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Calcineurin Mutants Render T Lymphocytes Resistant to Cyclosporin A

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SUMMARY

The immunosuppressants cyclosporin A (CsA) and FK506 have been widely used to prevent and treat graft rejection after human organ and tissue transplantations. CsA and FK506 associate with intracellular binding proteins (i.e., CsA with cyclophilin A and FK506 with FKBP12) to form protein/drug complexes that suppress the immune system by preventing activation of T cells in response to antigen presentation. The common target of CsA and FK506 is calcineurin, a Ca^{2+}/calmodulin-regulated, serine/threonine-specific protein phosphatase that regulates the nuclear import of a transcription factor, NF-AT, required for expression of T cell activation genes. In previous studies, we identified calcineurin mutations that block binding by the cyclophilin A/CsA or FKBP12/FK506 complexes and thereby render yeast cells resistant to the antifungal effects of CsA or FK506. In this report, we demonstrate that the corresponding mutations in murine calcineurin render the T cell receptor signal transduction cascade CsA resistant in human Jurkat T cells. Our findings support the recently determined calcineurin X-ray crystal structure, provide evidence that calcineurin is the only CsA-sensitive component limiting signaling from the T cell receptor to the nucleus, and suggest a means to render cells and tissues resistant to the toxic side effects of CsA and FK506.

CsA and FK506 suppress the immune system and prevent graft rejection by blocking intermediate steps in signal transduction pathways required for T cell activation (1–3). Both CsA and FK506 enter the cell and associate with intracellular binding proteins (i.e., CsA with cyclophilin A and FK506 with FKBP12) to form protein/drug complexes that are the active *in vivo* agents. The common target of the cyclophilin A/CsA and FKBP12/FK506 complexes is calcineurin, a Ca^{2+}/calmodulin-dependent, serine/threonine-specific protein phosphatase whose activity is inhibited by both protein/drug complexes (4, 5). Calcineurin plays a central role in sensing calcium increases in stimulated T cells and appropriately regulating gene expression required for T cell activation (6–8). In resting cells, calcineurin is an inactive heterodimer composed of the CNA and CNB subunits. After activation, the Ca^{2+}/calmodulin complex binds to a carboxyl-terminal domain of CNA, leading to conformational changes that release an autoinhibitory domain from the calcineurin-active site, resulting in the active heterotrimeric phosphatase.

The target of calcineurin is a transcription factor, NF-AT, required for T cell activation (9–14). NF-AT has two components: a cytoplasmic subunit, NF-ATc, and a nuclear subunit, NF-ATn, composed of Fos and Jun family members. Activation of the T cell receptor leads to an increase in intracellular Ca^{2+}, calcineurin activation, and dephosphorylation and nuclear import of NF-ATc. Subsequently, NF-ATc associates with the nuclear NF-AT subunit, and the genes encoding IL-2 and other factors required for T cell activation are transcribed.

CsA and FK506 are natural products of soil micro-organisms and have potent antimicrobial activities. We and others have analyzed their mechanism of action in the baker's yeast *Saccharomyces cerevisiae*, revealing that the mechanisms of CsA and FK506 antifungal and immunosuppressive action are remarkably similar (15–24). Yeast cyclophilin A and FKBP12 mediate CsA and FK506 action in yeast and are 65% and 54%, respectively, identical to the human homologs (25, 26). Furthermore, the X-ray crystal structures of human and yeast FKBP12 are virtually superimposable (27, 28). In yeast, as in human T cells, the cyclophilin A/CsA and FKBP12/FK506 complexes inhibit the protein phosphatase

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ABBREVIATIONS: CsA, cyclosporin A; CNAα1, catalytic A subunit of murine calcineurin; CNB, regulatory B subunit of calcineurin; PCR, polymerase chain reaction; SEAP, secreted alkaline phosphatase; PMA, 12-*O*-tetradecanoylphorbol-13-acetate phorbol ester; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

calcineurin (16, 18–20), whose subunits are also highly conserved in organisms from yeast to humans (29–32). Thus, CsA and FK506 have an extremely similar mechanism of action to inhibit the function of two different cell types: yeast and T cells.

