

The Immunophilin Ligands Cyclosporin A and FK506 Suppress Prostate Cancer Cell Growth by Androgen Receptor-Dependent and -Independent Mechanisms

Sumudra Periyasamy, Manya Warriar, Manoranjani P. M. Tillekeratne, Weinian Shou, and Edwin R. Sanchez

Department of Physiology and Pharmacology (S.P., M.W., M.P.M.T., E.R.S.), University of Toledo College of Medicine, Toledo, Ohio 43614-5804; and Herman B. Wells Center for Pediatric Research (W.S.), Section of Pediatric Cardiology, Department of Pediatrics, Indiana University School of Medicine, Indianapolis, Indiana 46202

The androgen receptor (AR) contributes to growth of prostate cancer even under conditions of androgen ablation. Thus, new strategies to target AR activity are needed. The AR interacts with the immunophilin FK506-binding protein 52 (FKBP52), and studies in the FKBP52 knockout mouse have shown that this protein is essential to AR activity in the prostate. Therefore, we tested whether the immunophilin ligand FK506 affected AR activity in prostate cancer cell lines. We also tested the hypothesis that the AR interacts with another immunophilin, cyclophilin 40 (Cyp40), and is regulated by its cognate ligand cyclosporin A (CsA). We show that levels of FKBP52, FKBP51, Cyp40, and a related co-chaperone PP5 were much higher in prostate cancer cell lines [LNCaP, PC-3, and DU145] compared with primary prostate cells, and that the AR of LNCaP cells can interact with Cyp40. In the absence of androgen, CsA caused inhibition of cell growth in

the AR-positive LNCaP and AR-negative PC-3 and DU145 cell lines. Interestingly, FK506 only inhibited LNCaP cells, suggesting a dependence on the AR for this effect. Both CsA and FK506 inhibited growth without inducing apoptosis. In LNCaP cells, CsA completely blocked androgen-stimulated growth, whereas FK506 was partially effective. Further studies in LNCaP cells revealed that CsA and FK506 were able to block or attenuate several stages of AR signaling, including hormone binding, nuclear translocation, and activity at several AR-responsive reporter and endogenous genes. These findings provide the first evidence that CsA and FK506 can negatively modulate proliferation of prostate cells *in vitro*. Immunophilins may now serve as new targets to disrupt AR-mediated prostate cancer growth. (*Endocrinology* 148: 4716–4726, 2007)

ANDROGENS ARE KNOWN to regulate the growth of malignant prostate epithelial cells (PrECs), as documented by the initial growth arrest of metastatic prostate tumors by androgen ablation (1). It is also now clear that hormonal stimulation of proliferation is mediated by the androgen receptor (AR) and that AR mutations are at least one cause of oncogenic transformation of the prostate (2, 3). Unfortunately, prostate cancer often develops adaptive mechanisms to growth in the presence of low androgens, historically referred to as androgen independence, but now more properly known as ablation resistance. A majority of ablation-resistant tumors still express ARs (4–6). Thus, it is

likely that factors other than androgens may activate the AR and contribute to prostate cancer progression.

Like other members of the steroid receptor family, the unliganded AR is primarily found in the cytoplasm as a heterocomplex containing the molecular chaperone heat shock protein 90 (Hsp90) (for review, see Ref. 7). In the better-studied glucocorticoid (GR), estrogen, and progesterone (PR) receptors, the existence of several tetratricopeptide repeat (TPR) proteins in receptor heterocomplexes has also been documented. These include the FK506-binding protein 52 (FKBP52), the highly similar FKBP51, cyclophilin 40 (Cyp40), and protein phosphatase 5 (PP5) (8, 9). To date, FKBP52 has reliably interacted with the AR (10–12), and one recent report has suggested an AR/FKBP51 interaction (13). Although, Cyp40 and PP5 have not yet been found with the AR, this is most likely due to a lack of effort than molecular differences. Thus, it has been our recent hypothesis that TPR heterogeneity of AR complexes may serve as important points of regulation for this receptor.

Immunophilins are proteins so named because they bind the immunosuppressive drugs, FK506 and cyclosporin A (CsA), the binding of which causes inhibition of the protein's peptidyl-prolyl cis-trans isomerase (PPIase) activity (14, 15). Immunophilins can be divided into two major categories: the small molecular weight immunophilins, such as FKBP12 and cyclophilin 18, that mediate immunosuppression at lymphocytes (16, 17); and the large molecular weight immunophi-

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Abbreviations: AR, Androgen receptor; CaN, calcineurin; CAT, chloramphenicol acetyl transferase; CsA, cyclosporin A; Cyp40, cyclophilin 40; DCC, dextran-coated charcoal-treated; DHT, dihydrotestosterone; DTT, dithiothreitol; FBS, fetal bovine serum; FKBP52, FK506-binding protein 52; GFP-AR, green fluorescent protein-androgen receptor; GR, glucocorticoid; hPrEC, human prostate epithelial cell; Hsp90, heat shock protein 90; KO, knockout; MMTV, mouse mammary tumor virus; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolone bromide; PP5, protein phosphatase 5; PPIase, peptidyl-prolyl cis-trans isomerase; PR, progesterone; PrEC, prostate epithelial cell; PSA, prostate-specific antigen; PSA-Luc, prostate-specific antigen luciferase; TPR, tetratricopeptide repeat; WCE, whole cell extract.

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lins, such as FKBP52 and Cyp40, that contain TPR domains necessary for interaction with Hsp90 (18). With their identification as components of inactivate steroid receptor complexes (19–23), the possibility was raised that receptor activity might be influenced by immunophilin ligands. Indeed, a variety of reports support this notion. For example, FK506 and CsA can potentiate GR-mediated transcription in mouse L929 fibroblast cells (24–26), whereas FK506 has a similar effect on PR expressed in yeast (27). Yet, these compounds can also be inhibitory. Thus, GR in A6 kidney cells are inhibited by both FK506 and CsA (28), whereas FK506 inhibits both PR and GR found in T47D breast cancer cells (29, 30). Therefore, it appears that immunophilin ligands can have dichotomous effects on hormone action that are cell-type and receptor-type dependent. Although somewhat confusing at present, such diversity of action holds the promise that these drugs can be used to selectively target hormonal responses.

A variety of molecular studies have shed some light on how immunophilins and their cognate ligands may control receptor-signaling processes. Early work by our laboratory (31) and Renoir *et al.* (32) showed that incubation of cell lysates with FK506 caused an increase in the hormone-binding affinities of GR and PR. In later work, we showed that the effect on hormone binding might be due to a mechanism in which FK506 promotes GR interaction with PP5 by preventing the binding of both FKBP52 and FKBP51 (26). Work by Reynolds (33) and Denny (34) *et al.* showed a linkage between FKBP51 and reduced GR binding affinity and GR resistance in squirrel monkeys. Similarly, overexpression of FKBP51 reduced progesterin responsiveness in cells transfected with PR (35). In contrast, FKBP52 appears to have the opposite effect on steroid receptors because overexpression of FKBP52 increases both GR hormone-binding affinity and reporter gene activity (26, 36). Although more limited, studies of Cyp40 also point to a functional role in steroid receptor regulation because a mutant strain of yeast deficient for Cpr7 (a Cyp40 homolog) has greatly reduced GR and estrogen activity (37). Meanwhile, a separate line of evidence has demonstrated a role for FKBP5s in the intracellular trafficking of receptors. In this case, the interaction of GR with FKBP52 appears to promote nuclear translocation of receptor through concomitant recruitment of the motor protein dynein (38).

