β-galactosidase activity was measured as described above. Means and standard errors for three independent assays are shown.

potentiation of progesterone action by FK506, we questioned whether calcineurin is involved. We were unable to demonstrate the specificity of FK506 using rapamycin, since rapamycin severely suppresses BJ3505 cell growth (Figure 3). To overcome this problem, we decided to test the involvement of calcineurin using an FK506 analogue, 15-*O*-desmethyl-FK520, which binds FKBP12 but is a poor inhibitor of calcineurin (Liu et al., 1992). First, the cytotoxicity of this analogue was examined. As shown in Figure 3, 15-*O*-desmethyl-FK520, like its parental compound, does not inhibit BJ3505 cell growth. As shown in Figure 4, at a submaximal concentration of R5020 (3 nM), 15-*O*-desmethyl-FK520 failed to mimic FK506 in increasing hPR-B transcriptional activity. These data suggest a role of calcineurin involved in the FK506 action in regulating PR function.

The putative involvement of calcineurin was further examined using calmidazolium, a calmodulin antagonist (Gietzen et al., 1981), since calcineurin is a Ca^{2+}- and calmodulin-regulated phosphatase. As shown in Figure 5, calmidazolium by itself was ineffective in inducing the expression of β-galactosidase. However, calmidazolium was as effective as FK506 in enhancing the transcriptional activity of hPR-B driven by submaximal concentrations of R5020. Furthermore, no additive effect was observed at maximal concentrations of calmidazolium and FK506, suggesting that these two agents share the same mechanism or inhibit the same calmodulin-associated enzyme, such as calcineurin.

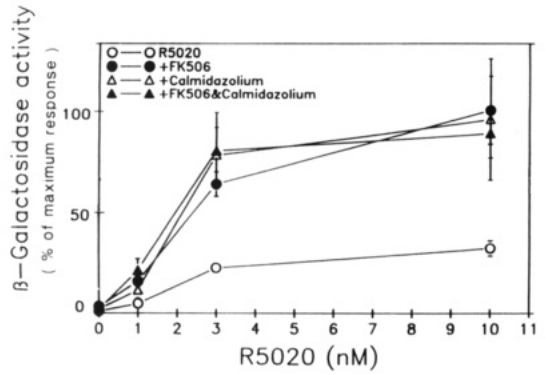

FIGURE 5: Effect of calmidazolium and FK506 on progesterone-mediated transactivation. Two milliliters of BJ3505 was preincubated with or without 10 μM FK506 or calmidazolium or a combination of the two agents for 90 min at 30 °C. Then 100 μM of $CuSO_4$ was added to initiate the expression of hPR-B. At the same time, 0–10 nM R5020 was added to initiate the expression of β-galactosidase. After 2 h of incubation, the transcriptional activity of hPR-B was assayed as described above. Means and standard errors of three independent experiments are shown.

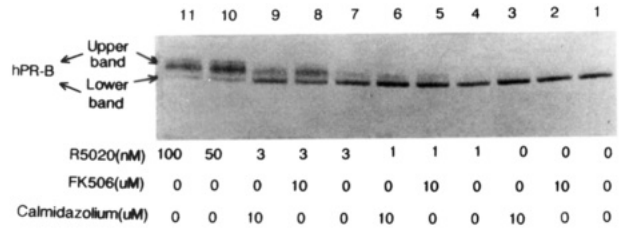

FIGURE 6: Immunoblot of hPR-B expressed in yeast under the influence of R5020, FK506, and calmidazolium. BJ3505 cells were grown and incubated with the reagents as indicated, 20 mL of cells per condition. Cell extract containing 50 μg of protein was applied into each well of a 10% polyacrylamide SDS gel and subjected to electrophoresis followed by immunoblot analysis. hPR-B was detected by anti-hPR monoclonal antibody, AT414. This experiment was performed 3 times with similar results.

Confirmation of this hypothesis requires evidence of at least one phosphoprotein in the progesterone signaling pathway, whose level of phosphorylation is increased by FK506 and calmidazolium.

