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Squirrel Monkey Immunophilin FKBP51 Is a Potent Inhibitor of Glucocorticoid Receptor Binding*

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ABSTRACT

Squirrel monkeys have high circulating cortisol to compensate for expression of low-affinity glucocorticoid receptors (GRs). We have demonstrated that the FK506-binding immunophilin FKBP51 is elevated in squirrel monkey lymphocytes (SML) and, in preliminary studies, have shown that squirrel monkey FKBP51 is inhibitory to GR binding. In this report, we have demonstrated that elevated FKBP51 is the unequivocal cause of glucocorticoid resistance in SML in the following ways: 1) FK506 increased GR binding in cytosol from SML in a concentration-dependent manner, an effect reproduced by rapamycin but not cyclosporin A. The apparent K_d (6.1 nM) and rank-order of steroid displacement of [^{3}H]dexamethasone binding in FK506-treated SML cytosol are characteristic of high-affinity GR binding. 2) cytosol from COS-7 cells expressing squirrel monkey FKBP51 inhibited GR binding in cytosol from human lymphocytes by 74%. Cytosol from COS-7 cells expressing human FKBP51 inhibited GR binding by 23%. 3) expression of squirrel monkey FKBP51 increased the median effective concentration (EC_{50}) for dexamethasone in GR transactivation studies in COS-7 cells by approximately 17-fold, compared with the EC_{50} in control cells. The expression of human FKBP51 increased the EC_{50} for dexamethasone in COS-7 cells by less than 3-fold, compared with control. Squirrel monkey FKBP51 shares 94% overall amino acid homology with human FKBP51, with 92% and 99% homology with human FKBP51 in the peptidyl-prolyl isomerase and the tetratricopeptide repeat domains, respectively. Amino acid differences in the more variable N- or C-terminal regions or in regions which join the highly homologous functional domains may be responsible for its more potent inhibitory activity. (*Endocrinology* **141:** 4107–4113, 2000)

SOME SPECIES OF neotropical primates, including squirrel monkeys, cotton-top tamarins, and owl monkeys, have markedly elevated plasma cortisol levels secondary to a generalized state of glucocorticoid resistance (1). We and others have shown that this results, at least in part, from the expression of glucocorticoid receptors (GRs) with low binding affinity (1, 2). However, the low binding affinity does not result from mutations in the receptor protein, a cause of some forms of glucocorticoid resistance in humans (3), as squirrel monkey GRs expressed in reticulocyte lysate exhibit high-affinity binding (2). Rather, we found that squirrel monkeys express a soluble inhibitory factor, which, in mixing studies of cytosol from squirrel monkey lymphocytes (SML) with mouse L929 cell cytosol, reduces GR binding affinity by 11-fold (4). Our recent goals have been to identify this factor and to determine how it affects GR binding.

The binding activity of the GR is dependent on the ordered assembly of a mature receptor heterocomplex (reviewed in Refs. 5 and 6). Four proteins (hsp90, hsp70, Hop, and hsp40) are thought to be essential for the conversion of GR to a hormone binding state, which is stabilized by the inclusion of p23 in the complex (7–9). The omission of any of these proteins might affect the formation of a GR with optimal binding activity. Indeed, altered expression of hsp90 or expression of mutant forms of hsp90 is associated with reduced responsiveness to glucocorticoids (10–12). We compared the levels of components of the GR heterocomplex in cytosol from SML with those in human lymphocytes (HL). We found that the levels of hsp90, hsp70, Hip, Hop, and p23 were quite similar in these cell types (4). On the other hand, the levels of some of the hsp90-associated immunophilins showed marked differences between squirrel monkey and human cells. Four such proteins have been shown to interact with the hsp90-GR complexes: the FK506-binding immunophilins, FKBP51 and FKBP52, cyclophilin 40 (CyP-40), and the protein phosphatase (PP5) (5). The level of FKBP51 was 13-fold higher in SML than in HL cytosol, whereas FKBP52 in SML was less than one-half that in human cells (4). The idea that an immunophilin might affect GR binding, however, is contrary to the conclusions of several studies regarding the role of these proteins in GR heterocomplexes. First, GR heterocomplexes with hormone-binding activity can be reconstituted in the absence of immunophilins (7, 8). Second, neither FK506 nor cyclosporin A, which inhibit the peptidyl-prolyl isomerase (PPIase) activities of FKBP51 and FKBP52 or CyP-40, respectively, affect GR binding (13, 14).