Until recently, there were few studies on the control of AR function by immunophilins. Most notable of the new reports is the observation by Cheung-Flynn *et al.* (12) that male mice with targeted ablation of FKBP52 are infertile due to the aberrant development of the penis and prostate gland. Our laboratory (39) has independently generated FKBP52 knockout (KO) mice and identified the developmental defect of the penis to be hypospadias, a condition common in human newborns and known to result from androgen insensitivity (40, 41). Interestingly, FKBP52 KO males also showed prostate dysgenesis, as well as dramatically reduced AR activity at both endogenous and heterologous genes (12, 39). These results are in good agreement with studies in the AR KO mouse, which also develops penile aberrations and dysgenic prostate (42, 43), suggesting that FKBP52 is an essential immunophilin controlling AR activity in these tissues. Whether FKBP52 or related immunophilins are involved in AR control of prostate oncogenesis remains to be seen. However, several

recent reports point in this direction. For example, overexpression of Cyp40 and FKBP52 was found in breast cancer tumors, with expression of each protein being controlled by estradiol (44). Using gene array and other approaches, several laboratories have shown that FKBP51 expression is significantly higher in prostate cancer samples compared with appropriate controls (45, 46). Moreover, reports now exist showing that FKBP51 is a highly sensitive AR-regulated protein (47, 48) whose up-regulation serves to increase AR activity (13). Thus, it is now feasible that dysregulated expression of FKBP51 could lead to overactivity on the part of the AR, perhaps with oncogenic consequences.

With these concepts in mind, we have explored the effects of immunophilins and immunophilin ligands on AR activity in three prostate cancer cell lines: the AR-positive (LNCaP) cells, and the AR-negative (PC-3) and (DU145) cells. We show that CsA was a highly effective inhibitor of both AR-dependent and -independent proliferation, whereas FK506 only inhibited AR-dependent growth. Further studies are presented that show targeting of AR activity by both CsA and FK506 in the LNCaP cells. To the best of our knowledge, these results are the first of their kind and provide potential new targets in the treatment of both early- and late-stage prostate cancer.

Materials and Methods

Cell culture

The human prostate cancer cell lines LNCaP, PC-3, and DU145 were routinely cultured and maintained in RPMI 1640 medium or DMEM containing 10% fetal bovine serum (FBS) and 0.05% vol/vol penicillin/streptomycin. The normal human prostate epithelial cells (hPrECs) were obtained from Clonetics Corp. (Cambrex Corp., San Diego, CA) at early passage (P2 or P3) and cultured in the prostate epithelial basal medium supplied by the company. Prostate epithelial basal medium was supplemented with human epidermal growth factor, triiodothyronine, transferrin, epinephrine, gentamicin sulfate, amphotericin B, bovine pituitary extract, bovine insulin, hydrocortisone, and retinoic acid additives provided by the manufacturer. The normal and cancerous prostate cell lines were grown at 37°C in a humidified atmosphere containing 5% CO₂, and allowed to reach 80% confluence before they were subcultured.

Growth inhibition assay

LNCaP cells (3×10^3 cells per well) were plated in 96-well culture plates and allowed to adhere to substrate for 24 h in RPMI 1640 medium containing 10% normal FBS. PC-3 (2×10^3 cells per well) and DU145 (2×10^3 cells per well) cells were plated in 96-well culture plates for 24 h in DMEM containing 10% normal FBS. Cells were incubated for 7 d in the absence and presence of CsA (0–10.0 μ M), FK506 (0–10.0 μ M), with a change of media and ligands on d 2, 4, and 6. To determine the effect of CsA and FK506 on androgen-stimulated cell growth, LNCaP cells grown in RPMI 1640 medium containing 10% charcoal-stripped serum were treated with increasing concentrations of dihydrotestosterone (DHT) (0–100 nM) in the absence and presence of CsA (5.0 μ M) and FK506 (10.0 μ M), with media and ligand changes on d 2, 4, and 6. At the end of d 7, a calorimetric assay using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to determine relative cell numbers, as previously described (49). Briefly, 0.1 mg (50 μ l of 2 mg/ml) MTT was added to each well and incubated at 37°C for 2 h. The MTT medium was then removed, and 150 μ l dimethylsulfoxide was added to each well and allowed to shake gently for 15 min at room temperature. Absorbance was measured at 595 nm using a Molecular Devices SOFTmax microplate reader (Molecular Devices Corp., Sunnyvale, CA).

DNA fragmentation analysis

Apoptosis was monitored by internucleosomal DNA degradation. Briefly, LNCaP, PC-3, and DU145 cells were cultured for 7 d in the presence and absence of CsA (5.0 μM) or FK506 (10.0 μM), with a change of media and ligands on d 2, 4, and 6. As a positive control, LNCaP cells were treated with wortmannin (500 nM) for 6 h. Genomic DNA was isolated using the Apoptotic Ladder Kit, according to the manufacturer's protocol (Roche Diagnostics Corp., Indianapolis, IN). Aliquots of DNA (10.0 μg) were electrophoresed through 1.5% agarose gels and visualized by ethidium bromide staining.

Transient transfection, luciferase, and chloramphenicol acetyl transferase (CAT) reporter assays

For transient transfection assay, LNCaP cells were plated either at a density of 5×10^5 in six-well plates or 1×10^6 in 6-cm culture dishes and incubated with RPMI 1640 medium containing 10% dextran-coated charcoal-treated (DCC) serum. At 95% confluence, the cells were transfected with 4 μg DNA [prostate-specific antigen luciferase (PSA-Luc) or activin-responsive element-containing luciferase plasmids] per well of a six-well plate or 8.0 μg DNA [mouse mammary tumor virus (MMTV)-CAT] per 6-cm dish using Lipofectamine 2000 reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol, and incubated for 6 h at 37 C in a serum-free RPMI 1640 medium. A β -galactosidase plasmid was cotransfected as an internal control to normalize for transfection efficiency. The cells were then fed 6 h after transfection with RPMI 1640 medium containing 10% DCC serum. Twenty-four hours after transfection, the cells were washed and refed with RPMI 1640 medium containing 10% DCC serum, and then treated with CsA (5.0 μM) or FK506 (10.0 μM) for 3 h, followed by increasing concentrations of R1881 (0.1–10.0 nM) for an additional 20 h. Cell extracts were prepared with reporter lysis buffer (Promega Corp., Madison, WI), and luciferase activity was measured using a luminometer and expressed as a percentage of control after normalization to β -galactosidase activity. CAT enzyme activity was performed according to the method of Nordeen *et al.* (50) with minor modification. CAT data were expressed as a percentage of control after normalization to β -galactosidase activity.