Mutations that render yeast cells resistant to the toxic effects of either CsA or FK506 were previously identified, including single amino acid substitutions in the CNA catalytic subunit that confer dominant drug resistance by preventing binding and inhibition by the cyclophilin A/CsA (T350K, T350R, Y377F) or by the FKBP12/FK506 complexes (W388C) (20). The X-ray crystal structures of a protein phosphatase 1-microcystin complex (33), of the calcineurin AB holoenzyme (34), and of the calcineurin AB/FK506/FKBP12 complex (34, 35), together with previous biochemical studies of okadaic acid and microcystin resistant and sensitive protein phosphatase 1 and 2A mutants (36, 37), suggest that the calcineurin residues altered in yeast drug resistant mutants are intimately involved in cyclophilin A/CsA and FKBP12/FK506 binding.

The mutations that render calcineurin CsA or FK506 resistant occur in calcineurin residues highly conserved or invariant in yeast to humans, suggesting that the corresponding mutations might render murine or human calcineurins appropriately drug resistant. In this report, we demonstrate that this is indeed the case. After transfection of human Jurkat T cells, the wild-type CNA and CNB genes shifted the dose-response curve for CsA inhibition of T cell receptor-dependent expression of an NF-AT-responsive reporter gene ~2.5-fold, as reported previously (6, 8). In contrast, expression of wild-type CNB in conjunction with either the V314R or the Y341F CNA mutants rendered signaling via the T cell receptor cascade CsA resistant. Our findings demonstrate that mutations originally identified in a yeast model system also function in mammalian cells, suggest that calcineurin is the only CsA-sensitive element of the signal transduction cascade linking the T cell receptor and NF-AT, and provide support for the recent calcineurin X-ray crystal structure (34, 35). Finally, expression of these mutants in other cell types, or in transgenic animals, should allow us to test whether calcineurin is the target for the toxic side effects of CsA and FK506 and, if so, to engineer drug-resistant organs for use in human transplant recipients.

Materials and Methods

Cell culture. The Jurkat human T cell leukemia cell line, which had been stably transfected with the simian virus 40 large T antigen (TAg Jurkat cells), was kindly provided by Rick Brams and Gerald Crabtree (Stanford University, Stanford, CA). TAg Jurkat cells were maintained in RPMI 1640 medium containing 10% (v/v) fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37° in an atmosphere composed of 5% CO_2/95% air.

Generation of murine calcineurin mutants. The genes encoding CNAα1 and CNB in the plasmid pBJ5 expressed from the SRα promoter (38), a hybrid of the simian virus 40 early promoter and the R-U5 region of human T cell leukemia virus type 1 long terminal repeat, were obtained from Neil Clipstone and Gerald Crabtree (Stanford University). Mutations were introduced by PCR overlap mutagenesis (Y341F) or using the Altered Sites II *in vitro* Mutagenesis System (Promega, Madison, WI) (V314R) as follows. For PCR overlap mutagenesis, outer primers were 217 5′-CCCTGAATTCTATCTCTCTCCCTCTTTTTTTATT and 218 5′-CCCTGAATTCGCGCCGGTGCGGTCGGGGTGTGCA, and inner primers were 201 5′-GAAATTTGGGAGCCAGAACGGATGCGGGGAGCA 202 5′-TGCTGGGGCCATCCGTTCTGGCTCCCACCTTTC. First-round PCR was with primers 202/217 and 201/218 with PCR conditions: 1 × 3 min at 94°, 35 × 30 sec at 94°, 30 sec at 55°, 2 min at 72°, and 1 × 5 min at 72°. The resulting 780- and 1020-bp PCR products were gel-purified and mixed, and the second-round PCR with primers 217 and 218 was performed with the above PCR protocol. The resulting 1.8-kb PCR product was purified, cleaved with *Eco*RI, and subcloned in plasmid pBJ5. For the V314R mutation, the murine CNA gene was subcloned as a 1.8-kb *Eco*RI fragment into the pAlter plasmid and site-directed mutagenized using the Altered Sites II kit (Promega) with the V314R mutagenic primer 324 5′-GTTATTGTATCGATCTAAGTAATTTGG-3′ containing a *Cla*I site. An isolate with the introduced linked *Cla*I site was subcloned into plasmid pBJ5. The resulting pBJ5-CNA-V314R and pBJ5-CNA-Y341F plasmids were sequenced to confirm that the desired mutations had been introduced and that no extraneous mutations were present.