However, we have preliminary evidence that the inhibitory effect on GR binding of SML cytosol is reproduced with cytosol from COS cells expressing squirrel monkey FKBP51 (4, 15). In this paper, we present data that unequivocally

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demonstrates that squirrel monkey FKBP51 is a potent inhibitor of GR binding and likely contributes to glucocorticoid resistance in this species. First, we showed that incubation with FK506 and rapamycin increased [^{3}H]dexamethasone binding in cytosol from SML by almost 5-fold. The binding affinity and rank-order of displacement by different steroids were characteristic of high-affinity GR binding. Second, we showed that squirrel monkey FKBP51 is 8-fold more potent than human FKBP51 in affecting GR binding in cytosol mixing studies and nearly 7-fold more potent than human FKBP51 in dexamethasone-induced transcriptional activity in COS cells transfected with human GR. Comparison of the squirrel monkey and human FKBP51 sequences indicates that the two proteins share 94% overall amino acid identity, 92% identity within the PPIase domains, and 99% identity within the tetratricopeptide repeat (TPR) domains. However, we have identified a number of potentially functionally important substitutions present in the N- and C-terminal regions and in sequences joining functional domains of squirrel monkey FKBP51 that may contribute to its species-specific inhibitory activity.

Materials and Methods

Materials

Culture medium was obtained from Life Technologies (Grand Island, NY). Defined and charcoal-dextran-treated FBS was purchased from HyClone Laboratories, Inc. (Logan, UT). FK506 and mifepristone were kindly provided by Fujisawa USA, Inc. (Deerfield, IL) and Roussel-Uclaf (Romainville, France), respectively. Rapamycin, dexamethasone, cortisol, progesterone, 17β-estradiol, L-proline, and ATP were purchased from Sigma, St. Louis, MO). [^{3}H]Dexamethasone (82–86 Ci/mmol) was from Amersham Pharmacia Biotech (Arlington Heights, IL). The antibody to human FKBP51 (Hi51) has been described previously (16). Horseradish peroxidase-labeled goat antimouse IgG was from American Qualex (San Clemente, CA). The GR-pcDNA1.1/Amp plasmid was constructed from hGR-pGEM7 (2) by excision with BamHI and XbaI and ligation into pcDNA1.1/Amp (Invitrogen, Carlsbad, CA). The mouse mammary tumor virus (MMTV) promoter-luciferase reporter vector was provided by Dr. R. M. Evans (Salk Institute, La Jolla, CA).

Cell cultures

The squirrel monkey B-lymphoblast cell line (SML) was transformed with Epstein-Barr virus (17). A HL cell line, also transformed with Epstein-Barr virus, was provided by Dr. David Brandon (Oregon Health Sciences University, Portland, OR). COS-7 cells were obtained from American Type Culture Collection (Manassas, VA). HL and SML were grown in suspension cultures in RPMI 1640 medium supplemented with 10% FBS, 50 U/ml penicillin G, and 0.05 mg/ml streptomycin. COS-7 cells were grown in monolayers in DMEM supplemented with 10% FBS and antibiotics. Cells were grown at 37 C in a humidified atmosphere of 5% CO_2-95% air.

GR binding analysis

Cells were washed in PBS and resuspended in HEM buffer [10 mM HEPES (pH 7.4), 2 mM EDTA, 20 mM sodium molybdate] and lysed by sonication. A soluble fraction (hereafter referred to as cytosol) was isolated by centrifugation at 100,000 $\times$ *g* for 1 h at 4 C. Protein concentrations were quantified by the method of Bradford (18). Fifty-microliter aliquots of cytosol were added to a mix containing [^{3}H]dexamethasone in the absence and presence of excess radioinert dexamethasone. In some experiments, the concentration of radioinert dexamethasone was varied or the effects of mifepristone, cortisol, progesterone, or 17β-estradiol (10^{-9} to 2×10^{-5} M) on binding were evaluated. After 18 h at 4 C, free steroid was removed by the addition of dextran-coated charcoal, and the radioactivity in the supernatants was determined. Specific binding was determined by subtracting nonspecific counts from total counts. Data were analyzed by nonlinear regression analysis and visualized by the method of Scatchard (19) using PRISM software (GraphPad Software, Inc., San Diego, CA).

Expression of FKBP51 in COS-7 cells

The construction of expression plasmids containing either squirrel monkey or human FKBP51 complementary DNA (cDNA) has been reported previously (4). The sequences of these inserts were determined across both strands using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and a PE Applied Biosystems 373XL sequencer (Foster City, CA). These plasmids were used in each of two transfection protocols. First, 24 h before transfection, COS-7 cells were plated in 100-mm dishes at 8×10^5 cells per dish. Cells were transfected with 5 μg/dish of either pCI-neo (control) or pCI-neo containing squirrel monkey or human FKBP51 cDNA (sm51-pCI-neo and h51-pCI-neo, respectively) using Superfect transfection reagent (QIAGEN, Valencia, CA). After 3 h, the medium was replaced, and the cells were incubated for 24 h before cytosol was collected. The samples were adjusted to the same protein concentration (2 mg/ml) and mixed for subsequent analysis of GR binding activity.