Enhancement of Yeast hPR-B Phosphorylation by FK506 and Calmidazolium. Thus, it was decided to test if PR phosphorylation is modified by FK506 and calmidazolium. Because the progesterone-induced upshift of PR on an SDS–polyacrylamide gel has been accepted as a reliable and sensitive procedure to monitor steroid-induced PR phosphorylation (Sheridan et al., 1989; Chung et al., 1992), Western blot analysis was performed. As shown in Figure 6, in the absence of hormone, PR was detected as a single band migrating as a 114 kDa protein on the SDS gel (lane 1). FK506 and calmidazolium alone did not change the intensity or the mobility of hPR-B (lanes 2 and 3). In the absence of FK506, a gradual increase in the intensity of an upper, broader band was observed when the concentrations of R5020 were increased from 1 to 3 to 50 nM (lanes 1, 7, and 10). This is consistent with the previous report that progesterone-induced an upshift of yeast hPR-B on the SDS gel and the upper band is the phosphorylated form (Vegeto et al., 1992). At 1 and 3 nM R5020, the intensities of the upper band were significantly increased by 10 μM FK506 and calmidazolium (comparing lane 4 to lanes 5 and 6 and lane 7 to lanes 8 and 9). These results are quite reproducible, since the same experiment was performed 3 times with similar results. These data suggest that both FK506 and calmidazolium cooperate with R5020 in increasing the level of hPR-B phosphorylation. A net increase in the level of hPR-B phosphorylation may have been the result of the activation of a kinase or the inhibition of a phosphatase activity. Since FK506 has been identified as a specific inhibitor of calcineurin (Liu et al., 1991), it is reasonable to propose that FK506 increases the level of hPR-B phosphorylation by protecting it from dephosphorylation, perhaps by calcineurin.

We believe that the results obtained from our yeast model system are physiologically relevant, because the phosphorylation patterns of chick PR expressed either endogenously or in yeast are identical, suggesting that the kinases and phosphatases which regulate PR phosphorylation are highly conserved throughout evolution (Poletti et al., 1993).

Calcineurin homologues in yeast have recently been identified (Cyert et al., 1991; Cyert & Thorner, 1992). Two genes, CNA1 and CNA2, encode the catalytic subunit, and one gene, CNB1, encodes the regulatory subunit of yeast calcineurin. Double mutants of cna1 and cna2 and single mutants of cnb1 fail to resume growth upon the challenge of mating pheromone, α-factor, suggesting calcineurin is involved in regulating yeast mating processes (Cyert & Thorner, 1992). However, the endogenous substrates of yeast calcineurin have not been elucidated. Most recently, it was shown by Foor et al. (1992) that in wild-type yeast, recovery from mating factor is sensitive to FK506. These studies from yeast are consistent with the observation in mammalian lymphocytes that calcineurin is a target of FK506 action. The increase in phosphorylation of hPR-B expressed in yeast by FK506 may provide new clues in the identification of the native calcineurin substrates in yeast.

Eliminating the Involvement of FKBP12 in Progesterone Transactivation. FK506 does not inhibit the calcineurin phosphatase activity unless it complexes with a cognate immunophilin. To date, four species of FKBP have been identified: FKBP12, FKBP13, FKBP25, and FKBP59. Among them, FKBP12 is the most extensively studied and has been considered as the most abundant FKBP. FKBP12 has the greatest binding affinity to FK506 (K_D = 0.4 nM). The K_i of FKBP12–FK506 inhibiting the phosphatase activity of calcineurin is approximately 8 nM (Rosen et al., 1993). The latter value is almost 3 orders of magnitude less than the present estimate for the ED_{50} of FK506 in potentiating the transcriptional activity of hPR-B (6 μM) (data not shown). Thus, it seems that FKBP12 is not involved in the FK506 action observed in our present study. To confirm this hypothesis, a yeast strain, JHY2-1b, which lacks FKBP12 by disruption of FKBP12 gene (Heitman et al., 1991), was transformed with the hPR-B and the reporter plasmids. Since the growth of the mutant transformants is not inhibited by rapamycin (shown in Figure 7), it permits us to compare the effect of FK506 and rapamycin in regulating the transcriptional activity of hPR-B. As shown in Figures 8 and 9, a potentiative effect of FK506 was observed in a submaximal concentration of R5020 (3 nM), while rapamycin is totally ineffective. These data strongly support the notion that FKBP12 is not involved in the effect of FK506 in potentiating progesterone transactivation. In addition, the FK506 specificity demonstrated in this rapamycin-resistant strain further indicates that calcineurin may be involved in the FK506 effect in regulating PR function.

