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Targets of immunophilin–immunosuppressant complexes are distinct highly conserved regions of calcineurin A

Maria E.Cardenas[1], R.Scott Muir[2], Tamara Breuder[2] and Joseph Heitman[1,2,3,4]

Departments of [1]Genetics and [3]Pharmacology, and [2]The Howard Hughes Medical Institute, Duke University Medical Center, Box 3546, Durham, NC 27710, USA

[4]Corresponding author

The immunosuppressive complexes cyclophilin A–cyclosporin A (CsA) and FKBP12–FK506 inhibit calcineurin, a heterodimeric Ca^{2+}-calmodulin-dependent protein phosphatase that regulates signal transduction. We have characterized CsA- or FK506-resistant mutants isolated from a CsA–FK506-sensitive *Saccharomyces cerevisiae* strain. Three mutations that confer dominant CsA resistance are single amino acid substitutions (T350K, T350R, Y377F) in the calcineurin A catalytic subunit CMP1. One mutation that confers dominant FK506 resistance alters a single residue (W430C) in the calcineurin A catalytic subunit CMP2. *In vitro* and *in vivo*, the CsA-resistant calcineurin mutants bind FKBP12–FK506 but have reduced affinity for cyclophilin A–CsA. When introduced into the CMP1 subunit, the FK506 resistance mutation (W388C) blocks binding by FKBP12–FK506, but not by cyclophilin A–CsA. Co-expression of CsA-resistant and FK506-resistant calcineurin A subunits confers resistance to CsA and to FK506 but not to CsA plus FK506. Double mutant calcineurin A subunits (Y377F, W388C CMP1 and Y419F, W430C CMP2) confer resistance to CsA, to FK506 and to CsA plus FK506. These studies identify cyclophilin A–CsA and FKBP12–FK506 binding targets as distinct, highly conserved regions of calcineurin A that overlap the binding domain for the calcineurin B regulatory subunit.

Key words: calcium/cyclophilin/cyclosporin/immunosuppression/yeast

Introduction

The immunosuppressants cyclosporin A (CsA) and FK506 inhibit the function of T lymphocytes by blocking signal transduction cascades required for T-cell activation (reviewed by Heitman *et al.*, 1992; Schreiber and Crabtree, 1992; Cardenas *et al.*, 1994a). CsA and FK506 are both natural products produced by soil microorganisms (reviewed by Hemenway and Heitman, 1993). Their relevant roles in nature may be reflected in their potent toxicity towards microorganisms, including fungi and parasites.

CsA binds to a family of proteins termed cyclophilins, whereas FK506 binds to a different protein family termed FKBPs. These two distinct protein families are highly conserved and are likely to participate in protein folding via their ability to catalyze *cis–trans* peptidyl–prolyl isomerization. Genetic studies in yeast demonstrated that the cytoplasmic immunophilins cyclophilin A and FKBP12 respectively mediate CsA and FK506 actions in yeast (Foor *et al.*, 1992; Breuder *et al.*, 1994; Cunningham and Fink, 1994). Moreover, neither cyclophilin A nor FKBP12 are essential in yeast (Heitman *et al.*, 1991a). These findings, and studies of immunosuppressant action in mammals, led to the model that immunophilin–drug complexes are the active intracellular agents (Tropschug *et al.*, 1989; Bierer *et al.*, 1990; Dumont *et al.*, 1990; Heitman *et al.*, 1991b).

CsA and FK506 bind cyclophilin A and FKBP12 to form structurally distinct complexes that inhibit a common target, the Ca^{2+}-calmodulin-regulated serine threonine phosphatase calcineurin (Liu,J. *et al.*, 1991, 1992). CsA and FK506 were known to inhibit a calcium-dependent intermediate signal transduction step required for T cells to respond to antigen (Mattila *et al.*, 1990). Calcineurin has been shown to be a critical T-cell signaling component that responds to elevated calcium levels (Clipstone and Crabtree, 1992; O'Keefe *et al.*, 1992). The findings that the cytoplasmic subunit of the transcription factor NF-AT is a substrate for calcineurin *in vitro* (Jain *et al.*, 1993a) and that CsA and FK506 inhibit the appearance of NF-AT in nuclear extracts of activated T cells, suggest that dephosphorylation by calcineurin may trigger nuclear import of NF-AT, thereby permitting expression of genes required for T-cell activation (Flanagan *et al.*, 1991; Jain *et al.*, 1993a,b; McCaffrey *et al.*, 1993; Northrop *et al.*, 1994; Rao, 1994). Calcineurin has also been identified in the yeast *Saccharomyces cerevisiae* (Cyert *et al.*, 1991; Kuno *et al.*, 1991; Liu,Y. *et al.*, 1991; Cyert and Thorner, 1992; Nakamura *et al.*, 1992; Ye and Bretscher, 1992), where it is required for recovery from pheromone-induced cell cycle arrest (Cyert *et al.*, 1991; Cyert and Thorner, 1992; Foor *et al.*, 1992), growth in the presence of extracellular cations (Nakamura *et al.*, 1993; Breuder *et al.*, 1994), regulation of calcium pump function (Cunningham and Fink, 1994) and, in certain strains, for viability (Parent *et al.*, 1993; Breuder *et al.*, 1994). As in mammalian cells, the cyclophilin A–CsA and FKBP12–FK506 complexes inhibit calcineurin functions in yeast. CsA and FK506 are now being increasingly employed to analyze calcineurin function in a variety of cells, tissues and organisms (Hendey *et al.*, 1992; Dawson *et al.*, 1993; Goldfeld *et al.*, 1994; Lieberman and Mody, 1994; Lyons *et al.*, 1994; Mulkey *et al.*, 1994).