PCR analysis of FKBP51

LNCaP cells were treated with R1881 (1.0 nM) in the presence and absence of CsA (5.0 μM) or FK506 (10.0 μM) for 20 h. After treatment, total RNA was extracted using Trizol (Invitrogen) reagent and was treated with RNase-free DNase (Promega). After spectrophotometric quantification, RT (2 μg total RNA) was performed by the 1st Strand cDNA Synthesis Kit (Roche) using oligo(dT) primer. First-strand cDNA (2 μl) was amplified using PCR master mix (Promega). For PCR, specific primers against hFKBP51 (forward primer: TTCCCCAACTCAGGACAGAAC; reverse primer: GTAAAACCTAACAGCCCCACATTG) at a final concentration of 1 μM were used. PCR conditions were: 95 C for 5 min, 95 C for 1 min, 56.1 C for 1 min, 72 C for 2 min, and 72 C for 10 min. Primers were designed based on the cDNA sequence obtained from GenBank (HSU42031). 18S RNA was also amplified as an internal control. Amplified PCR products were electrophoresed on 1% agarose gels and visualized using ethidium bromide staining.

Steroid binding assay

LNCaP cells were treated with ethanol (0.01%), or CsA (5.0 μM) or FK506 (10.0 μM) for 24 h, and the cell pellets were washed twice with PBS and resuspended in ice-cold homogenization buffer [10 mM HEPES, 1.5 mM EDTA, and 10 mM sodium molybdate (pH 7.4)] with protease inhibitors. Cell pellets were lysed by Dounce homogenization, followed by centrifugation for 10 min at $16,000 \times g$. The resulting supernatants (cytosol) were used for binding assay without freezing. In a typical binding assay, 150 μl cytosol (~ 2.0 mg/ml) was incubated with 10.0 nM mibolerone (72α -methyl- ^3H , 70.0 ci/mmol) for 20 h at 0 C. Mibolerone is a synthetic high-affinity ligand for the AR. Nonspecific binding of [^3H]mibolerone was determined separately by adding a 1000-fold excess of unlabeled mibolerone (10 μM) to the incubate. After incubation, the protein bound radioactivity was separated from free radioactivity by 200

μl 1% DCC in 10 mM HEPES buffer (pH 7.4). Data are expressed as dpm bound hormone/mg cytosol protein.

Preparation of whole cell extract (WCE)

WCEs of prostate cells were prepared as previously described (51). Briefly, 2×10 -cm dishes of each cell type were washed with cold PBS, harvested, and centrifuged at $3000 \times g$ for 10 min. The resulting pellets were resuspended in cold WCE buffer [20 mM HEPES, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, and 0.5 mM dithiothreitol (DTT) (pH 7.9)] with protease inhibitors and centrifuged at $100,000 \times g$ for 10 min. The supernatants were either stored at -80 C or used immediately for Western analysis to determine AR and TPR protein expression levels. To determine FKBP51 protein expression levels, LNCaP cells were treated with R1881 (1.0 nM) in the presence and absence of CsA (5.0 μM) or FK506 (10.0 μM) for 20 h.

Green fluorescent protein-androgen receptor (GFP-AR) localization by confocal fluorescence microscopy

Subcellular localization of GFP-AR was analyzed by confocal fluorescence microscopy. LNCaP and PC-3 cells (5×10^5 cells) were plated onto laminin-coated coverslips in six-well plates in RPMI 1640 or DMEM medium containing 5% DCC serum. At 80% confluence, cells were transfected with GFP-AR plasmid using Lipofectamine 2000 transfection agent according to the manufacturer's protocol and incubated for 6 h in a reduced serum OPTI-MEM I medium. The cells were fed 6 h after transfection with RPMI 1640 or DMEM containing 5% DCC serum. At 24 h after transfection, cells were washed and refed with RPMI 1640 or DMEM containing 5% DCC serum, and pretreated with or without CsA (5.0 μM) for 2 h, followed by R1881 (1.0 nM) for an additional 3 h. After treatment, the cells were fixed with methanol, and the coverslips were mounted onto microscope slides. Image visualization was performed with a Leica TCS-SP2 laser-scanning microscope (Leica, Mannheim, Germany).

Purification of AR/Cyp40 complex

Immunoaffinity purification of the AR complex was performed as described by Veldscholte *et al.* (10). Briefly, LNCaP cells were suspended in ice-cold homogenization buffer [10 mM HEPES, 3.0 mM EDTA, 12 mM α -thioglycerol, 10 mM DTT, 20 mM sodium molybdate, and 10% weight/volume glycerol (pH 7.4)] with protease inhibitors, and homogenized with a glass/Teflon (DuPont Co., Wilmington, DE) homogenizer on ice and centrifuged at $800 \times g$ for 5 min. The supernatant was then centrifuged for 30 min at $105,000 \times g$ at 3 C. The supernatant (cytosol) was used without freezing or storage for immunoprecipitation of the AR complexes. Aliquots (typically 400 μl) of cytosol were treated for 2 h on ice with CsA (5.0 μM) or vehicle control, followed by an additional

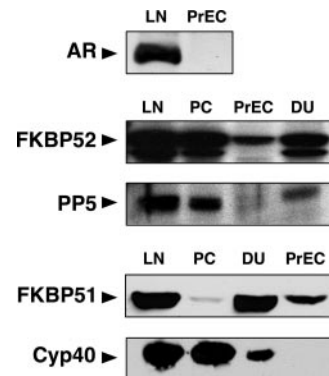
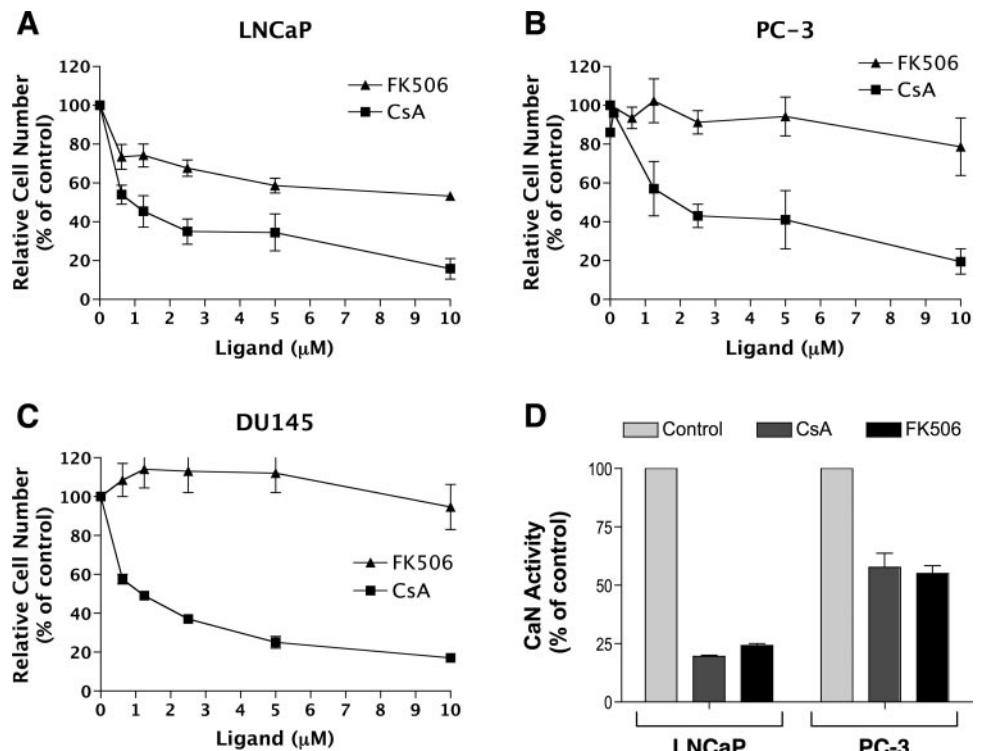


FIG. 1. Expression levels of AR and TPR proteins in normal and cancerous prostate cell lines. Fifty micrograms of WCEs from normal hPrECs and cancerous human prostate cell lines [LNCaP (LN), PC-3 (PC), and DU145 (DU)] were subjected to Western blotting with antibodies specific to FKBP52, PP5, FKBP51, Cyp40, and AR, as described in *Materials and Methods*. Results are representative of three independent experiments.