With FKBP12 eliminated, another candidate is FKBP13. FKBP13 has been proposed to be membrane-associated, located in the endoplasmic reticulum of cells (Fretz et al., 1991; Nielsen et al., 1992). Although it is possible that the FKBP13–FKBP complex may inhibit the phosphatase activity

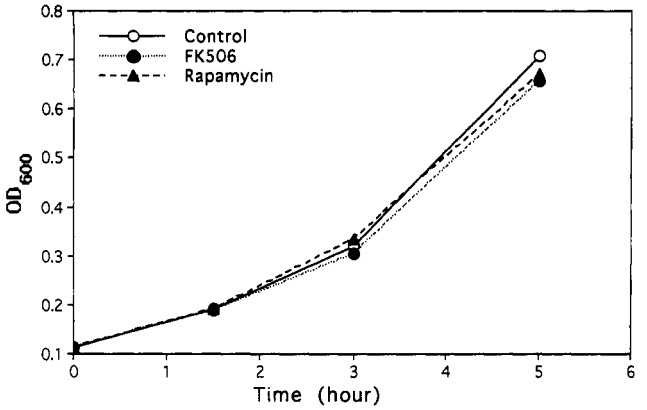

FIGURE 7: Rapamycin and FK506 do not suppress the growth of JHY2-1b, an FKBP12-deficient yeast strain. Cells were grown in the absence (○—○) and presence of 10 μM FK506 (●···●) or rapamycin (▲---▲) for 5 h. Cell density was monitored at 0, 1.5, 3, and 5 h.

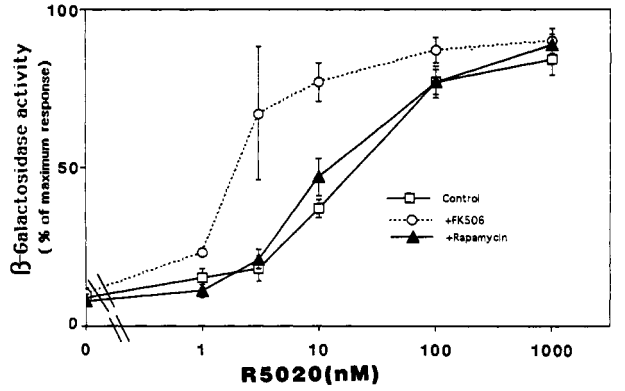

FIGURE 8: Differential effect of FK506 and rapamycin on progesterone transactivation in JHY2-1b, an FKBP12-deficient yeast strain. JHY2-1b cells expressing hPR-B and the reporter were incubated without (□—□) or with (○···○) 10 μM FK506 or (▲—▲) rapamycin for 3 h. $CuSO_4$ and various concentrations of R5020 were simultaneously added with the immunosuppressants. The transcriptional activity of hPR-B was measured as described. Means and standard errors of three independent experiments are shown.