Calcineurin is a heterodimer composed of a catalytic A subunit and a regulatory B subunit (Klee *et al.*, 1988). In response to intracellular rises in Ca^{2+}, calmodulin–Ca^{2+} complexes bind to a C-terminal segment of the calcineurin A catalytic subunit, thereby inducing conformational

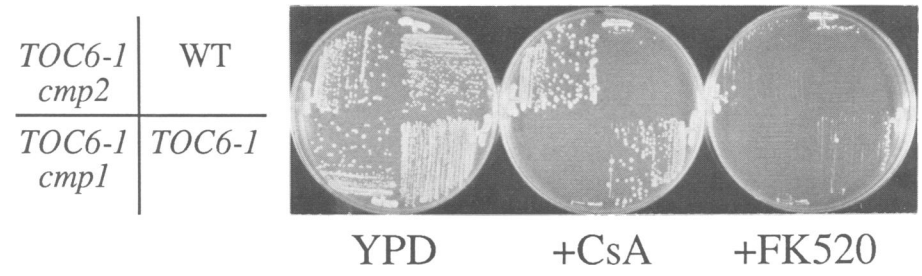

Fig. 1. Mutation in the calcineurin A catalytic subunit confers cyclosporin resistance. Isogenic strains were grown for 106 h at 30°C on YPD medium containing drug vehicle alone (YPD), 100 μg/ml CsA (+CsA) or 1 μg/ml of the FK506 analog, FK520 (+FK520). Isogenic strains were: wild-type TB23 (WT), CsA-resistant TOC6 mutant (*TOC6-1*), TOC6 mutant lacking calcineurin CMP1 (*TOC6-1 cmp1*, strain TB72), and TOC6 mutant lacking calcineurin CMP2 (*TOC6-1 cmp2*, strain TB73). Identical findings were obtained with either FK520 or FK506 (not shown).

changes that release the C-terminal autoinhibitory domain of calcineurin from the active site of the enzyme, leading to activation of calcineurin activity (Hashimoto *et al.*, 1990). The precise binding sites of the immunophilin–drug complexes within the calcineurin AB–calmodulin complex are not yet known. It is known that the cyclophilin A–CsA and FKBP12–FK506 complexes competitively bind to calcineurin and thus bind the same or overlapping sites (Liu,J. *et al.*, 1991). Immunophilin–drug complexes can bind to truncated calcineurins lacking the C-terminal regulatory domain of calcineurin A, indicating that neither this portion of calcineurin nor calmodulin are required for inhibition (Liu *et al.*, 1992; Swanson *et al.*, 1992; Parsons *et al.*, 1994). Previous studies have resulted in conflicting views as to the importance of the calcineurin A and B subunits in associating with immunophilin–drug complexes (Haddy *et al.*, 1992; Li and Handschumacher, 1993; Ryffel *et al.*, 1993; Husi *et al.*, 1994; Milan *et al.*, 1994; Woerly *et al.*, 1994). While some studies reveal cross-linking of immunophilin–drug complexes to only the B subunit (Li and Handschumacher, 1993), others implicate the A subunit (Ryffel *et al.*, 1993; Husi *et al.*, 1994; Woerly *et al.*, 1994). Notably, neither FKBP12–FK506 nor cyclophilin A–CsA binds calcineurin B alone (Liu *et al.*, 1992).

In this study, we have taken a genetic approach to define the binding sites of the immunophilin–drug complexes within calcineurin. Our approach was to isolate mutations that confer CsA or FK506 resistance in an unusual strain of *S.cerevisiae* whose growth is CsA–FK506 sensitive. Several of these mutations confer dominant drug resistance and result from single amino acid substitutions in the calcineurin A catalytic subunit. These studies identify distinct highly conserved regions of calcineurin as targets of the cyclophilin A–CsA and FKBP12–FK506 immunosuppressive complexes. A detailed view of immunophilin–drug targets on calcineurin should foster the design of novel calcineurin inhibitors as possible immunosuppressants. In addition, drug-resistant calcineurin mutants should prove valuable reagents to probe calcineurin functions *in vivo* and, potentially, to render cells and tissues resistant to CsA and FK506 actions.

Results

Isolation of cyclosporin A-resistant yeast mutants

Whereas most *S.cerevisiae* strains are resistant to CsA and FK506, we recently reported that in an unusual CsA–FK506-sensitive strain, IL993/5c, calcineurin is essential and cyclophilin A and FKBP12 respectively mediate CsA and FK506 toxicity (Breuder *et al.*, 1994). We previously described 32 mutants resistant to 100 μg/ml CsA that were isolated from two IL993/5c isogenic derivatives of opposite mating type and complementary auxotrophic mutations (Breuder *et al.*, 1994). Here, additional CsA- and FK506-resistant mutants were isolated to identify cyclophilin A–CsA and FKBP12–FK506 targets on calcineurin. The majority of CsA-resistant mutants (291/300) were resistant to CsA and to FK506, possibly because calcineurin is no longer essential or is resistant to both drugs. Nine mutants were resistant to CsA but not to FK506. Of these, eight were recessive cyclophilin A mutations complemented by the cyclophilin A gene (Cardenas, Lim and Heitman, in preparation).

Calcineurin mutations confer CsA resistance

In contrast to the recessive cyclophilin A mutations, the *TOC6-1* (for Target of CsA) mutation confers dominant CsA resistance and is not a cyclophilin A mutation (Breuder *et al.*, 1994). The *TOC6-1* mutation confers at least a 40-fold increase in resistance to CsA, growing at 1000 μg/ml CsA compared to a minimum inhibitory concentration (MIC) of 25 μg/ml in the isogenic wild-type strain, and confers no resistance to FK506. To determine if the *TOC6-1* mutation affects calcineurin, we disrupted the genes encoding either of the two redundant calcineurin A catalytic subunits, CMP1 or CMP2 (Cyert *et al.*, 1991; Liu,Y. *et al.*, 1991; Ye and Bretscher, 1992). Disruption of the *CMP1* gene abolished the CsA-resistant phenotype of the *TOC6-1* mutation, whereas disruption of the *CMP2* gene did not (Figure 1). The *CMP1* gene was PCR amplified in duplicate from wild-type and *TOC6-1* mutant genomic DNA. The *CMP1* gene cloned from the *TOC6-1* mutant conferred CsA resistance when expressed from a low copy number centromeric plasmid, whereas the wild-type *CMP1* gene did not (Figure 2A). Thus the *TOC6-1* mutation is a *CMP1* allele, hereafter referred to as *CMP1-1*.