Second, the effects of expression of squirrel monkey and human FKBP51 on ligand-induced transcriptional activation of GR in COS-7 cells were evaluated. A modification of the method of Bodwell *et al.* (20) was used. COS-7 cells were maintained as above, except that 39.5 μg/ml L-proline was added to the medium. Cells in log-phase growth were washed in PBS and suspended in electroporation buffer [10 mM piperazine diethanesulfonic acid (pH 7.4), 137 mM NaCl, 2.7 mM KCl, 2.7 mM EGTA, 5.6 mM glucose, 1 mM ATP] at a density of 2.0×10^7 cells/ml, on ice, for 15 min. Aliquots (300 μl) of the cell suspension were mixed with 50 μl TE buffer [10 mM Tris (pH 7.5), 1 mM EDTA] containing 3 μg GR-pcDNA1.1/Amp plasmid, 5 μg MMTV luciferase reporter vector, and 10 μg of either sm51-pCI-neo, h51-pCI-neo, or empty pCI-neo vector and were electroporated using a Gene Pulser II with Capacitance Extender Plus and Pulse Controller Plus modules (Bio-Rad Laboratories, Inc. Hercules, CA) with the PulseTrac system activated and set to deliver a pulse of 174 V. Capacitance (2.0–2.4 millifarads) was adjusted to give a time constant of approximately 140 msec. Electroporated cells were diluted in DMEM with 10% charcoal/dextran-treated FBS, 39.5 μg/ml L-proline, and antibiotics, as above, and plated in 60-mm dishes at a density of 8×10^5 cells/dish. After 18 h, the medium was replaced, and the cells were treated with dexamethasone (0.1–100 nM). Cell extracts were prepared after a further 24-h incubation using 300 μl/dish reporter lysis buffer (PharMingen, San Diego, CA) and were assayed for luciferase activity, as described previously (21). To confirm that the expression of transfected plasmids was similar in each set of cells, the levels of GR and FKBP51 were determined by Western blot analysis, as described previously (4). The endogenous levels of hsp90 were used as an internal standard.

Results

Effect of FK506 on GR binding in SML

The goal of these studies was to substantiate that the FK506-binding protein FKBP51 is responsible for low GR binding in squirrel monkey cells. First, we examined the effects of FK506 on GR binding in SML cytosol. SML cytosol was incubated with various concentrations of FK506 for 2 h, on ice, before the determination of GR binding. Specific binding of [^{3}H]dexamethasone, which was low in untreated SML cytosol, was increased 4.5-fold by FK506 with a median effective concentration (EC_{50}) of approximately 250 nM (Fig. 1), consistent with the interaction of FK506 with a large-molecular-weight immunophilin (22). FK506 had no effect on [^{3}H]dexamethasone binding in cytosol from HL (data not shown), consistent with most, but not all, previous studies showing that FK506 does not affect steroid binding activity of high-affinity GR complexes (13, 23, 24). We also evaluated

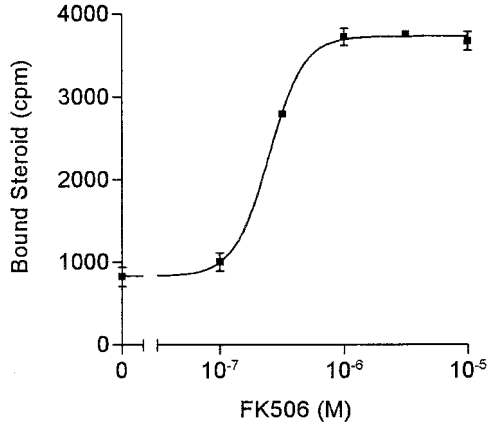

FIG. 1. Effect of FK506 on GR binding in SML cytosol. SML cytosol (3 mg/ml) was incubated for 2 h, on ice, in the presence of varying concentrations of FK506 and was assayed for GR binding using 10 nM [^{3}H]dexamethasone in the presence and absence of 500-fold excess dexamethasone. This plot represents the mean ± range of two separate experiments.

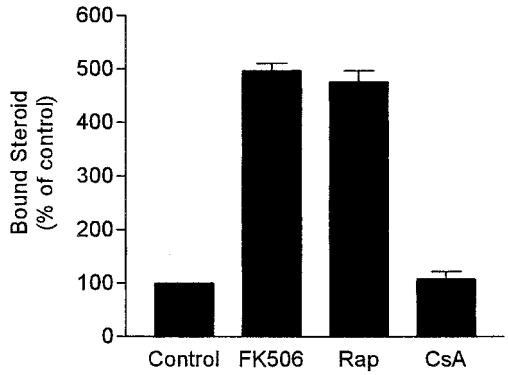

FIG. 2. Effects of FK506, rapamycin, and cyclosporin A on GR binding in SML cytosol. SML cytosol (1 mg/ml) was incubated for 2 h, on ice, in the presence of 10 μM FK506, 1 μM rapamycin (Rap), or 10 μM cyclosporin A (CsA) and was assayed for GR binding using 10 nM [^{3}H]dexamethasone in the presence and absence of 500-fold excess dexamethasone. This plot represents the mean ± SEM of three separate experiments.