FIG. 2. Effects of FK506 and CsA on androgen-independent growth and CaN activity in LNCaP, PC-3, and DU145 cells. LNCaP (3000 cells per well) (A), PC-3 (2000 cells per well) (B), and DU145 (2000 cells per well) (C) cells were incubated with increasing concentrations of CsA or FK506 for 7 d, with a change of media and ligands on d 2, 4, and 6. Cell numbers on d 7 were determined by MTT colorimetric assay. All panels are representative of three independent experiments. In each experiment, cell densities in three replicate wells were measured per condition. Thus, each value corresponds to the mean \pm SEM of nine wells, with 100% growth representing values derived from ethanol-treated control cells. D, LNCaP and PC-3 cells were treated with CsA (5.0 μ M), FK506 (10.0 μ M), or vehicle (0.01% ethanol) for 20 h before preparation of lysates. CaN-specific phosphatase activity was measured by phosphate released from the CaN-specific RII phosphopeptide. Results are the means \pm SEM of three independent experiments, each performed in triplicate.



incubation for 1 h at 37 C. After rechilling, anti-AR antibody or nonimmune IgG was added and incubated on ice for 2 h, followed by rotation with 30 μ l protein-A Sepharose at 4 C overnight. The beads were washed three times with TEG buffer (10 mM Tris; 3 mM EDTA; 10% glycerol; and 50 mM NaCl, pH 7.4) with 10 mM sodium molybdate, followed by extraction with 2 \times sodium dodecyl sulfate sample buffer.

Gel electrophoresis and Western blotting

Samples were resolved by denaturing SDS-PAGE using either 7 or 10% polyacrylamide gels, followed by transfer to Immobilon polyvinylidene difluoride membranes and immunoblotting, as previously described (24). The human anti-AR antibody (SC-815) was used to probe for receptor, whereas various antibodies were used to probe for FKBP52 (UPJ56), FKBP51 (PA0-021), PP5, and Cyp40 (PA3-022). The blots were washed and incubated with the appropriate peroxidase- and 125 I-conjugated counter antibodies, followed by color development and autoradiography.

Calcineurin (CaN) assay

Cell extraction and CaN assay were performed with the QuantiZyme Assay System (AK-816) according to the manufacturer's protocol (BIOMOL, Intl., LP, Plymouth Meeting, PA). Briefly, control (0.01% ethanol), CsA (5.0 μ M), and FK506-treated (10.0 μ M) LNCaP and PC-3 cells were washed in ice-cold Tris-buffered saline [20 mM Tris (pH 7.2) and 150 mM NaCl], and lysed in 50 μ l lysis buffer [50 mM Tris (pH 7.5), 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.2% NP-40] containing protease inhibitors. Cell lysis was facilitated by passing through a 16-g needle, and the lysates were centrifuged for 30 min at 100,000 \times g at 4 C. Supernatants were transferred to fresh tubes in aliquots on ice, and frozen in dry ice/ethanol and stored at -80 C. Before assay, free phosphate was removed from the supernatants by gel filtration. CaN was measured as units of free phosphate released from the CaN-specific RII phosphopeptide in the presence of okadaic acid to inhibit other phosphatases.

Results

Analysis of AR and immunophilin content in prostate cells

As an initial test that immunophilins might play a role in AR control of prostate cell growth, we examined by Western blot analysis the protein expression levels of the AR and immunophilins in WCEs obtained from normal hPrECs and cancerous human prostate cell lines LNCaP, DU145, and PC-3. LNCaP is a well-established, androgen-responsive cell line obtained from the lymph node metastasis of a patient with advanced prostate cancer (10, 11). LNCaP cells express AR (52) and a number of androgen-inducible genes [e.g. prostate-specific antigen (PSA), FKBP51, and kallikrein-2]. In contrast, the PC-3 and DU145 cell lines do not express AR, yet were obtained from bone and brain metastasis, respectively, of two prostate cancer patients. As expected, AR protein was detected in LNCaP cells (Fig. 1), but not in PC-3 and DU145 cell lines (data not shown). We also could not detect AR protein in PrECs. Although it is not clear why PrECs fail to express AR, prior studies have suggested that PrECs partially dedifferentiate under culture conditions, leading to loss, not only of AR, but also PSA expression (53). Figure 1 also shows high levels of expression for FKBP52, PP5, and Cyp40 in the LNCaP, PC-3, and DU145. Although overexpression of these TPRs suggests a possible role for all three in prostate cell oncogenesis, confirmation of this fact will require analysis of a larger set of prostate-derived cell lines, as well as prostate tissues. FKBP51 expression was also high in LNCaP and DU145 but was much lower in PC-3 cells. This exception would suggest that FKBP51 overexpression is not a requisite marker of prostate cancer. However, because FKBP51 is thought to contribute principally through mod-

ulation of AR activity, it may still be an important factor in AR-positive prostate cells, such as LNCaP.

Differential effects of CsA and FK506 on androgen-independent prostate cell growth

CsA and FK506 are potent inhibitors of cell growth in the lymph system (14, 54). To the best of our knowledge, no reports yet exist on the growth inhibitory properties of these ligands on prostate cells. To establish these properties, we measured prostate cell growth rates in the presence and absence of CsA or FK506 (Fig. 2). The results show CsA to be a highly effective inhibitor of prostate cell growth in the LNCaP, PC-3, and DU145 cell lines, achieving approximately 80% inhibition at 10 μM ligand. CsA inhibition was concentration dependent, yielding IC_{50} values of 1.0–2.5 μM in the three cell lines. In contrast, FK506 only inhibited growth in the LNCaP cells, with an IC_{50} value of 10.0 μM . It was interesting that FK506 had no inhibitory effect on the PC-3 and DU145 cells. This would suggest that growth inhibition by FK506 in prostate cells requires the presence of the AR.

Because suppression of T-cell growth by CsA and FK506 is mediated by Cyp18 and FKBP12, respectively, leading to inhibition of CaN activity, we considered the possibility that both ligands acted through the same targets in prostate cells. However, this did not seem likely because FK506 was a less potent inhibitor than CsA in LNCaP cells (~4–10 times more active than CsA), even though it is 10–100 times more potent than CsA as an immunosuppressive agent (16). Nonetheless, we directly tested this possibility by measuring CaN activity in LNCaP and PC-3 cells (Fig. 2D). The results show approximately equal potency for CaN inhibition by CsA and FK506 in LNCaP cells, and, more importantly, about equal efficacies in the PC-3 cells. Thus, in prostate cells, the degree of CaN inhibition by CsA and FK506 does not correlate with their respective effects on proliferation. Moreover, FK506 appears to be as effective as CsA for CaN inhibition in PC-3 cells, yet does not exhibit growth suppression in that cell line. Therefore, we conclude that the primary target of growth suppression in prostate cells is not the Cyp18/FKBP12/CaN pathway.