The *CMP1-1* mutation was mapped to the C-terminal two-thirds of the gene by exchanging restriction fragments of wild-type and mutant *CMP1* genes (Figure 2). Moreover, truncating the calcineurin A C-terminal regulatory domains did not abolish CsA resistance conferred by the CMP1-1 protein (Δ1, Δ2, Δ3, Figure 2), with the exception of one deletion that extends into the calcineurin B binding domain of calcineurin A and renders calcineurin non-functional,

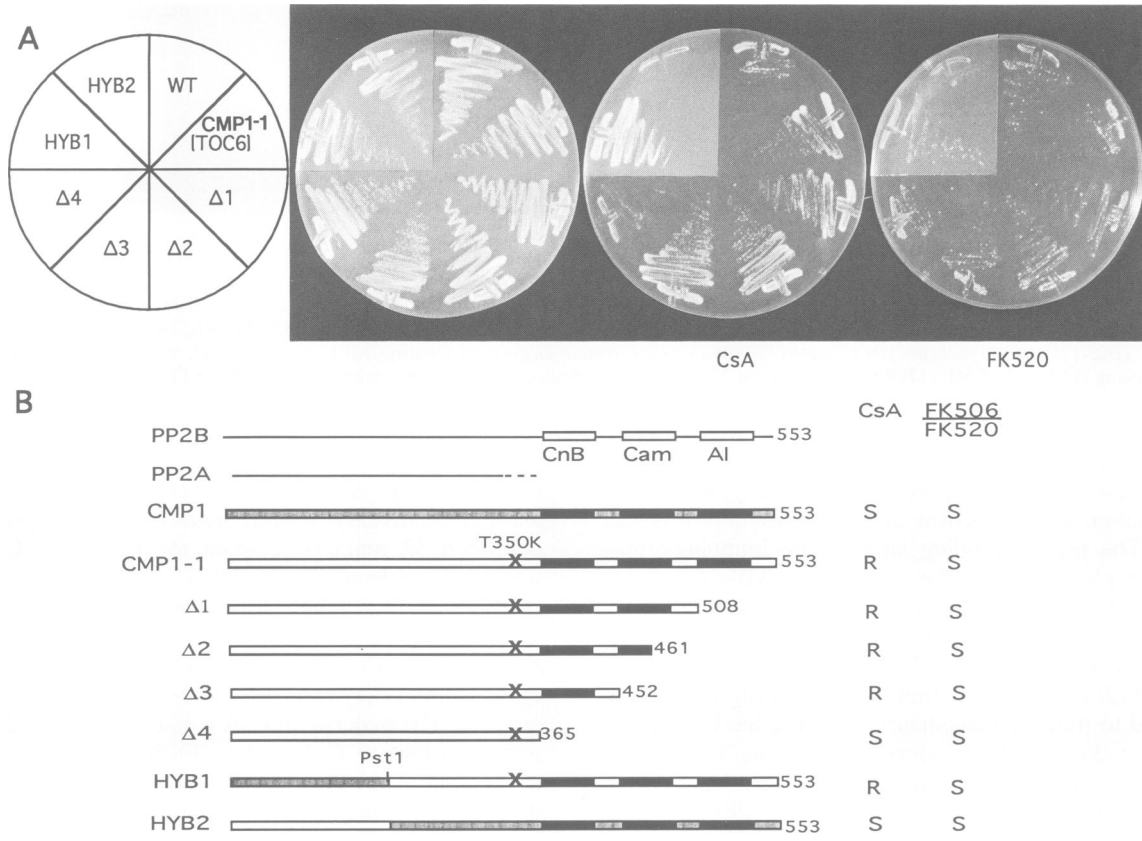

Fig. 2. Mutations that render calcineurin A CsA resistant map near or within the calcineurin B binding domain. (**A**) The wild-type CMP1 protein (CMP1), the CsA-resistant CMP1-1 protein [CMP1-1 (TOC6)], truncated forms of CMP1-1 retaining residues 1–508 (Δ1), 1–461 (Δ2), 1–452 (Δ3) or 1–365 (Δ4), or hybrid proteins comprised of the N-terminal one-third (HYB1) or the C-terminal two-thirds of the CMP1 protein and the remainder from the CMP1-1 protein, were expressed from the *CEN URA3* vector pRS316 (CMP1, CMP1-1, Δ1, Δ2, Δ3, Δ4) or from the *CEN LEU2* vector pRS315 (HYB1, HYB2) in CsA–FK520-sensitive strain TB23 (see Materials and methods) and grown for 6 days at 30°C on synthetic medium lacking leucine or uracil and containing drug vehicle alone (–), 100 μg/ml CsA (+CsA) or 1 μg/ml FK520 (+FK520). Strains expressing drug-resistant mutants yielded abundant colonies whereas those that do not gave rise to only a few colonies resulting from spontaneous drug-resistant mutants. (**B**) Alignment of primary structures indicating that protein phosphatases PP2B (calcineurin) and PP2A share identity in the N-terminal catalytic region but not in the C-terminal regulatory domains. The calcineurin B binding domain (CnB), calmodulin binding domain (Cam) and autoinhibitory region (AI) of calcineurin are indicated as boxes within the line depicting the primary sequence. The structures of, and phenotypes conferred by, wild-type, CsA-resistant, truncated and hybrid forms of the calcineurin A catalytic subunit CMP1, shown in panel (A), are summarized. In these cases, dark boxes indicate the CnB, Cam and AI domains, stippling indicates wild-type CMP1 protein sequences, open regions the CMP1-1 CsA-resistant protein sequences, and the black X the site of the T350K substitution in the CMP1-1 mutant protein. S = sensitive to CsA or FK506; R = resistant to CsA or FK506. Genes were cloned, truncated, and spliced to produce hybrid products as described in Materials and methods.

as measured by failure to complement a *cmp1 cmp2* mutant strain (not shown) (Δ4, Figure 2). Thus, the *CMP1-1* mutation maps to the middle one-third of the *CMP1* gene. DNA sequence analysis of the entire *CMP1-1* gene revealed a single nucleotide substitution within this region (ACA→AAA), which results in a lysine substitution at residue threonine 350 (T350K). This residue lies between the active site and the calcineurin B binding domain in a region highly conserved between calcineurins from different species (Figure 3).