the effects of rapamycin and cyclosporin A. FK506 and rapamycin bind small and large immunophilins, including FKBP51 and FKBP52, whereas cyclosporin A binds CyP-40 (22). Rapamycin was as efficacious as FK506 in stimulating the specific binding of [^{3}H]dexamethasone in SML cytosol, but cyclosporin A had no effect (Fig. 2). The binding activity of FK506-treated SML cytosol was further evaluated to confirm that the dramatic increase in [^{3}H]dexamethasone binding observed with FK506 and rapamycin represents bona fide GR binding. First, nonlinear regression analysis of saturation curves was performed on [^{3}H]dexamethasone binding in SML cytosol after incubation with vehicle or 2.3 μM FK506. Specific binding in control SML cytosol was sufficiently poor that we were unable to obtain reproducible K_d values. However, in FK506-treated SML cytosol we obtained an apparent K_d for [^{3}H]dexamethasone binding of 6.1 ± 0.4 nM (n = 5), approximating that determined in cytosol from FK506-treated HL (4.3 ± 0.4 nM, n = 5) used as a high-affinity GR control. Second, we generated displacement curves with different steroid hormones to confirm that [^{3}H]dexamethasone binding in FK506-treated SML cytosol exhibited rank-order competition consistent with binding to high-affinity GR. [^{3}H]Dexamethasone binding was displaced by dexamethasone ≈ mifepristone > cortisol > progesterone > estradiol, a rank-order identical to that observed for [^{3}H]dexamethasone binding in FK506-treated cytosol from HL (Fig. 3). These results show that [^{3}H]dexamethasone binding, induced by FK506 in SML cytosol, represents binding to high-affinity GR; and this provides support for the role of an FK506-binding protein in mediating the low binding activity of GR in squirrel monkey cells.

Expression of FKBP51 and GR activity

We demonstrated that the FK506-binding protein responsible for low GR binding in squirrel monkey cells is FKBP51, in two ways: first, we measured [^{3}H]dexamethasone binding in mixtures of cytosol from HL and COS-7 cells expressing squirrel monkey FKBP51. The effect of squirrel monkey FKBP51 was compared with that of human FKBP51. Mixtures were of equal volumes (150 μl) of HL cytosol and

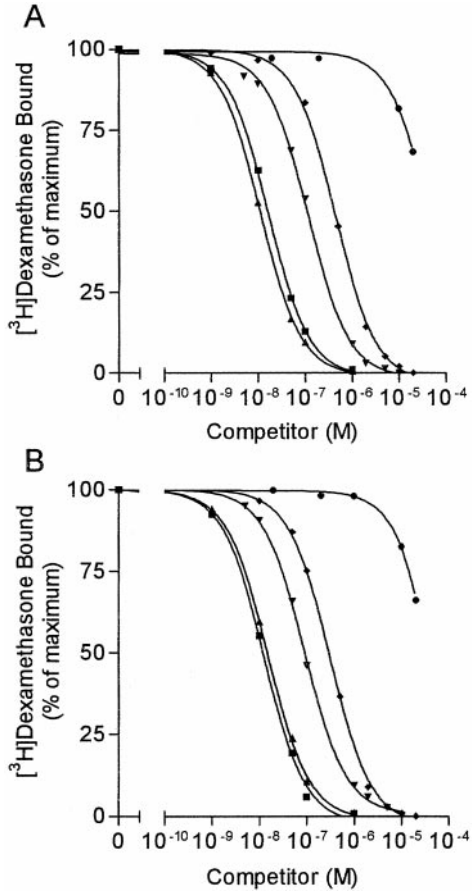

FIG. 3. Typical [^{3}H]dexamethasone displacement curves obtained with dexamethasone and other steroid hormones. [^{3}H]Dexamethasone (10 nM) binding was to cytosol from either SML (A) or HL (B), treated with 2.3 μM FK506. The data are expressed as the percent of specific [^{3}H]dexamethasone bound in the absence of competing steroids (set at 100%). Nonspecific binding was defined by binding in the presence of 5 μM dexamethasone. -■-, Dexamethasone; -▲-, mifepristone; -▼-, cortisol; -◆-, progesterone; -●-, 17β-estradiol.

cytosol from COS-7 cells, which were made up of the indicated volumes of cytosol from COS-7 cells transfected with squirrel monkey or human FKBP51, and the remainder made up of cytosol from control COS-7 cells. Cytosol extracts from COS-7 cells expressing squirrel monkey FKBP51 inhibited GR binding in HL, with the highest volume resulting in a 74% reduction in specific binding (Fig. 4). Extracts from COS-7 cells expressing human FKBP51 were much less inhibitory, achieving only a 23% inhibition of GR binding when the highest concentration of human FKBP51 was included in the assay. Because the levels of expression of squirrel monkey and human FKBP51 were similar in transfected COS-7 cells (typical Western blots of cell cytosols are shown in Fig. 5A), we could conclude that squirrel monkey FKBP51 is approximately 8-fold more potent than human FKBP51 in inhibiting GR binding.