Detection of murine CNA and CNB, FKBP12, and cyclophilin A in transfected human TAg Jurkat T cells. TAg Jurkat cells (5×10^7) were transiently cotransfected by electroporation (250 V, 960 μF) with 4 μg of the pBJ5 expression plasmid expressing wild-type or mutant murine CNA (pBJ5-CNA, pBJ5-CNA VR, or pBJ5-CNA YF) and 4 μg of plasmid pBJ5 expressing murine CNB (pBJ5-CNB). For controls, 8 μg of pBJ5 vector DNA alone were introduced. Cells were harvested 48 hr after transfection, washed once with phosphate-buffered saline, and crude proteins were extracted with lysis buffer (25 mM HEPES, pH 7.2, 100 mM NaCl, 2 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 0.5% Triton X-100). Equal amounts of proteins were separated by 12% and 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and expression of the murine CNAα1, CNB, FKBP12, and cyclophilin A was determined by Western blot analyses with enhanced chemiluminescence detection using rabbit polyclonal antisera against the A and B subunits of bovine CN(UBI), human FKBP12 (provided by Andy Marks, Mt. Sinai Medical Center, New York, NY), and human cyclophilin A (Affinity Bioreagents, Golden, CO).

Assay of SEAP activity in cotransfected human TAg Jurkat cells. Jurkat cells (5×10^7) were cotransfected by electroporation with 4 μg each of plasmid pBJ5 expressing wild-type or mutant CNA (pBJ5-CNA, pBJ5-CNA VR, or pBJ5-CNA YF), 4 μg of plasmid pBJ5 expressing the wild-type CNB regulatory subunit (pBJ5-CNB), and 4 μg of the pNFAT-SX reporter plasmid bearing multiple NF-AT response elements upstream of the SEAP reporter gene (6, 39) (kindly provided by Gerald Crabtree). Transfected cells were activated with 10 ng/ml PMA and 0.5 μM ionomycin in the absence or presence of various concentrations of CsA at 12-hr post-transfection incubation. After 12-hr activation, SEAP activity was assayed in the cell culture medium after inactivation of endogenous SEAP activity by heat treatment at 65° for 1 hr. Portions of heated culture supernatants (100 μl) were mixed with 100 μl of 2× SEAP assay buffer (2 M diethanolamine, 1 mM $MgCl_2$, and 20 mM L-homoarginine), transferred to flat-bottom microtiter plates, and incubated at 37° for 10 min. Then, 20 μl of substrate (120 mM paranitrophenolphosphate in 1× SEAP buffer) was added per well, and A_{405nm} was measured using an enzyme-linked immunosorbent assay reader, and levels of SEAP activity were calculated at points when changes in absorbance were linear with respect to time.

Results

Introduction of yeast CsA-resistant calcineurin mutations into murine calcineurin. We previously identified three mutations in the CNA catalytic subunit (T350K, T350R, Y377F) that prevent binding by the cyclophilin A/CsA complex and thereby render yeast cells resistant to CsA (20). As shown in Fig. 1, these mutations result from single amino