FK506 and CsA have induced apoptosis in certain cell types (54–58). To test whether apoptosis was the mechanism by which CsA and FK506 caused growth inhibition, we measured apoptosis by DNA fragmentation after CsA and FK506 treatment. As a positive control, LNCaP cells were also treated with wortmannin, a known inducer of apoptosis in LNCaP cells (59). Treatment of LNCaP (Fig. 3), PC-3, and DU145 cell lines (data not shown) with either CsA or FK506 did not induce DNA fragmentation. In contrast, treatment of LNCaP cells with wortmannin promoted fragmentation (Fig. 3). These findings indicate that the growth inhibitory properties of CsA and FK506 in prostate cells were not due to induction of cell death.

CsA and FK506 suppress androgen-dependent growth of LNCaP cells

Prior studies have shown androgen activation of the AR to play an important role in progression of prostate cancer (1, 60). To determine whether CsA and FK506 modulate andro-

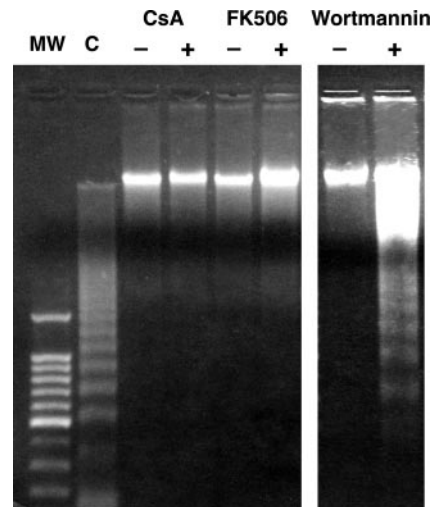


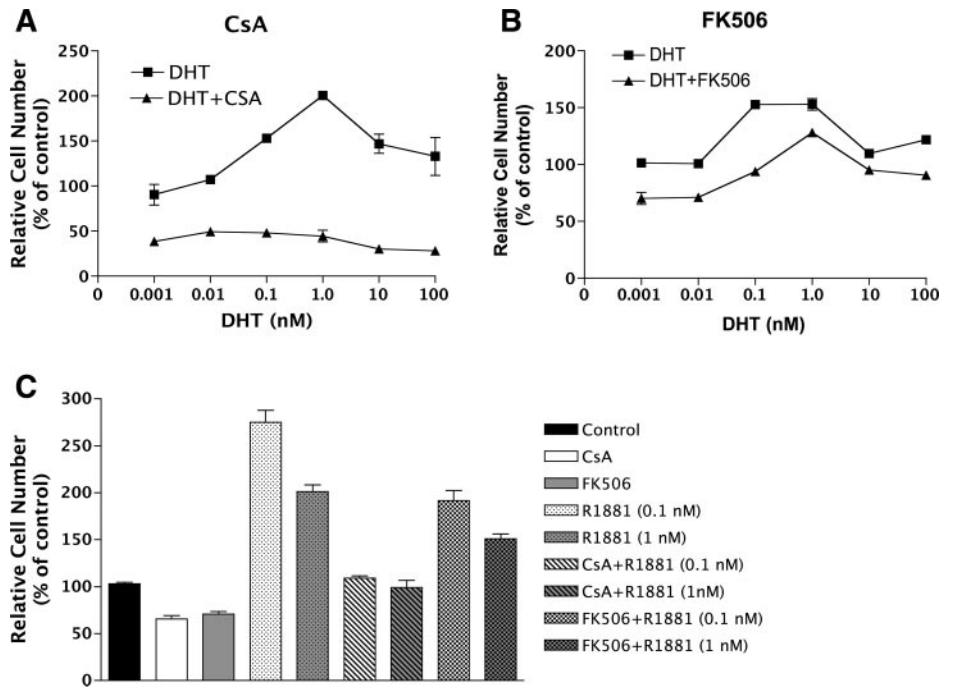
FIG. 3. CsA and FK506 do not induce apoptosis. LNCaP cells were cultured in the presence and absence of CsA (5.0 μM) or FK506 (10.0 μM) for 7 d, with a change of media and ligands on d 2, 4, and 6. DNA was isolated, subjected to agarose gel electrophoresis, and visualized by ethidium bromide staining. DNA from wortmannin-treated LNCaP cells and camptothecin-treated (C) U937 cells (provided by manufacturer) were used as positive controls. Results are representative of three independent experiments. Similar results were obtained in PC-3 and DU145 cells. MW, Molecular weight markers.

gen-mediated prostate cell growth, LNCaP cells were treated with increasing concentrations of the natural androgen DHT in the presence and absence of immunophilin ligand (Fig. 4, A and B). In the absence of CsA and FK506, DHT showed a stimulatory effect on growth at low concentrations of hormone (0.01–1.0 nM) that decreased at higher concentrations (10–100 nM). These data are consistent with reports showing androgen stimulation of LNCaP cells to be biphasic with respect to concentration (61–63). The results of Fig. 4, A and B, also show that CsA completely inhibited the androgen-induced mitogenic response, whereas FK506 was partially effective (maximal inhibition = 38% at 0.1 nM DHT). To confirm the partial activity of FK506, growth assays were rerun using R1881 (Fig. 4C). Once again, CsA demonstrated 100% inhibition at 0.1 and 1.0 nM R1881, whereas FK506 showed approximately 40% inhibition at 0.1 and 1.0 nM R1881, respectively.

CsA and FK506 suppress AR transcriptional activity in LNCaP cells

To explore the molecular mechanism of growth inhibition, we tested the effects of CsA and FK506 on androgen-regulated gene expression by transfecting LNCaP cells with various AR-controlled reporter constructs. To compare the effect on AR transcriptional activity with the inhibitory effect on LNCaP growth response, reporter gene activity was measured using the IC_{50} growth-inhibitory values of ligands. We first used the minimal promoter containing two synthetic androgen response elements linked to the adenovirus E1B TATA box and luciferase reporter (pARE2E1B-Luc) (Fig. 5, A and B). The results show 80 and 35% inhibition of R1881-induced pARE2E1B-Luc activity by CsA and FK506, respectively. Neither CsA nor FK506 had any effect on pARE2E1B-

FIG. 4. CsA and FK506 inhibit androgen-induced growth in LNCaP cells. LNCaP cells were incubated with increasing concentrations of DHT (A and B) or R1881 (C) for 7 d in the presence and absence of CsA (5.0 μ M) or FK506 (10.0 μ M). Change of media and ligands occurred on d 2, 4, and 6. Cell numbers on d 7 were determined by MTT assay. The data are representative of two independent experiments. In each experiment, cell densities in four replicate wells were measured per condition. Thus, each value corresponds to the mean \pm SEM of eight samples, with 100% growth representing ethanol-treated control cells.