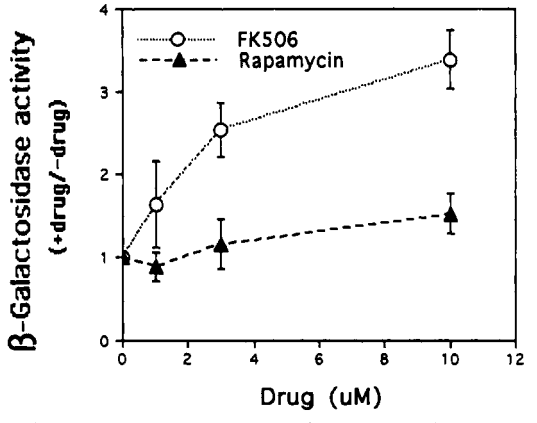

FIGURE 9: Dose–response curves of FK506 and rapamycin in potentiation of PR-dependent transcriptional activity in JHY2-1b. Cells were incubated with 0, 1, 3, and 10 μM FK506 (○···○) or rapamycin (▲---▲) for 3 h. β-galactosidase activity induced by 3 nM R5020 was assayed as described above. The ratios of the transcriptional activity of hPR-B in the presence and absence of various concentrations of FK506 or rapamycin are expressed to show the ability of the drug as a potentiation of hPR-B activity.

of calcineurin (K_i = 1.5 μM), on the basis of its cellular localization it seems unlikely that FKBP13 mediates the transcriptional potentiation effects of FK506 (Hung & Schreiber, 1992). FKBP25–FK506 may also act as an inhibitor for calcineurin (K_i = 150 nM). In addiiton, FKBP25 has a putative nuclear localization sequence. Thus, it may mediate the effects of FK506 observed in this study. However, the fact that FKBP59 is a common component of the nontransformed steroid receptor and an FKBP59–hsp90–hsp70 complex has been detected in yeast (Tai et al., 1993) makes FKBP59 an attractive candidate for mediating FK506's action of modifying the structure and function of hPR-B. Naturally, this hypothesis has to be confirmed by demonstrating that FKBP59–FK506 can form a complex with calcineurin. Attempts to establish the formation of an FKB59–FK506–calcineurin complex have failed. Difficulties may arise from weak binding between FKBP59 and calcineurin as reflected in our present estimate that the ED_{50} for FK506 in potentiating progesterone transactivation is about 6 μM. Alternatively, FKBP59 may associate with different forms of calcineurin. The primary structures and the cellular localizations of these isoforms may have limited similarity to those of calcineurin identified to date.

Speculation on the Progesterone Signaling Pathways. To summarize the data presented in this study, a speculative model which illustrates the initial steps in progesterone-mediated signaling pathways is presented. The unliganded progesterone receptor is bound by hsp70, hsp90, and FKBP59, and the signaling pathway is initiated by binding of hormone to its cognate receptor. This initiation effect is exclusively dependent on hormone, since none of the reagents tested in this study, such as FK506, calmidazolium, or okadaic acid (data on okadaic acid not shown), is able to bypass hormone in activating transcription. The hormone–receptor interaction may induce a conformational change of PR that exposes potential phosphorylation sites on the surface of the receptor. Then the receptor may be phosphorylated by a receptor kinase (RK) which is situated in the vicinty of the heteromeric complex. Subsequently, the phosphorylated PR dissociates from the hsp90–hsp70–FKBP59 complex. Either phosphorylated PR, RK, or other kinases that regulate RK kinase activity may serve as a substrate for calcineurin or its isoform. In the presence of FK506, calmodulin and calcineurin (or its isoform) bind the FK506–FKBP59 complex, resulting in the inhibition of phosphatase activity. Thus, the phosphorylation on RK or PR is locally protected by FK506. As a result, PR exists in a state of greater phosphorylation which functions as a more efficient transcription factor.

Effects of FK506 on Other Steroid Hormone Receptors. Very recently, two reports have appeared in the literature related to this area of investigation. First, Ning and Sanchez (1993) suggested that both FK506 and rapamycin enhanced GR-mediated transactivation. This common effect of FK506 and rapamycin seems to be in contradiction with the immunosuppressant literature, since FK506 and rapamycin function as reciprocal antagonists in immune cells (Sigal & Dumont, 1992) and in yeast (Heitman et al., 1991). Then, in the second paper, Hutchinson et al. (1993) stated that FK506 does not affect the functions of GR. The discrepancy existing between these two reports is due to the fact that maximal concentrations of dexamethasone were used in the second study. To explain the common effect of FK506 and rapamycin in potentiating GR-mediated transactivation, Ning and Sanchez speculated that binding of FK506 and rapamycin to FKBP59 may have a direct effect on receptor transformation, presumably by inducing receptor dissociation from the heat shock protein–FKBP59 complex. In our present study, we used 15-*O*-desmethyl-FK520 and rapamycin to demonstrate the specificity of FK506 in rapamycin-sensitive and -resistant yeast