We further evaluated the apparent difference in inhibitory activity of squirrel monkey and human FKBP51, by investigating the effects of FKBP51 expression on GR activation in cell culture. COS-7 cells were transfected with a plasmid expressing human GR, an MMTV-luciferase reporter plasmid, and either an empty vector or vector containing squirrel monkey or human FKBP51 cDNA. COS-7 cells were chosen for these experiments because they are routinely used for GR transactivation studies and normally express very low levels of FKBP51. The activation of the reporter plasmid was determined by assaying the cells for luciferase activity after treatment with dexamethasone for 24 h. In each experiment, the levels of expression of squirrel monkey and human FKBP51, as well as GR, were determined by Western blot analysis of cell lysates and were found to be the same (typical Western blots are shown in Fig. 5B). Treatment with dexamethasone induced the transactivation of GR in COS-7 cells transfected with empty pCi-neo vector with an EC_{50} of 0.8 ± 0.2×10^{-9} M (n = 3) (Fig. 6). Expression of human FKBP51 increased the EC_{50} by almost 3-fold (2.2 ± 0.2 × 10^{-9} M, n = 3) and changed the shape of the dexamethasone-induction curve. The expression of squirrel monkey FKBP51 had a greater effect on the sensitivity to dexamethasone, increasing the EC_{50} by 17-fold, compared with control (EC_{50} of 13.7 ± 0.2 × 10^{-9} M, n = 3) (Fig. 6). The expression of squirrel monkey FKBP51 also affected the shape of the dexamethasone-induction curve, compared with control.

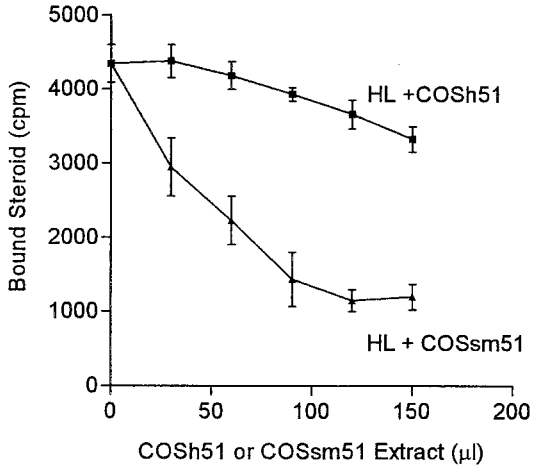

FIG. 4. The effect of cytosol from cells expressing FKBP51 on GR binding in HL cell cytosol. Cytosol was isolated from HL, control COS-7 cells, or COS-7 cells expressing either squirrel monkey FKBP51 (COSsm51) or human FKBP51 (COSh51). The cytosols were adjusted to 2 mg/ml and mixed on ice for 2 h. Mixtures were made up of equal volumes (150 μl each) of HL cytosol and the indicated volumes of COSsm51 or COSh51 cell cytosol, the remainder made up of cytosol from control cells. GR binding was assayed using 10 nM [^{3}H]dexamethasone in the presence and absence of 500-fold excess dexamethasone. Each *point* represents the mean ± SEM of three separate experiments.

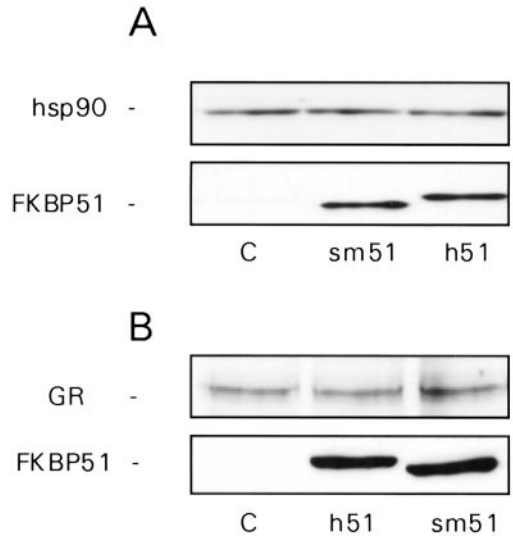

FIG. 5. Expression of human and squirrel monkey FKBP51, hsp90, and GR in COS-7 cells. A, COS-7 cells were transfected using Superfect with empty pCI-neo (C) or pCI-neo containing squirrel monkey (sm51) or human FKBP51 (h51) cDNAs and, after 24 h, collected for Western blot of hsp90 and FKBP51; B, COS-7 cells were transfected by electroporation with GR-pcDNA1.1/Amp and MMTV-luciferase plasmids and either empty pCI-neo (C) or pCI-neo containing human (h51) or squirrel monkey FKBP51 (sm51) cDNAs and, after 24 h, collected for Western blot of GR and FKBP51.