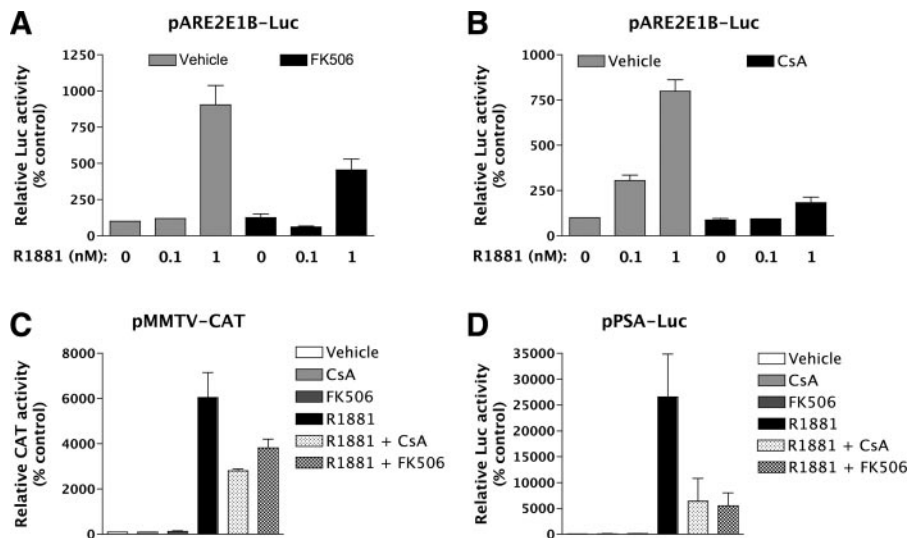


Luc activity when used alone. Moreover, CsA and FK506 had no effect on AR protein levels (see Fig. 7A). Because promoter context can influence transcriptional efficiency and selectivity, we tested the effects of CsA and FK506 on two other reporter constructs: (pMMTV-CAT) and (pPSA-Luc). The pMMTV-CAT construct is driven by the natural MMTV long-terminal repeat promoter containing several AR response elements (64). Similar to pARE2E1B-Luc results, R1881-induced pMMTV-CAT activity (Fig. 5C) was inhibited by CsA more effectively (54%) than FK506 (35%). To determine whether the inhibitory effects of CsA and FK506 extend to prostate-specific genes, we used the pPSA-Luc reporter. Expression of PSA in prostate is principally regulated by

androgens acting through the AR (65–67). As shown in Fig. 5D, AR transcriptional activity at pPSA-Luc was once again inhibited by both CsA and FK506, except in this case, each immunophilin ligand had about equal efficacy.

As a last test, we measured CsA and FK506 activity on expression of the endogenous gene FKBP51, a highly responsive target of AR transcriptional activity (13, 45, 48). In LNCaP cells, both CsA and FK506 had an inhibitory effect on expression of androgen-induced FKBP51 mRNA and protein (Fig. 6). Therefore, taken as a whole, the results of Figs. 2–6 show a good correlation between the ability of FK506 and CsA to differentially inhibit LNCaP cell AR transcriptional activity and the inhibitory effects of these drugs on the androgen-induced mitogenic response.

FIG. 5. CsA and FK506 suppress androgen-induced AR transcriptional activity in LNCaP cells. LNCaP cells were transiently transfected with pARE2E1B-Luc minimal reporter (A and B), pMMTV-CAT reporter (C), or pPSA-Luc reporter (D) along with a β -galactosidase plasmid, as control. Twenty hours later, cells were exposed to various combinations of R1881, CsA, and FK506, as described below. Lysates were prepared and analyzed for luciferase or CAT enzyme activities. The results shown represent the means \pm SEM of three to six independent experiments. A and B, Cells were treated with or without FK506 (10.0 μ M) or CsA (5.0 μ M) for 2 h, followed by increasing concentrations of R1881 (0–1.0 nM) for 20 h. C and D, Cells were treated with CsA (5.0 μ M) for 20 h, FK506 (10 μ M) for 20 h, R1881 (1.0 nM) for 20 h, CsA (5.0 μ M) or FK506 (10.0 μ M) for 2, h followed by R1881 (1.0 nM) for 20 h.



CsA decreases AR hormone-binding capacity in LNCaP cells without disrupting the Cyp40/AR interaction

To determine the stage of AR signaling affected by CsA and FK506, we initiated a series of experiments targeting distinct steps of AR action. First and foremost among these was to determine whether the immunophilin ligands affected the ability of the AR to bind hormone. This was achieved by measuring the hormone-binding capacity in cytosols derived from LNCaP cells treated with CsA or FK506 (Fig. 7, A and B). Western blotting of the cytosols revealed equal amounts of AR protein in control and ligand-treated cells. Thus, reduced AR transcriptional activity is not attributable to loss of receptor. However, CsA and FK506 did reduce specific binding of the AR agonist [³H]mibolerone by 30 and 38%, respectively, suggesting that attenuation of this function is at least partly responsible for the loss of AR transcriptional activity.

To test whether the effect of CsA on AR hormone-binding function was due to alterations in the AR heterocomplex, we measured the Cyp40 content of AR complexes after CsA treatment of LNCaP cell cytosol. The results of Fig. 7C show

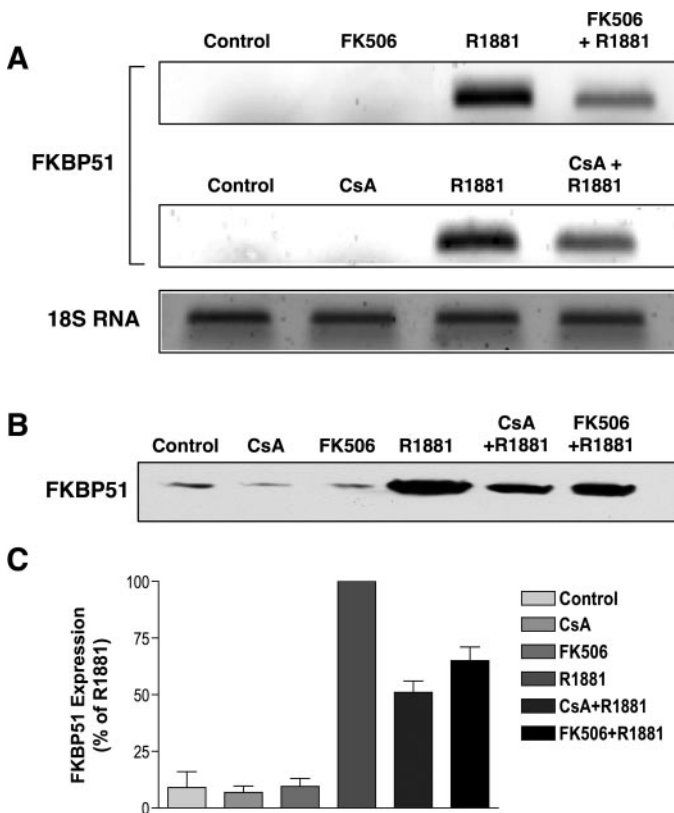


FIG. 6. CsA and FK506 suppress androgen-induced expression of FKBP51. **A**, LNCaP cells were treated with R1881 (1.0 nM) in the presence and absence of CsA (5.0 μ M) or FK506 (10.0 μ M) for 20 h. Total RNA was extracted, reverse transcribed, and amplified. Amplified PCR products were electrophoresed on 1% agarose gels and visualized by ethidium bromide staining. Ribosomal 18S RNA was used as an internal control. **B**, LNCaP cells were treated with R1881 (1.0 nM) in the presence and absence of CsA (5.0 μ M) or FK506 (10.0 μ M) for 20 h. Whole cell lysates were prepared and analyzed by Western blot for FKBP51 protein. **C**, Bands corresponding to FKBP51 protein were analyzed by densitometric scanning of the films. Results are representative of two independent experiments.

that the AR complex from control cytosol contains Cyp40 and that CsA treatment does not disrupt this interaction. To the best of our knowledge, this is the first report of Cyp40 association with the AR. More importantly, the data indicate that CsA has no effect on the Cyp40/AR interaction, suggesting that inhibition by CsA of the Cyp40 PPlase function may be the primary event, rather than altered protein-protein interaction.