strains, respectively. The specificity of FK506 in potentiating progesterone transactivation has led to the suggestion that calcineurin may be involved in regulating PR function. Furthermore, our preliminary data showed that FK506 has no significant effect in potentiating androgen- and estrogen-mediated transcription in yeast cells expressing human androgen and estrogen receptors (AR and ER) (data not shown). These data suggest that the effect of FK506 is specific for PR but not for AR and ER. Thus, it seems unlikely that the transcriptional activities of AR and ER are regulated by calcineurin. Our data also argue against the possibility that binding of FK506 or rapamycin to FKBP59 may have a direct effect on receptor dissociation, since FKBP59 is a common protein associated with AR, ER, PR, and GR (Tai et al., 1986). Our present data support the notion that in the steroid signaling pathways, receptor dissociation is a step downstream from that of steroid-induced receptor phosphorylation which is presumably tightly controlled by different kinases and phosphatases acting on different receptors. This is consistent with the results of the recent phosphorylation studies that all the progesterone-induced phosphorylation sites of PR contain the consensus sequence for proline-directed protein kinase (PDPK) (Denner et al., 1990; Poletti et al., 1993). Such a consensus sequence is apparently lacking in the phosphorylation sites of AR and ER (Orti et al., 1992). These data suggest that, *in vivo*, PDPK may be responsible for the steroid-induced phosphorylation of PR but not for that of AR and ER.

In conclusion, although the nontransformed PR, AR, and ER are associated with their common component, the hsp90–hsp70–FKBP59 complex, the signal of each receptor is initiated by its own steroid and propagated through its specific transductive pathways. Use of FK506 and its analogues has helped us to identify the involvement of calcineurin in progesterone signaling. We believe that FK506 may continue to serve as a valuable tool to clarify the identities of other components in the progesterone signaling pathways.