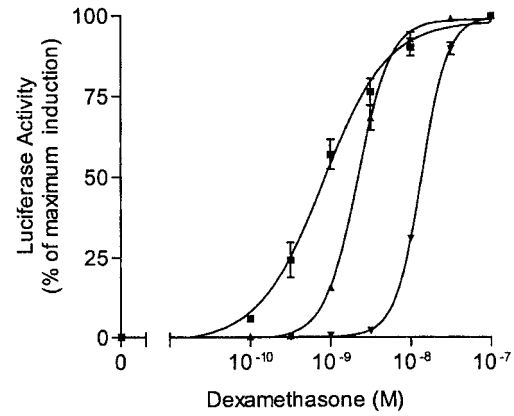

FIG. 6. The effect of expression of FKBP51 on transactivation of GR by dexamethasone in COS-7 cells. COS-7 cells were electroporated with GR-pcDNA1.1/Amp, MMTV-luciferase, and either empty pCI-neo (control) or pCI-neo containing squirrel monkey or human FKBP51 cDNAs and treated with the indicated concentrations of dexamethasone. After 24 h, the cells were collected for assay of luciferase activity. Each *point* represents the mean ± SEM of three separate experiments. -■-, Control; -▲-, human FKBP51; -▼-, squirrel monkey FKBP51.

Primary amino acid sequence of squirrel monkey FKBP51

The difference in activities of expressed squirrel monkey and human FKBP51 on receptor binding and transactivation assays prompted a comparison of their respective amino acid sequences. Squirrel monkey FKBP51 cDNA was sequenced across both strands, and the deduced amino acid sequence was compared with that of human FKBP51 (Fig. 7). The mouse FKBP51 was included for comparison. The squirrel monkey FKBP51 cDNA sequence has been deposited in GenBank with the accession number AF140759. Squirrel monkey FKBP51 is 94% identical to human FKBP51 and 84% identical to mouse FKBP51. The TPR domains, made up of three TPR units, and necessary for the interaction of immunophilins with hsp90 (14, 16, 25, 26), exhibited striking homology between squirrel monkey and human FKBP51 sequences (Fig. 7, *double underline*). Squirrel monkey and human FKBP51 differed in only one amino acid (residue 285) in this domain. The PPIase domains of squirrel monkey and human FKBP51s exhibited greater diversity, sharing 92% homology (Fig. 7, *single underline*) but are identical in conserved residues required to bind FK506 (22). The consensus calmodulin-binding motif of squirrel monkey FKBP51 (Fig. 7, *dashed underline*) differed in one amino acid (residue 408), compared with the human sequence. Amino acid differences between squirrel monkey and human FKBP51, which significantly alter charge and/or polarity, are highlighted (Fig. 7, *shaded residues*). Eight amino acid differences in squirrel monkey FKBP51 represent substitutions that impart a more basic charge, which may be responsible for the more rapid migration of squirrel monkey FKBP51 on SDS-polyacrylamide gels (Fig. 5). Regardless, detailed site-directed mutagenesis may be necessary to determine the structural basis for the increased inhibitory activity of squirrel monkey FKBP51, compared with the human protein.

Discussion

These results show that squirrel monkey FKBP51 is a potent inhibitor of GR binding, and they support our contention that increased expression of the FK506-binding immunophilin FKBP51 is a major cause of glucocorticoid resistance in squirrel monkeys. We had previously shown that the levels of FKBP51 are higher in squirrel monkey cells (4) and now have demonstrated that expression of squirrel monkey FKBP51 significantly reduces the ability of dexamethasone to bind to and activate GR. Furthermore, squirrel monkey FKBP51 is more potent than human FKBP51 in inhibiting GR activity. Thus, changes in both expression and structure of FKBP51 occurred during the course of independent evolution of the squirrel monkey on the South American continent and led to end-organ resistance to glucocorticoids. This must have prompted a number of compensatory physiological and biochemical changes in squirrel monkeys to maximize the levels of free cortisol. For example, high levels of ACTH drive increased synthesis and secretion of cortisol from the adrenal gland (27, 28). Second, squirrel monkeys express corticosteroid-binding globulin with remarkably low affinity for cortisol (29, 30). Third, there is a low rate of metabolic clearance of cortisol (27), and also peripheral 11β-hydroxysteroid dehydrogenase 1 in squirrel monkeys favors conversion of cortisone to cortisol (31). However, the secretion of cortisol seems to be regulated quite normally in squirrel monkeys. For example, cortisol secretion in squirrel monkeys is appropriately stimulated by chair restraint and social and environmental disturbances (32, 33). Thus, squirrel monkeys