Additional mutations that confer resistance to up to 1000 μg/ml CsA but not to FK506 were isolated from a CsA–FK506-sensitive strain containing the cyclophilin A gene on a 2μ multicopy plasmid. In this strain, any recessive cyclophilin A mutations that arise are complemented and fail to confer drug resistance. By this means, two additional mutations that confer dominant CsA resistance were obtained. In both cases, disruption of the calcineurin *CMP1* gene abolished drug resistance whereas disruption of the *CMP2* gene had no effect (data not shown). DNA sequence analysis revealed that both contain single amino acid substitutions in the *CMP1* gene, T350R (*CMP1-2* allele, ACA→AGA) and Y377F (*CMP1-3* allele, TAC→TTC) (Figure 3). Thus, substitution of T350 by either lysine or arginine renders calcineurin CsA resistant. By Western blot analysis with antisera against the CMP1 subunit (Materials and methods), the *CMP1-1, CMP1-2* and *CMP1-3* mutations do not alter the size or reduce the expression or stability of the calcineurin A CMP1 subunit (Figure 5C).

All three CsA-resistant calcineurin A CMP1 mutant proteins are functional because they support viability of the calcineurin-dependent host strain when the gene encoding the redundant CMP2 subunit is disrupted (Figure 1 and not shown). This is especially significant for the Y377F *CMP1-3* mutation, in which the amino acid substitution lies within the calcineurin B binding domain of calcineurin A (Figure 3) but does not compromise calcineurin function. One mechanism by which these mutations might confer CsA resistance would be to render calcineurin A independent of calcineurin B, especially given that calcineurin B readily cross-links to cyclophilin