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                                        Calcineurin B                    CsA
Human A1.. TIFSAPNYLDVYNNKAAVLKYENNVMNIRQFNCsPHPYWLPNFMDVFTWSLPFVGEKVTEMLVnvLsICSdDEL
Human A2.. TIFSAPNYLDVYNNKAAVLKYENNVMNIRQFNCsPHPYWLPNFMDVFTWSLPFVGEKVTEMLVnvLsICSdDEL
Murine Aα1 TIFSAPNYLDVYNNKAAVLKYENNVMNIRQFNCsPHPYWLPNFMDVFTWSLPFVGEKVTEMLVnvLNICSdDEL
D.melanog. TIFSAPNYLDVYNNKAAVLKYENNVMNIRQFNCsPHPYWLPNFMDVFTWSLPFVGEKVTEMLVnILNICSdDEL
N. crassa. TIFSAPNYLDVYNNKAAVLKYENNVMNIRQFNCtPHPYWLPNFMDVFTWSLPFVGEKiTdMLiaILstCSeeEL
A.nidulans TIFSAPNYLDVYNNKAAVLKYENNVMNIRQFNCtPHPYWLPNFMDVFTWSLPFVGEKiTdiviaILNtCSkeEL
S.c.  CMP1 TmFSAPNYLDtYhNKAAVLKYEeNVMNIRQFhmsPHPYWLPdFMDVFTWSLPFVGEKVTsMLVsILNICSeqEL  S
CMP1-1.... ..........K...............................................................  R
CMP1-2.... ..........R...............................................................  R
CMP1-3.... .....................................F....................................  R
CMP1       ........350..........................377..................................
S.c.  CMP2 TlFSAPNYLDtYNNKAAiLKYENNVMNIRQFNmtPHPYWLPdFMDVFTWSLPFVGEKVTEMLVaILNICteDEL  S
CMP2-3.... .....................................F....................................  R
CMP2       ....................................419...................................
Murine Aα1 TIFSAPNYLDVYNNKAAVLKYENNVMNIRQFNCsPHPYWLPNFMDVFTWSLPFVGEKVTEMLVnvLNICSdDEL
Murine VR  ..........R...............................................................  R
Murine YF  .....................................F....................................  R
Murine Aα1 ........314..........................341..................................
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Fig. 1. CsA-resistant calcineurin mutations are in highly conserved residues. Portions of the primary amino acid sequence of the CNA catalytic subunits from several species are aligned [with amino acids (*single letter abbreviations*)]. *Capital letters*, residues invariant or highly conserved. *Calcineurin B*, region of CNA that binds the CNB regulatory subunit (*bold and overlined*). Substitutions that render the yeast CNA subunit CMP1 CsA resistant are shown below the wild-type sequence as the single letters *K*, *R*, and *F*, representing the T350K (*CMP1-1*), T350R (*CMP1-2*), and Y377F (*CMP1-3*) CsA-resistant mutants. Substitutions that render the yeast CNA CMP2 subunit CsA resistant are shown below the wild-type sequence as the single letter *F*, representing the Y419F (*CMP2-3*) CsA-resistant mutant. *S* or *R*, sensitivity or resistance to CsA, respectively. These substitutions occur in an invariant residue (Y377F) or within a region of marked identity (T350K or R, where T350 is valine in other CNA proteins). The corresponding mutations in murine CNAα1 gene, V314R and Y341F, are indicated by the single letters *R* and *F* below the wild-type sequence. Sequences for calcineurins that are human or murine or from *Drosophila melanogaster*, *Neurospora crassa*, *Aspergillus nidulans*, and *S. cerevisiae* (CMP1 and CMP2) are from Refs. 29 and 48–52, respectively.

acid substitutions in a region of CNA that is highly conserved between yeast, murine, and human calcineurins. Two occurred in a residue between the active site and the CNB binding domain (T350R, T350K), and the other occurred within the CNB binding domain (Y377F). T350 in yeast CNA corresponds to V314 in humans, and the only difference between the side chains of valine and threonine is a single hydroxyl group. Y377 is conserved as Y341 in human calcineurin, and substitution in the yeast CsA-resistant mutant by phenylalanine deletes only the phenolic hydroxyl group, suggesting this is a critical contact to either CsA or cyclophilin A.

Based on these previous studies, we introduced by site-directed mutagenesis two different mutations into the murine CNAα1 gene (Fig. 1 and Materials and Methods). The murine CNA gene was expressed under the control of the SRα promoter in the expression plasmid pBJ5, as described previously (6). The specific amino acid substitutions were V314K and Y341F, based on the earlier identification of the T350K and Y377F mutations in the corresponding residues of yeast CNA. DNA sequence analysis confirmed that the appropriate mutations had been introduced and that no extraneous mutations had occurred.

Expression of wild-type and mutant calcineurins in transfected human T cells. We first determined whether the V314R (VR) and Y341F (YF) calcineurin mutant enzymes could be stably expressed in mammalian cells, as is the case with the T350K and Y377F mutant enzymes expressed in yeast cells (20). We used a Jurkat human T cell line that has been stably transfected with the simian virus 40 T antigen, TAg cells, which support replication of plasmids with the simian virus 40 origin of replication, such as the pBJ5 expression plasmid used here.