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smFKBP51 MTTDEGAKNS RGNPAATVAE QGEDVTSKKD RGVLKIVKRV GHGEETPMIG DRVYVHYNGK  60
 hFKBP51 ---------N EES-T----- ----I----- ---------- -N-------- -K-----K--  60
 mFKBP51 ------TS-N GE-----MT- ----I-T--- ---------- -TSD-A--F- -K-----K-M  60

smFKBP51 LANGKKFDSS HDRNEPFVFS IGKGQVIKAW DIGVATMKKG EICHLLCKPE YAYGATGSLP 120
 hFKBP51 -S-------- ---------- L--------- ---------- ---------- -----SA---- 120
 mFKBP51 -SD------- ---KK--A-- L-Q------- ----S----- ---------- -----SA-H-Q 120

smFKBP51 KIPSNATLFF EVELLDFKGE DLLEDGGIIR RTKRRGEGYS NPNEGARVQI HLEGRCGGRV 180
 hFKBP51 ---------- -I-------- --F------- ----K----- ------T-E- ---------M 180
 mFKBP51 ---------- -I-------- --F--S-V-- -I--K----- ------T-KV ----C----T 180

smFKBP51 FDCRDVAFTV GEGEDHDIPI GIDKALEKMQ REEQCILHLG PRYGFGEAGK PKFGIEPNAE 240
 hFKBP51 ---------- ---------- ---------- -------Y-- ---------- ---------- 240
 mFKBP51 ------V-V- ---------- ------V--- -------Y-- ---------- -----D---- 240

smFKBP51 LIYEVTLKSF EKAKESWEMD TKEKLEQAAI VKEKGTVYFK GGKYVQAVIQ YGKIVSWLEM 300
 hFKBP51 ---------- ---------- ---------- ---------- ----M----- ---------- 300
 mFKBP51 -M-------- ---------- -----T---- ---------- ----T----- -R-------- 300

smFKBP51 EYGLSEKESK ASESFLLAAF LNLAMCYLKL REYTKAVECC DKALGLDSAN EKGLYRRGEA 360
 hFKBP51 ---------- ---------- ---------- ---------- ---------- ---------- 360
 mFKBP51 ---------- ---------- ---------- ---N------ ---------- ---------- 360

smFKBP51 QLLMNEFESA KGDFEKVLEV NPQNKAARLQ IFMCQKKAKE HNERDRRIYA NMFKKFAEQD 420
 hFKBP51 ---------- ---------- ---------- -S-------- -------I-- ---------- 420
 mFBBP51 -----D---- ---------A- ----R----- -S---R---- ---------V-- --------R- 420

smFKBP51 AKEEANKAMS KKTSEGVTNE KLTASHAVEE EKPEGHV                          457
 hFKBP51 ---------G ---------- -G-D-Q-M-- -------                          457
 mFKBP51 -----S--G- --AV--AAG. -QHE-Q-M-- G-AK---                          456
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FIG. 7. Comparison of the amino acid sequences of squirrel monkey, human, and mouse FKBP51. Human and mouse FKBP51 sequences were obtained from GenBank (accession numbers U71321 and U36220, respectively). The squirrel monkey FKBP51 cDNA sequence has been deposited in GenBank with the accession number AF140759. Identical amino acids are indicated by a *hyphen*. *Dots* indicate gaps. The N-terminal PPIase domain homologous to FKBP12 is indicated with a *single underline*, and the three TPR regions in the C-terminal half of each sequence (amino acids 271–304, 323–353, and 354–387) are indicated by *double underlines*. The consensus calmodulin-binding motif (amino acids 397–413) is indicated with a *dashed underline*. Residues that are different in squirrel monkey and human FKBP51 and represent significant changes in charge or polarity are indicated by *shading*.

enjoy a relatively normal pituitary adrenal physiology, albeit at a much higher hormonal set point.