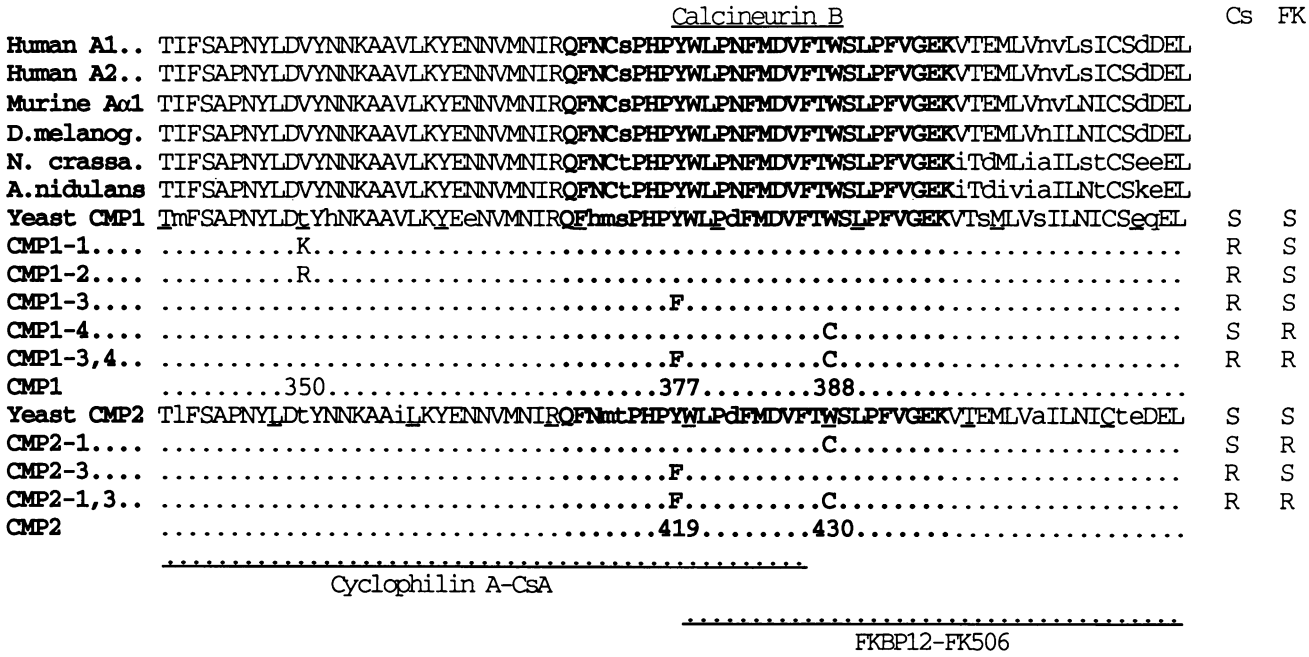

Fig. 3. Alignment of calcineurin A sequences encompassing the calcineurin B binding domain. Portions of the primary amino acid sequence of the calcineurin A catalytic subunits from several species are aligned with amino acids indicated by single letter abbreviations. Residues invariant or conserved in 5–7 of the eight sequences are shown in capital letters. The region of calcineurin A that binds the calcineurin B regulatory subunit is shown in bold, overlined, and designated Calcineurin B. Substitutions that render the yeast calcineurin A CMP1 subunit CsA resistant, FK506 resistant or both CsA and FK506 resistant are shown below the wild-type CMP1 sequence as the single letters K, R, F, C, and F and C, representing the T350K (CMP1-1), T350R (CMP1-2) and Y377F (CMP1-3) CsA-resistant mutants, the W388C (CMP1-4) FK506-resistant mutant and the Y377F, W388C (CMP1-3,4) CsA- and FK506-resistant mutant. Substitutions that render the yeast calcineurin A CMP2 subunit FK506 resistant, CsA resistant or both FK506 and CsA resistant are shown below the wild-type CMP2 sequence as the single letters C, F, or F and C, representing the W430C (CMP2-1) FK506-resistant mutant, the Y419F (CMP2-3) CsA-resistant mutant and the Y419F, W430C (CMP2-1,3) FK506- and CsA-resistant mutant. Sensitivity or resistance to CsA (Cs) or FK506 (FK) are indicated by an S or an R, respectively. These substitutions either occur in invariant residues (Y377F, W430C) or within a region of marked identity (T350K, T350R, where T350 is valine in other calcineurin A proteins). Two substitutions occur within the calcineurin B binding site on calcineurin A (Y377F, W430C). Proposed binding sites of the immunophilin–drug complexes on calcineurin A are indicated by underlining and the notations Cyclophilin A–CsA and FKBP12–FK506. Sequences for calcineurins from human, murine, *Drosophila melanogaster*, *Neurospora crassa*, *Aspergillus nidulans* and *S.cerevisiae* (CMP1 and CMP2) are from Guerini and Klee (1989), Kincaid *et al.* (1990), Guerini *et al.* (1992), Higuchi *et al.* (1991), Rasmussen *et al.* (1994) and Cyert *et al.* (1991).

A in the complex (Li and Handschumacher, 1993). However, we found that, when expressed in two different calcineurin B mutant strains (SMY7 and JHY58, Cardenas *et al.*, 1994b), the T350K, T350R and Y377F CsA-resistant calcineurin A CMP1 mutant proteins did not confer LiCl resistance and therefore do not provide calcineurin function (data not shown). Thus, all three CsA-resistant calcineurin A mutant proteins still require calcineurin B to function.

Isolation of FK506-resistant calcineurin mutants

By a similar protocol, we also isolated mutants resistant to FK506, but not to CsA, from the CsA–FK506-sensitive yeast strain expressing FKBP12 from a multicopy number 2μ plasmid. Three independent mutations that conferred resistance to FK506, but not to CsA, were obtained. These mutations confer at least a 200-fold increase in FK506 resistance and grow at 100 μg/ml FK506 compared to an MIC of 0.5 μg/ml in the isogenic wild-type strain. All three mutations confer dominant FK506 resistance when mated to isogenic wild-type strains. In all three mutant strains, disruption of the calcineurin A *CMP1* gene had no effect, whereas disruption of the *CMP2* gene abolished FK506 resistance. By DNA sequence analysis, all three contain single nucleotide substitutions in the *CMP2* gene. These mutations substitute different nucleotides within the same codon of the calcineurin A *CMP2* gene (TGG→TGT or TGC), resulting in a single amino acid substitution, W430C (Figure 3). Residue W430 in the calcineurin A subunit CMP2 corresponds to residue W388 in the calcineurin A CMP1 subunit (Figure 3). As with the CsA-resistant CMP1 mutant proteins, the FK506-resistant W430C CMP2 mutant protein did not provide calcineurin function to strains lacking calcineurin B (not shown) and is therefore still dependent on calcineurin B for activity.

CsA- or FK506-resistant calcineurin mutants have reduced affinity for cyclophilin A–CsA or FKBP12–FK506

We next tested if these calcineurin A mutations render calcineurin CsA or FK506 resistant by preventing binding by the cyclophilin A–CsA and FKBP12–FK506 complexes. For this purpose, we followed *in vivo* and *in vitro* approaches employing the yeast two-hybrid system (Fields and Song, 1989) and immunophilin–ligand affinity chromatography, respectively.

For the first method, yeast FKBP12 was fused to the GAL4 DNA binding domain [GAL4(BD)–FKBP12] and the yeast calcineurin A CMP1 catalytic subunit (lacking the C-terminal autoinhibitory domain to eliminate the requirement of calmodulin for interaction) was fused to the GAL4 transcriptional activation domain [GAL4(AD)–