Blockade of AR nuclear translocation by CsA

Because FK506 reduced AR hormone binding to approximately the same extent as the effect of this ligand on androgen-induced growth (Fig. 4) and AR-mediated transcription (Fig. 5), it seemed reasonable to conclude that the primary stage of FK506 action is at the level of AR hormone-binding function. In contrast, the moderate reduction of hormone binding by CsA cannot fully explain this compound's more potent effect on prostate cell growth and AR transactivation. Therefore, we reasoned that CsA is either working by two completely distinct mechanisms, or that it can inhibit AR activity at multiples stages of action. To test this, we measured the hormone-induced nuclear localization of the AR in cells treated with CsA. This was achieved by transfecting LNCaP and PC-3 cells with GFP-AR plasmid. Subcellular localization of GFP-AR was examined by laser scanning confocal microscopy. As expected, unliganded GFP-AR in LNCaP cells was predominantly localized to the cytoplasm, and CsA alone did not affect this distribution (Fig. 8). In response to R1881, GFP-AR translocated to the nucleus in 90.3% of cells examined (SEM = \pm 5.78 for three independent transfection experiments). Interestingly, in cells treated with CsA, R1881 caused GFP-AR nuclear translocation in only

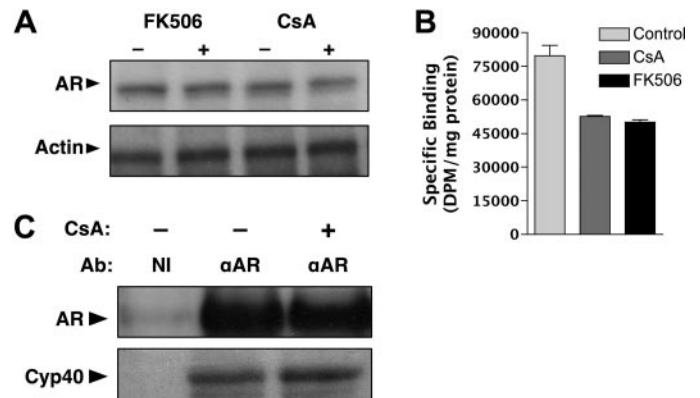


FIG. 7. Effects of CsA and FK506 on AR hormone binding capacity and Cyp40 interaction. **A**, LNCaP cells were treated with or without CsA (5.0 μ M) or FK506 (10.0 μ M) for 20 h, and whole cell lysates were prepared and analyzed by Western blot for total AR protein or actin (loading control). **B**, Cytosolic extracts from similarly treated LNCaP cells were incubated at 4 C with 10 nM [³H]mibolerone for 20 h. Specific binding is expressed as dpm/mg protein. Results shown are the means \pm SEM of three independent experiments, each performed in triplicate. **C**, To assess Cyp40 interaction with the AR and the effect of CsA, aliquots of LNCaP cytosol were treated on ice for 2 h with CsA (5.0 μ M) or vehicle, followed by incubation for 1 h at 37 C. After rechilling, AR complexes were immunoadsorbed with nonimmune antibody (NI) or antibody specific for AR (α AR), and analyzed for the presence of AR and Cyp40 by Western blotting. Results are representative of four independent experiments.

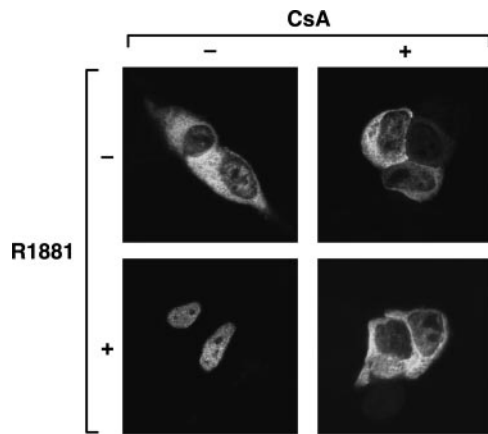


FIG. 8. CsA blocks androgen-induced GFP-AR nuclear translocation. LNCaP cells were transiently transfected with GFP-AR expression plasmid. After 24 h, cells were treated with or without CsA ($5.0 \mu\text{M}$) for 2 h, followed by treatment with or without R1881 (1.0 nM) for 3 h. Fluorescent images of GFP-AR expression were captured by confocal laser scanning microscopy. Images are representative of three independent transfection experiments. Similar results were obtained in PC-3 cells transfected with GFP-AR.

10.7% of cells ($\text{SEM} = \pm 6.36$). Because CsA had only a partial effect to reduce AR hormone-binding function (Fig. 7), this would suggest that CsA acts to inhibit the AR at more than one stage of signaling, with the major effect perhaps being blockade of nuclear translocation. Similar results were obtained when PC-3 cells were transfected with GFP-AR (data not shown).

Discussion

Previous studies have shown contributions by FKBP52, FKBP51, PP5, and Cyp40 to protein folding, ligand binding, and nuclear localization of GR and PR receptors (18, 68). In this report we studied the roles of these proteins in AR action by measuring their expression levels in prostate cell lines and the effects of immunophilin ligands on AR-induced prostate cell growth. We show for the first time that prostate cancer cells (LNCaP, PC-3, and DU145) have a general pattern of overexpression for FKBP52, FKBP51, Cyp40, and PP5 compared with normal PrECs, and that FK506 and CsA inhibit androgen-induced prostate cell growth by inhibiting AR activity. These results represent an alternative to androgen ablation in the treatment of prostate cancer. Of course, the well-characterized ability of FK506 and CsA to suppress the immune system via FKBP12 and Cyp18 (14, 16) is presently an obstacle to this approach. However, recent findings describing FKBP52-deficient mice (12, 39) suggest that this approach is worth pursuing. In this case, loss of FKBP52 caused male infertility through a selective abrogation of AR activity, whereas apparently leaving all other steroid receptor processes in the male unchanged. Moreover, only select AR-controlled tissues were affected in male FKBP52 KO animals, most notably the prostate gland. Thus, if immunophilin ligands can be developed that discriminate between FKBP12 and FKBP52, or Cyp18 and Cyp40, then selective targeting of AR-controlled prostate cell growth without side effects on the immune system or other steroid-regulated physiology should be possible.