The first evidence presented here implicating a role for an FK506-binding protein in glucocorticoid resistance in squirrel monkeys is the dramatic induction of GR binding in squirrel monkey cells by FK506 and rapamycin. Effects of FK506 on GR activity have been reported by several laboratories. Taken together, they showed that FK506 and rapamycin, as well as cyclosporin A, potentiated the induction of a reporter gene by dexamethasone in a cell line derived from L929 cells (34, 35). However, it has subsequently been demonstrated that all three agents cause the accumulation of dexamethasone in L929 cells, suggesting that the potentiation of the hormone response results from a higher intracellular concentration of steroid (36, 37). The studies presented here were performed with cytosol preparations, eliminating a trivial explanation for our finding. The effect of FK506 was reproduced by rapamycin but not cyclosporin A, indicating that this is not a nonspecific action of the immunosuppressants. Furthermore, the effect of FK506 occurred at concentrations consistent with an interaction involving the large-molecular-weight rather than small-molecular-weight immunophilins, such as FKBP12, which occurs with a much lower K_d (22). Rather, we favor the idea that FK506 and rapamycin cause dissociation of FKBP51 from the GR heterocomplex. Because of the relatively low levels of GR in SML and the lack of antibodies for GR immunoprecipitation and analysis of the GR heterocomplex in SML, we do not have direct proof that this occurs. However, FK506 can prevent the association of FKBP51 with GR heterocomplexes. The interaction of squirrel monkey FKBP51 with GR from L929 cells and the inhibitory effect of SML cytosol on GR binding were both inhibited by FK506 when cytosol from L929 cells was mixed with cytosol from SML (4). These results are consistent with studies using FK506 affinity columns, which showed that FKBP51 is not adsorbed from HeLa cell extracts unless it is dissociated from steroid receptor heterocomplexes (38). Thus, the FK506-binding pocket is inaccessible when FKBP51 is associated with the heterocomplex. However, steroid heterocomplexes are in a state of constant disassembly and reassembly; and FKBP51 in SML cytosol, once dissociated from the heterocomplex, cannot reassemble in the presence of FK506. The results of these studies indicate that the presence of squirrel monkey FKBP51 in the GR heterocomplex confers low-affinity binding and that FK506 inhibits this interaction.

On the other hand, most laboratories, including ours, have failed to find any effect of FK506 on GR binding in three cell lines that express high affinity GR (13, 23). In contrast to the low-affinity GR heterocomplex in squirrel monkey cells, high-affinity GR-hsp90 hetercomplexes contain either FKBP52, CyP-40, or the protein phosphatase PP5 (39). Although the presence of PP5 in the heterocomplex has been shown to affect GR activity (40, 41), PP5 has only low-affinity FK506 binding activity (39), and CyP-40 does not bind FK506 (22). FK506 obviously binds FKBP52, but it does so differently than FKBP51. For example, GR heterocomplexes are retained by an FK506 affinity matrix, suggesting that FK506 binds FKBP52, whether in the heterocomplex or not (42). FK506 binding to the GR heterocomplex did not cause activation or dissociation of the complex (42). The results of these studies indicate that FK506 binds to FKBP52 within the mature GR heterocomplex but does not cause functional changes in the receptor. In contrast with this and previous studies, Ning and Sanchez (24) showed that incubation of cytosol from mouse S49 lymphocyte cells with FK506 resulted in a small, but reproducible, increase in the binding affinity of relatively high-affinity GR. However, the molecular basis for this effect was not determined, and the relative levels of FKBP51 and FKBP52 in S49 cells have yet to be determined.

We show here that squirrel monkey FKBP51 is approximately 8-fold more potent than human FKBP51 in two assay systems measuring GR binding and activity. Because the expression of FKBP51 mRNA is increased by glucocorticoids (17, 43), we had hypothesized that the regulation of FKBP51 represents a short-feedback loop by which the sensitivity to glucocorticoids is influenced by previous exposure to hormone (4). We were surprised that squirrel monkey FKBP51 is more potent than the human protein, considering that the two are 94% identical. It is possible that amino acid differences within their functional domains may be responsible for the difference in activity. We have not measured the PPIase activity of squirrel monkey FKBP51, which differs, in seven amino acids in the PPIase domain, from human FKBP51. However, the intrinsic PPIase activity of the immunophilins is not thought to play a role in steroid receptor function (13, 44), suggesting that any difference in PPIase activity of the two proteins likely does not contribute to the differences in the inhibitory activities described here. Rather, the difference in GR inhibitory activity of squirrel monkey and human FKBP51 most likely results from substitutions in other domains. It is unlikely to reside within the TPR domain, which is necessary for the interaction of FKBP51 with hsp90 (16, 45) but is virtually identical between human and squirrel monkey FKBP51. However, sequences in addition to the TPR domain are required for stable association of both FKBP51 and CyP-40 with hsp90 and steroid receptor complexes (45, 26). C-terminal amino acids beyond the TPR domains of the immunophilins are important for preferred association of FKBP51 over FKBP52 in progesterone receptor complexes (45), and it is this region and the N-terminal region that are the most variable between human and squirrel monkey FKBP51.

However, the view that the only important amino acid differences between the two proteins are those that affect the interaction of the TPR domain of FKBP51 with the GR-hsp90 complex may be oversimplistic. Silverstein *et al.* (46) recently demonstrated that FKBP52 not only interacts with the GR through hsp90 but also binds directly to the GR in the absence of hsp90. They also showed that the interaction with GR did not occur through either the PPIase or TPR domains of FKBP52, although the region of FKBP52 that is involved was not defined. We do not know whether FKBP51 also interacts with the GR directly. However, we noted that the expression of both human and squirrel monkey FKBP51 in COS-7 cells not only shifted, but also changed, the shape of the dexamethasone dose-response curves, suggesting a more complex interaction of this immunophilin with the receptor heterocomplex.

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