Jurkat TAg cells were transiently cotransfected by electroporation with plasmid pBJ5 encoding wild-type or mutant murine CNA (pBJ5-CNA, pBJ5-CNA VR, or pBJ5-CNA YF) and plasmid pBJ5 expressing wild-type murine CNB (pBJ5-CNB) as described in Materials and Methods. Cell extracts were prepared from the transfectants, and expression of calcineurin was analyzed by Western blot. As shown in Fig. 2, both CNA and CNB were readily detectable in lysates from T

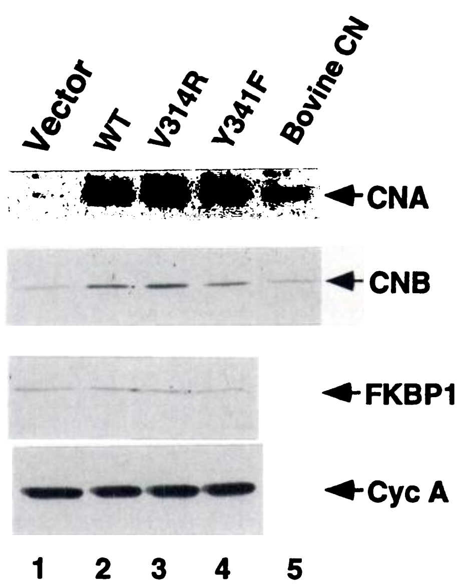

Fig. 2. Expression of murine CNA and CNB, FKBP12, and cyclophilin A in human TAg Jurkat T cells. TAg Jurkat T cells were transfected with vector alone (*Vector, lane 1*), with wild-type CNB plus wild-type CNA (*WT, lane 2*), or with CNB plus the CNA mutants V314R (*lane 3*) and Y341F (*lane 4*), and 20 μg of extracted total protein was separated by 12% (CNA, CNB) and 15% [FKBP12, cyclophilin A (*Cyc A*)] sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and analyzed by Western blot for expression of CNA and CNB. Purified bovine calcineurin (*Bovine CN*) (Sigma) was electrophoresed (*lane 5*) as a control. Endogenous FKBP12 and cyclophilin A were also detected, both to assess their expression levels and to control for equal loading. By comparison with molecular mass standards, the proteins migrated as ~60 kDa (CNA), ~18 kDa (CNB), ~12-15 kDa (FKBP12), and ~20 kDa (cyclophilin A).

cells transfected with either the wild-type or mutant CNA and CNB genes but not from control cells transfected with the expression vector pBJ5 alone (compare *lanes 2–4* with *lane 1*, Fig. 2). Authentic bovine calcineurin (Sigma Chemical, St. Louis, MO) was analyzed in parallel (Fig. 2, *lane 5*), demonstrating that the rabbit polyclonal antisera against bovine calcineurin detects both CNA and CNB and that the ~60-kDa and ~18-kDa proteins detected by this antisera in transfected T cells comigrate with the purified CNA and CNB standards. Both subunits of the wild-type and mutant calcineurin enzymes were expressed to equivalent extents, and thus the V314R and Y341F mutations do not alter the expression level or stability of CNA. That equivalent amounts of protein were expressed allowed direct comparisons of the physiological responses of T cells transfected with wild-type and mutant calcineurins, as described below.

Murine CNA mutants render T cell signaling CsA resistant. To determine whether expression of the V314R and Y341F CNA mutant proteins would render T cells CsA resistant, as occurred with the corresponding mutant proteins expressed in yeast cells (20), we adopted an assay developed by Clipstone and Crabtree (6). In this assay, TAg Jurkat cells are transiently transfected with a reporter gene in which multiple NF-AT response elements regulate expression of the SEAP reporter gene. Activation of the T cell receptor cascade is accomplished by the addition of PMA to activate protein kinase C and calcium ionophore (ionomycin) to increase intracellular calcium. These reagents mimic the production of diacylglycerol and inositol trisphosphate that normally occurs during T cell activation via activation of phospholipase Cγ. In this assay system, overexpression of wild-type calcineurin renders signaling via the T cell receptor in Jurkat T cells relatively more resistant to CsA and FK506, shifting the dose-response curve ~5-fold (6, 8).