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REFERENCES

Auricchio, F. (1989) *J. Steroid Biochem. 32*, 613–622.
Beato, M. (1989) *Cell 56*, 335–344.
Carson-Jurica, M. A., Schrader, W. T., & O'Malley, B. W. (1990) *Endocr. Rev. 11*, 201–220.
Chung, J., Juo, C. J., Crabtree, G. R., & Blenis, J. (1992) *Cell 69*, 1227–1236.
Clipstone, N. A., & Crabtree, G. R. (1992) *Nature 357*, 695–697.
Cyert, M. S., & Thorner, J. (1992) *Mol. Cell. Biol. 12*, 3460–3469.
Cyert, M. S., Kunisawa, R., Kaim, D., & Thorner, J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7376–7380.
Denner, L. A., Shrader, W. T., O'Malley, B. W., & Weigel, N. L. (1990) *J. Biol. Chem. 265*, 16548–16555.
Evans, R. M. (1988) *Science 240*, 889–895.
Foor, F., Parent, S. A., Morin, N., Dahl, A. M., Ramadan, N., Chrebet, G., Bostian, K. A., & Nielsen, J. B. (1992) *Nature 360*, 682–684.
Fretz, H., Albers, M. W., Galat, A., Standaert, R. F., Burakoff, S. J., Bierer, B. E., & Schreiber, S. L. (1991) *J. Am. Chem. Soc. 113*, 1409–1411.
Gietzen, K., Wuthrich, A., & Bader, H. (1981) *Biochem. Biophys. Res. Commun. 101*, 418–425.
Heitman, J., Movva, N. R., Heistand, P. C., & Hall, M. N. (1991) *Science 253*, 905–909.
Hung, D. T., & Schreiber, S. L. (1992) *Biophys. Biochem. Res. Commun. 184*, 733–738.
Ito, H., Fukada, Y., Murata, K., & Kimura, A. (1983) *J. Bacteriol. 153*, 163.
Kost, S. L., Smith, D. F., Sullivan, W. P., Welch, W. J., & Toft, D. O. (1989) *Mol. Cell. Biol. 9*, 3829–3838.
Kunz, J., Henriquez, R., Schneider, U., Deuter-Reinhard, M., Movra, N. R., & Hall, M. N. (1993) *Cell 73*, 585–596.
Lebeau, M.-C., Massol, N., Herrick, J., Faber, L. E., Renoir, J.-M., Radanyi, C., & Baulieu, E.-E. (1991) *J. Biol. Chem. 267*, 4281–4284.
Liu, J., Farmer, J. D., Jr., Lane, W. S., Friedman, J., Weissman, I., & Schreiber, S. L. (1991) *Cell 66*, 807–815.
Liu, J., Albers, M. W., Wandless, T. J., Luan, S., Alberg, D. G., Belshaw, P. J., Cohen, P., MacKintosh, C., Klee, C. B., & Schreiber, S. L. (1992) *Biochemistry 31*, 3896–3901.
Moudgil, V. K. (1990) *Biochim. Biophys. Acta 1055*, 242–258.
Nielsen, J. B., Foor, F., Siekierka, J. J., Hsu, M.-J., Ramadan, N., Morin, N., Shafiee, A., Dahl, A., Brizuela, L., Cherebet, G., Bostian, K. A., & Parent, S. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 7471–7475.
Ning, Y.-M., & Sanchez, E. R. (1993) *J. Biol. Chem. 268*, 6073–6076.
Orti, E., Bodwell, J. E., & Munck, A. (1992) *Endocr. Rev. 13*, 105–128.
Peattie, D. A., Harding, M. W., Fleming, M. A., DeCenzo, M. T., Lipke, J. A., Livingston, D. J., & Benasutti, M. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10974–10978.
Poletti, A., Conneely, O. M., McDonnell, D. P., Schrader, W. T., O'Malley, B. W., & Weigel, N. L. (1993) *Biochemistry 32*, 9563–9569.
Price, D. J., Grove, J. R., Calvo, V., Avruch, J., & Bierer, B. E. (1992) *Science 257*, 973–977.
Rosen, M. K., Yang, D., Martin, P. K., & Schreiber, S. L. (1993) *J. Am. Chem. Soc. 115*, 822–823.
Sanchez, E. R., Toft, D. O., Schlesinger, M. J., & Pratt, W. B. (1985) *J. Biol. Chem. 260*, 12398–12401.
Schreiber, S. L. (1991) *Science 251*, 283–287.
Schreiber, S. L., & Crabtree, G. R. (1992) *Immunol. Today 13*, 136–142.
Sheridan, P. L., Francis, M. D., & Horwitz, K. B. (1989) *J. Biol. Chem. 264*, 7054–7058.
Sigal, N. H., & Dumont, F. J. (1992) *Annu. Rev. Immunol. 10*, 519–560.
Tai, P.-K. K., Maeda, Y., Nakao, K., Wakim, N. G., Duhring, J. L., & Faber, L. E. (1986) *Biochemistry 25*, 5269–5275.
Tai, P.-K., K., Albers, M. W., Chang, H., Faber, L. E., & Schreiber, S. L. (1992) *Science 256*, 1315–1318.
Tai, P.-K. K., Chang, H., Albers, M. W., Schreiber, S. L., Toft, D. O., & Faber, L. E. (1993) *Biochemistry 32*, 8842–8847.
Traish, A., & Wotiz, H. (1990) *Endocrinology 127*, 1167–1175.
Vegeto, E., Allan, G. F., Schrader, W. T., Tsai, M.-J., McDonnell, D. P., & O'Malley, B. W. (1992) *Cell 69*, 703–713.
Wickersham, L. J. (1951) *Tech. Bull.—U.S. Dep. Agric. 1029*.
Yem, A. W., Tomasselli, A. G., Heinrikson, R. L., Zurcher-Neely, H., Ruff, V. A., Johnson, R. A., & Deibel, M. R., Jr. (1992) *J. Biol. Chem. 267*, 2868–2871.