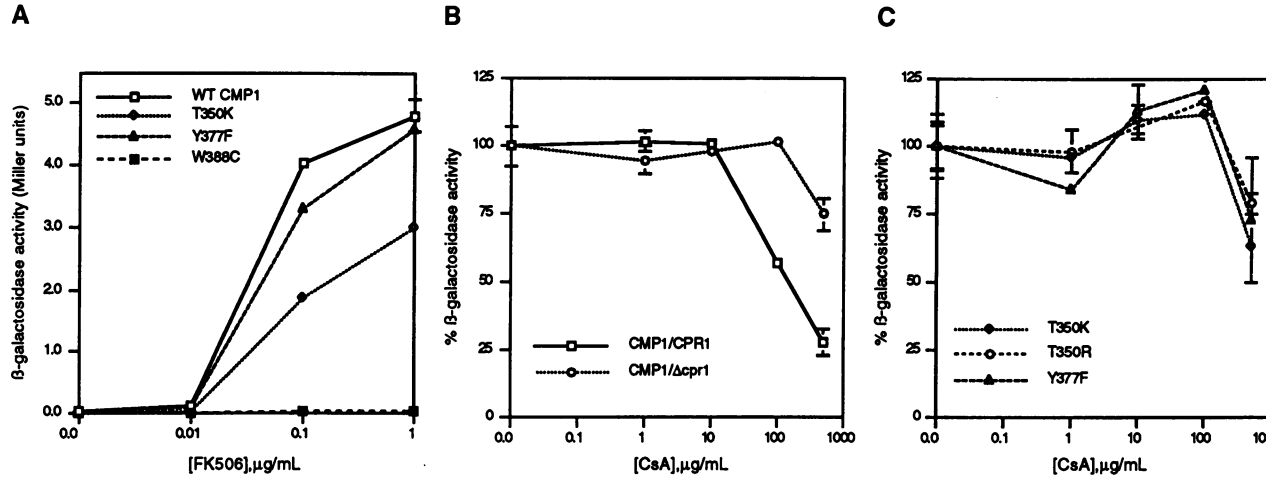

Fig. 4. Binding of CsA- and FK506-resistant calcineurin A mutants to FKBP12–FK506 and cyclophilin A–CsA in the yeast two-hybrid system. Cultures were inoculated in duplicate in synthetic medium lacking tryptophan and leucine. Medium contained either FK506, to detect FKBP12–FK506 binding to calcineurin (panel A), or 100 ng/ml FK506 plus CsA at the indicated concentrations to assay cyclophilin A–CsA binding to calcineurin by its ability to compete FKBP12–FK506 binding to calcineurin (panels B and C). Following growth for 24 h at 30°C, samples were removed, β-galactosidase expression determined by CPRG assay, averaged for duplicate samples, and mean values were plotted with error bars. The GAL4(BD)–FKBP12 fusion protein was co-expressed with the GAL4(AD) fused to the wild-type CMP1 protein, or to the CsA-resistant CMP1-1 (T350K), CMP1-2 (T350R) and CMP1-3 (Y377F), or the FK506-resistant CMP1-4 (W388C) mutant proteins, each of which lacked the calcineurin autoinhibitory domain (thereby bypassing the calmodulin requirement for interaction). Fusion proteins were co-expressed in isogenic two-hybrid host strains which were wild-type (strain Y190) (panels A, B, and C) or lacked cyclophilin A (Δcpr1 strain CHY1) (panel B). In control strains lacking either the GAL4(BD)–FKBP12 or the GAL4(AD)–CMP1 protein, the level of β-galactosidase expressed was no higher than the background level alone (<0.1 units). (**A**) FK506 stimulates FKBP12 binding to wild-type CMP1 (squares), and to CsA-resistant mutant proteins CMP1-1 (diamonds) and CMP1-3 (triangles). FK506 did not stimulate FKBP12 binding to an FK506-resistant CMP1-4 mutant protein (dotted squares). (**B**) Cyclophilin A–CsA complex competes binding of GAL4(BD)–FKBP12–FK506 to the GAL4(AD)–CMP1 fusion protein (squares). CsA-dependent competition was markedly decreased in a cyclophilin A-deficient host strain (circles); thus, the cyclophilin A–CsA complex competes FKBP12–FK506 binding to calcineurin. (**C**) Cyclophilin A–CsA competition of FKBP12–FK506 binding to CMP1 is greatly decreased by the CsA resistance mutations CMP1-1 (T350K, diamonds), CMP1-2 (T350R, circles) and CMP1-3 (Y377F, triangles), indicating that these mutations decrease cyclophilin A–CsA binding to calcineurin CMP1.

CMP1]. FK506 promoted an interaction between the GAL4(BD)–FKBP12 and the wild-type GAL4(AD)–CMP1 fusion proteins in the yeast two-hybrid system (Figure 4A). When the T350K, T350R and Y377F CsA-resistant mutations were introduced into the GAL4(AD)–CMP1 fusion protein, the interaction with the GAL4(BD)–FKBP12–FK506 complex was equivalent to that observed with wild-type CMP1 for the Y377F CMP1-3 mutant fusion protein, and reduced by ~2-fold for the T350K CMP1-1 mutant fusion protein (Figure 4A). Interaction with the T350R CMP1-2 mutant was similar to that with the T350K mutant (data not shown). Because neither the CMP1-1 nor the CMP1-2 mutants confer FK506 resistance *in vivo* (Figures 2 and 6A), the 2-fold reduction in FKBP12–FK506 binding to the CMP1-1 and CMP1-2 fusion proteins is not sufficient to confer FK506 resistance. When the FK506-resistant CMP2-1 mutation W430C was introduced at the corresponding position in CMP1 (W388C), the resulting GAL4(AD)–CMP1-4 mutant fusion protein did not interact with FKBP12–FK506 to any extent (Figure 4A). The GAL4(AD)–CMP1 mutant fusion proteins conferred appropriate drug resistance when expressed in a CsA–FK506-sensitive host strain, indicating that the fusion proteins are functionally expressed (data not shown). Thus, an FK506-resistant CMP1 mutant (CMP1-4) does not bind FKBP12–FK506, whereas CsA-resistant CMP1 mutants (CMP1-1, CMP1-2 and CMP1-3) do bind FKBP12–FK506.

Despite numerous attempts, we have thus far been unable to construct GAL4–cyclophilin A fusion proteins that interact with the GAL4–calcineurin fusion protein in the presence of CsA. As an alternative approach, we took advantage of the finding that FKBP12–FK506 and cyclophilin A–CsA compete to bind calcineurin (Liu,J. *et al.*, 1991). Thus, we monitored the ability of the cyclophilin A–CsA complex to reduce binding of the GAL4(BD)–FKBP12–FK506 complex to the GAL4(AD)–CMP1 fusion protein in the two-hybrid assay. With the wild-type GAL4(AD)–CMP1 protein, CsA bound to endogenous yeast cyclophilin A effectively and competed the FKBP12–FK506–CMP1 complex by 45 and 75% at 100 and 500 μg/ml CsA, respectively (Figure 4B). The ability of CsA to compete FKBP12–FK506 binding to CMP1 requires cyclophilin A, and accordingly was markedly reduced in a strain lacking cyclophilin A (Figure 4B). The ability of CsA to compete FKBP12–FK506 binding to the CMP1-1, CMP1-2 and CMP1-3 CsA-resistant mutant proteins was greatly decreased (Figure 4C), consistent with the interpretation that these mutations reduce cyclophilin A–CsA binding to CMP1.The FK506-resistant W388C CMP1-4 mutant protein could not be tested for cyclophilin A–CsA binding by competition of FKBP12–FK506 binding in the two-hybrid system (Figure 4B and C), because the W388C mutant protein does not bind FKBP12–FK506 in the two-hybrid assay (Figure 4A).