We tested the effects of overexpression of both wild-type and the presumptive CsA-resistant mutant forms of CNA in conjunction with CNB. As described above, Jurkat cells were cotransfected with the expression plasmid pBJ5 expressing wild-type or mutant CNA (pBJ5-CNA, pBJ5-CNA VR, or pBJ5-CNA YF), pBJ5 expressing the wild-type CNB regulatory subunit (pBJ5-CNB), and the plasmid bearing the NF-AT responsive SEAP reporter gene (NFAT-SX). The transfected cells were activated with 10 ng/ml PMA and ionomycin (0.5 μM) in the absence or presence of various concentrations of CsA. Although Jurkat cells transfected with the control vector alone were markedly sensitive to inhibition by CsA, as monitored by a decreased expression of SEAP activity (Fig. 3), cells transfected with both wild-type CNA and CNB become relatively more resistant to CsA in that the IC_{50} for drug action was increased ~2.5-fold, from ~0.4 to ~1 ng/ml. These findings are consistent with earlier studies from Clipstone and Crabtree (6) and O'Keefe *et al.* (8) that established calcineurin as a critical T cell signaling molecule. Remarkably, transfection with either the V314R or the Y341F mutant murine CNA genes, in combination with wild-type CNB, sufficed to render T cell signaling virtually resistant to CsA inhibition, shifting the IC_{50} for CsA action from ~0.4 to >100 ng/ml, a >250-fold increase. Because equal amounts of wild-type and mutant calcineurins were expressed in the transfected Jurkat cells (Fig. 2), we conclude that these effects are attributable to the mutations introduced into the murine CNA gene.

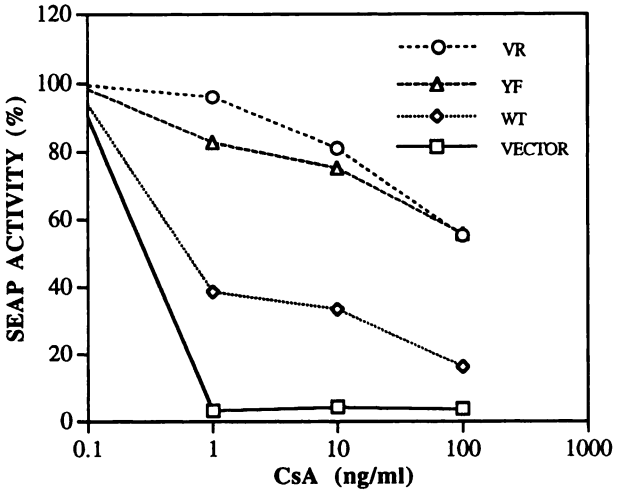

Fig. 3. Expression of murine CNA mutants renders T cell signaling CsA resistant. TAg Jurkat cells (5×10^7 cells) were transfected with the reporter plasmid NFAT-SX alone (*VECTOR*) or with the reporter plasmid NFAT-SX together with pBJ5 expressing wild-type CNA and CNB (*WT*), the CNA V314R mutant and CNB (*VR*), or the CNA Y341F mutant and CNB (*YF*). After 12-hr post-transfection incubation, transfected cells were activated with 10 ng/ml PMA and ionomycin (0.5 μM) in the absence or presence of various concentrations of CsA. After 12-hr activation, SEAP activity was assayed in cell extracts and expressed here as percentage of maximal reporter construct activity. The results presented are representative of three similar experiments.