Because FK506 and CsA are known to act via low-molecular weight immunophilins to inhibit CaN activity in T cells, it is possible that FK506 and CsA inhibit androgen-induced prostate cell growth by blocking CaN rather than by interactions with the TPR-containing immunophilins found in the AR complex. However, at least in LNCaP cells, our results do not support this model. First and foremost is our finding that Cyp40, like FKBP52 (69), is indeed a component of the AR in LNCaP cells. We also showed a good correlation between FK506 and CsA inhibition of LNCaP proliferation with decreased AR activity at the hormone-binding, nuclear translocation, and transactivity stages of receptor action. Moreover, if CaN were the primary target of FK506 and CsA in prostate cells, we would expect these drugs to show the same relative efficacies published for inhibition of T-cell activation. This is not the case because the IC_{50} value for inhibition of T-cell proliferation is 100 nM for CsA and 1.0 nM for FK506 (70, 71), yet we find CsA to be a more potent inhibitor than FK506 with respect to LNCaP proliferation ($1.0\text{--}2.5$ vs. $10.0 \mu\text{M}$, respectively). Finally, FK506 and CsA inhibited CaN activity not only in LNCaP cells, but also in PC-3 cells, a cell line whose growth is not inhibited by FK506. Thus, we conclude that the most likely targets of CsA and FK506 action as inhibitors of prostate cell growth are the AR-associated immunophilins.

In addition to inhibition of androgen-mediated AR function, we have also observed androgen-independent growth inhibition by CsA and FK506 in prostate cancer cell lines. In the case of FK506, androgen-independent inhibition was seen only in the AR-positive LNCaP cells, suggesting that the AR is required for this effect. Because AR protein even in the absence of hormone is known to stimulate proliferation in LNCaP cells (6, 72, 73), it is plausible that FK506 acting through FKBP51 or FKBP52 may control the hormone-free actions of the AR. An alternative is that FKBP51 or FKBP52 contributes to prostate cell growth independent of the AR through some unknown mechanism. However, this seems unlikely because FK506 did not inhibit growth in the PC3 or DU145 cell lines, even though both cell lines express as much FKBP52 as LNCaP cells, whereas DU145 cells express high levels of FKBP51 (Fig. 1). In contrast to FK506, CsA was able to inhibit effectively androgen-independent growth in all three cell lines, suggesting that it must act on a target distinct from that of FK506 and that the target can control proliferation independent of the AR. The obvious candidate would be Cyp40, and the fact that LNCaP, PC3, and DU145 all express high levels of Cyp40 supports this notion. The only other known target of CsA is Cyp18, in which case suppression of growth could occur, as already mentioned, through the CaN pathway. We did observe inhibition of CaN activity by CsA in LNCaP and PC-3 cells, but CaN in PC-3 cells was also inhibited by FK506, even though this compound did not inhibit PC-3 growth. Thus, it is more likely that CsA controls prostate cell growth either via Cyp40 or a novel target.

This issue aside, there is evidence for the downstream effects of CsA and Cyp40 on cancer growth. For example, CsA stimulates TGF- β expression (74, 75), which is known to inhibit prostate growth (76–78). Furthermore, in both pancreatic acinar AR42J cells and kidney epithelial LLC-PK1 cells, CsA induces cell-cycle arrest through reduction of cy-

clin D1 levels (58, 79). This is relevant because elevated cyclin D1 is associated with prostate cancer progression (80–82). With respect to Cyp40, it has been overexpressed in breast tumors (83), and a recent study has shown that it negatively regulates the progrowth transcription factor c-Myb (84). Finally, deletion of Cpr7, the Cyp40 homolog in yeast, generates a slow-growth phenotype that correlates with reduced activity of the Hsp90-dependent oncogene PP60V-Src (37).

Other laboratories (10, 13) have shown FKBP52 and FKBP51 to be a part of AR complexes in LNCaP cells. Here we show that Cyp40 also interacts with the LNCaP cell AR (Fig. 7), suggesting a role for this TPR in AR action. Through the use of CsA, we have inferred at least two roles for Cyp40: regulation of AR hormone-binding function and hormone-induced nuclear translocation. Interestingly, although CsA caused a decrease in AR hormone-binding capacity (Fig. 7), no dissociation of Cyp40 from the AR was noted. This is in contrast to our work with GR, in which FK506 treatment caused an increase in receptor hormone-binding affinity by a mechanism that displaces both FKBP51 and FKBP52, and allows replacement with PP5 on the TPR acceptor site of Hsp90 (26). Thus, the lack of displacement by Cyp40 may explain why AR hormone-binding activity is compromised by CsA because compensation by another member of the TPR family is not possible. This result also suggests that the PPIase function of Cyp40 is important to the hormone-binding process. The dramatic blockade of AR nuclear translocation (Fig. 8) was also surprising because our work with the GR of L929 cells showed a potentiation of this receptor function by FK506 (24, 26). In this case, potentiation of translocation could be explained, not only by the increase in GR hormone-binding affinity, but also by the ability of the recruited PP5 to bind the motor protein dynein (85). Because Galigniana *et al.* (85, 86) have shown a strong interaction between Cyp40 and dynein, we can speculate that CsA blocks AR translocation by disrupting the Cyp40/dynein interaction, in a manner that does not allow compensation by another dynein-interacting TPR protein, such as PP5 or FKBP52.

Here we show that FK506 inhibits androgen-induced proliferation of LNCaP cells by abrogating AR activity. Based solely on our present data and the fact that both FKBP51 and FKBP52 can associate with LNCaP cell AR, we do not yet know if FK506 acts on both FKBP51 and FKBP52 or whether it is selective for one over the other. Recent data from other laboratories point to FKBP51 as a logical target for FK506 in prostate cells. For example, microarray analysis showed elevated expression of FKBP51 in prostate cancer xenografts compared with benign prostate hyperplasia (47). It is now also clear that FKBP51 is an AR-regulated gene (45, 48), and at least one study has shown that overexpression of FKBP51 potentiates AR transcriptional activity in LNCaP cells (13). Thus, FKBP51 may not only be a potential marker of prostate cancer but may also serve as a therapeutic target. However, recent work from our laboratory suggests that FKBP52, not FKBP51, is the major regulator of AR action in the prostate. Like the Smith laboratory (12), we have also generated FKBP52 KO mice that show prostate dysgenesis. In the same work, we also describe FKBP51 KO animals, which show no abnormalities of prostate development (39). Indeed, as far as we know, no AR-

regulated physiology has been affected in FKBP51 KO males. Of course, it is possible that the two FKBP5s play dual roles, with FKBP52 responsible for AR-regulated organogenesis of the prostate, whereas FKBP51 plays a yet-to-be-defined role in the adult prostate that when dysregulated, perhaps leading to overexpression, causes aberrant activity on the part of AR.

In summary, we have shown that the immunophilin ligands FK506 and CsA are effective inhibitors of both androgen-dependent and -independent prostate cell growth. Although we do not yet know their exact mechanisms of action, it is clear that AR signaling is targeted in LNCaP cells. Further understanding of the mechanisms involved will require more refined approaches, such as mouse models of prostate-specific overexpression of FKBP52, FKBP51, and Cyp40, as well as the development of ligands specific for each TPR.

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Address all correspondence and requests for reprints to: Dr. Sumudra Periyasamy, Department of Physiology and Pharmacology, University of Toledo College of Medicine, 3035 Arlington Avenue, Toledo, Ohio 43614-5804. E-mail: Sumudra.Periyasamy@utoledo.edu.

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