For the *in vitro* approach, yeast cyclophilin A and FKBP12 were affinity tagged with a stretch of six histidines and purified by Ni^{2+} affinity chromatography. The purified proteins were coupled to affigel-10 to produce FKBP12 and cyclophilin A affinity matrices. Both affigel-10-immobilized proteins exhibited proline isomerase activity that was inhibited by CsA or FK506, respectively (Cardenas *et al.*, 1994b). These FKBP12 and cyclophilin A affinity matrices were incubated with yeast extracts in

the absence or presence of FK506 and CsA, respectively. Bound proteins were eluted and analyzed by Western blot with antisera directed against calcineurin A or calcineurin B. Because the anti-calcineurin A antisera is directed against a region of the calcineurin A CMP1 protein which is highly conserved in the CMP2 isoform, this antisera strongly reacts with CMP1 but also weakly cross-reacts with the CMP2 subunit (Ye and Bretscher, 1992; Cardenas *et al.*, 1994b). Therefore, to avoid unambiguous results, we prepared cell extracts from strains deleted for the *CMP2* gene and expressing wild-type, CsA-resistant, or FK506-resistant CMP1 protein. By Western blot analysis, wild-type and mutant cell extracts employed for immunophilin affinity chromatography contained equal amounts of calcineurin A (CMP1) and calcineurin B (CNB1) subunits (Figure 5C). These results demonstrate that the wild-type and immunosuppressant-resistant mutant calcineurin proteins are equally stable. Because previous studies have established that both the calcineurin A and B subunits are required for calcineurin binding to cyclophilin A–CsA or FKBP12–FK506 (Liu *et al.*, 1992; Li and Handschumacher, 1993; Cardenas *et al.*, 1994b; Zhu, Cardenas and Heitman, submitted), the calcineurin A subunit CMP1 and the calcineurin B subunit CNB1 are expected to bind immunophilin–drug affinity matrices as a heterodimer.

As previously observed (Cardenas *et al.*, 1994b), the wild-type calcineurin A catalytic subunit CMP1 and the calcineurin B regulatory subunit CNB1 from total crude protein cell extracts readily bound to the FKBP12–FK506 complex (lane 2, Figure 5A). This binding is specific since it was not observed with affigel-10 beads lacking FKBP12 (not shown, Cardenas *et al.*, 1994b). The CsA-resistant T350K, T350R and Y377F CMP1 mutant proteins bound FKBP12–FK506 to the same extent as wild-type CMP1, as detected by binding of both CMP1 (upper panel) and CNB1 (lanes 4, 6 and 8, Figure 5A). Conversely, with the FK506-resistant W388C CMP1-4 mutant protein, no binding of calcineurin A (upper panel) or calcineurin B (lower panel) to FKBP12–FK506 was detected (lane 10, Figure 5A), indicating that the W388C mutation prevents interaction with FKBP12–FK506 *in vitro*, as observed in the two-hybrid assay *in vivo* (Figure 4). Thus, *in vitro,* CsA-resistant calcineurin mutant proteins interact with FKBP12–FK506 whereas an FK506-resistant mutant protein does not. With extract from a *cmp1*-deleted wild-type *CMP2* strain, the strong CMP1-reactive signal was absent (lane 12, Figure 5A, upper panel). We note that, in the absence of CMP1, the CMP2 subunit effectively