In parallel, we also examined the effects of FK506, which also inhibits calcineurin. Although TAg Jurkat cells transfected with the control vector pBJ5 and the NF-AT-SEAP reporter plasmid were FK506 sensitive (IC_{50} = <0.001 ng/ml FK506; data not shown), as described previously (6, 8), cotransfection with even wild-type CNA and CNB rendered these cells FK506 resistant (IC_{50} = >1 ng/ml FK506), with a >1000-fold shift in the IC_{50}. Similar observations have been recently reported by Lam *et al.*, (40), who further demonstrated that FKBP12 is limiting in Jurkat cells and that after cotransfection with the gene encoding human FKBP12, FK506 sensitivity is restored to Jurkat cells overexpressing CNA and CNB. Apparently, when CNA and CNB are overexpressed, insufficient FKBP12 is present in the cell to effectively inhibit calcineurin despite high concentrations of FK506. Our observations, including Western blot analysis of FKBP12 levels (Fig. 2), are consistent with this interpretation and suggest that overexpression of even wild-type CNA and CNB should suffice in some cells and tissues to confer FK506 resistance.

Discussion

We demonstrated that mutations in the CNA catalytic subunit that were originally identified via their ability to render yeast calcineurin resistant to the antifungal effects of CsA are functional in the context of a mammalian calcineurin expressed in a mammalian cell. These findings provide genetic and physiological support for the recent X-ray crystal structure of bovine calcineurin (34, 35). Notably, the residues identified here, V314 and Y341, lie on the surface of calcineurin that interacts with FKBP12/FK506. Although these residues do not bind FKBP12/FK506 or confer FK506 resistance when mutated, our findings, and the previous observation that the cyclophilin A/CsA and FKBP12/FK506 com-

plexes compete for calcineurin (4), suggest these residues bind either CsA or cyclophilin A.

In related studies, we identified a CNA mutation that renders yeast calcineurin resistant to FKBP12/FK506 but not to cyclophilin A/CsA (20). The recent X-ray crystal structures of the bovine calcineurin AB/FK506/FKBP12 complex reveal that the affected residue, W352 in bovine calcineurin, is a critical contact to FK506, making two unusual bifurcated hydrogen bonds from the ϵ amino group of the tryptophan imidazole ring to the C13 and C15 methoxy oxygens of FK506 (34, 35). In recent complementary studies by Kawamura and Su (41), three other neighboring residues of CNA (T351, L354, and K360) were identified that when mutated, confer resistance to FKBP12/FK506 *in vitro* and *in vivo* in transfected T cells. Taken together, these studies define highly conserved regions of the CNA catalytic subunit as the targets for cyclophilin A/CsA and FKBP12/FK506 complexes in yeast and humans.

Previous studies had produced conflicting views as to the importance of the CNA and CNB subunits as targets of cyclophilin A/CsA and FKBP12/FK506. For example, the CNB subunit was shown to be required for calcineurin binding and inhibition by both cyclophilin A/CsA and FKBP12/FK506 (42, 43). FKBP12/FK506 and cyclophilin A/CsA complexes bound to the calcineurin AB complex readily cross-link to CNB but not to CNA (44, 45). Finally, residues of CNB implicated in binding of both inhibitors were recently identified and led to the proposal that CNB alone was the target of CsA and FK506 (46). In contrast, our previous studies in yeast led us to propose that the CNA catalytic subunit plays a critical role in drug action and that the cyclophilin A/CsA and FKBP12/FK506 complexes act by binding to both CNA and CNB (20). This model is further supported by our studies in T cells presented here and by recent complementary studies of FK506-resistant CNA mutants (41). Furthermore, the two recent independent solutions of the calcineurin AB/FK506/FKBP12 complex reveal that, as predicted, the FKBP12/FK506 complex makes contacts with both CNA and CNB.

In addition to their beneficial immunosuppressive effects, CsA and FK506 have a number of dose-limiting toxic side effects, most notably, nephrotoxicity. Because the only common feature of CsA and FK506 is the ability to inhibit calcineurin, CsA and FK506 nephrotoxicity likely results from calcineurin inhibition in the kidney. In previous studies, the immunosuppressive and nephrotoxic activities of CsA analogs were inseparable (47). Thus, the identification of additional drugs that target calcineurin is unlikely to ameliorate CsA and FK506 nephrotoxicity. Our findings provide a means to directly test whether calcineurin is the relevant CsA target in the kidney by expressing CsA-resistant calcineurin mutants in transgenic mice. If so, it might prove feasible to modify human organs before transplantation or to genetically engineer animals to serve as donors for CsA-resistant xenotransplants.

Acknowledgments

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