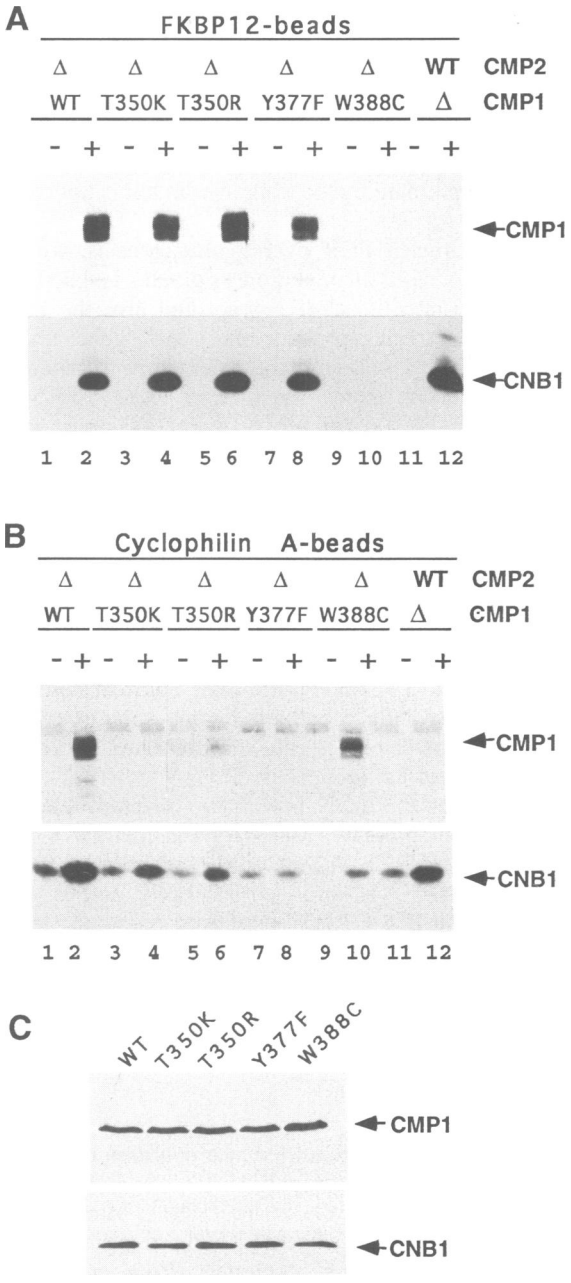

Fig. 5. Binding of CsA- and FK506-resistant calcineurin A mutants to FKBP12–FK506 and cyclophilin A–CsA *in vitro*. (**A**) Binding assays with the His6–FKBP12–affigel affinity matrix and equal amounts (1 mg) of protein extracts from the wild-type *CMP1 Δcmp2* strain (WT, strain TB71), isogenic CsA-resistant mutants lacking CMP2 and expressing CMP1 mutant proteins T350K (CMP1-1), T350R (CMP1-2), and Y377F (CMP1-3) (strains TB73, TB118 and TB119), a *Δcmp1 Δcmp2* strain expressing the CMP1-4 FK506-resistant mutant protein from the centromeric plasmid pCN1F (W388C, strain TB174) and an isogenic *CMP2 Δcmp1* strain (strain TB88) were performed in the absence (–, lanes 1, 3, 5, 7, 9 and 11) or the presence of 20 μM FK506 (+, lanes 2, 4, 6, 8, 10 and 12). The figure shows Western blots of material eluted from the beads and probed with affinity-purified antisera directed against the yeast CMP1 calcineurin A subunit (upper panel) or the CNB1 calcineurin B regulatory subunit (lower panel). Arrows to the right in panels A and B indicate the positions of migration of the CMP1 and CNB1 proteins. (**B**) Binding assays with the His6–cyclophilin A–affigel affinity matrix and equal amounts (1 mg) of protein extracts from the strains indicated in (**A**) were performed in the absence (–, lanes 1, 3, 5, 7, 9 and 11) or the presence of 100 μM CsA (+, lanes 2, 4, 6, 8, 10 and 12), and eluted material probed with the anti-CMP1 (upper panel) and anti-CNB1 (lower panel) antisera, as in (A). Variation in the amount of CNB1 protein associated with cyclophilin A and cyclophilin A–CsA is most likely attributable to reduced affinity conferred by the CMP1 mutations, because Western blot confirmed that equal amounts of CNB1 were present in each binding assay (panel C). Moreover, there is residual binding of calcineurin to cyclophilin that was not detectable by the affinity-purified anti-CMP1 antisera but which could be detected by the much higher affinity anti-CNB1 antisera. (**C**) Western blot analysis with anti-CMP1 (upper panel) and anti-CNB1 (lower panel) antisera demonstrated that equivalent amounts of the CMP1 and CNB1 proteins were present in the wild-type and mutant protein extracts employed for the immunophilin affinity chromatography. For this analysis, 40 μg of total protein were loaded for each strain.

supports binding of calcineurin B to FKBP12–FK506 (lane 12, Figure 5A, lower panel).

CsA stimulated binding between the wild-type calcineurin A CMP1 subunit and cyclophilin A (lane 2, Figure 5B, upper panel). This binding is specific since it was not detected when a Δ*cmp1* strain was used (lane 12, Figure 5B, upper panel). In contrast to wild-type CMP1, CsA stimulated only partial binding of the T350R CMP1-2 mutant protein, trace binding of the T350K CMP1-1 mutant protein and no binding of the Y377F CMP1-3 mutant calcineurin protein to cyclophilin A (see respectively lanes 6, 4 and 8, Figure 5B, upper panel). Because the cell extracts employed for affinity chromatography contained equal amounts of wild-type CMP1, and of the CMP1-1, CMP1-2 and CMP1-3 mutant proteins (Figure 5C), this decrease in binding is attributable to decreased affinity of the mutant calcineurins for the cyclophilin A–CsA complex. Thus, *in vivo* and *in vitro*, the T350K, T350R and Y377F CMP1 mutant proteins bind FKBP12–FK506 but their affinity for cyclophilin A–CsA is reduced, explaining why these mutants render cells resistant to CsA but not to FK506.

In the absence of CsA, wild-type CMP1, the T350K, T350R, Y377F and W388C CMP1 mutant proteins, and wild-type CMP2, supported binding of calcineurin B to cyclophilin A, albeit to a lesser extent for the mutant CMP1 proteins when compared to wild-type CMP1 (lanes 1, 3, 5, 7, 9 and 11, Figure 5B). With longer exposure, calcineurin A is also apparent in these ligand-independent complexes with cyclophilin A (Cardenas *et al.*, 1994b). CsA stimulated binding of calcineurin B to cyclophilin A in wild-type CMP1 extracts (lane 2, Figure 5B), and also with the T350K and T350R CMP1 mutant proteins (lanes 4 and 6, Figure 5B), albeit to a lesser extent than with wild-type CMP1. In contrast, CsA did not stimulate calcineurin B binding to cyclophilin A with the Y377F CMP1-3 mutant protein (compare lanes 7 and 8, Figure 5B). These variations in CNB1 protein associated with cyclophilin A and cyclophilin A–CsA are most likely to be attributable to reduced affinity conferred by the CMP1 mutant calcineurin subunit, because Western blot analysis (Figure 5C) confirmed that equal amounts of CNB1 protein were present in each binding assay. Moreover, although the mutations in CMP1 profoundly reduce affinity for association of calcineurin to the cyclophilin affinity matrix, there is a residual binding not detectable by the anti-CMP1 antisera which can be detected by the much higher affinity anti-CNB1 antisera (Figure 5B). The finding that the T350R and T350K mutations reduce, but do not abolish, CsA-dependent binding of cyclophilin A to both the calcineurin A and B subunits suggests that these mutations may confer CsA resistance by affecting both binding and some subsequent step required for inhibition.

In the case of the FK506-resistant CsA-sensitive CMP1-4 mutant protein, we detected no *in vitro* association of calcineurin CMP1-4 or CNB1 with the FKBP12–FK506 complex (lane 10, Figure 5A, upper and lower panels). Conversely, CsA stimulated binding of the CMP1-4 mutant calcineurin to cyclophilin A, as detected by Western blot analysis of either the CMP1-4 calcineurin A mutant subunit (compare lanes 9 and 10, Figure 5B, upper panel) or of the associated calcineurin B subunit CNB1 (lanes 9 and 10, Figure 5B, lower panel). Thus, the FK506-resistant W388C CMP1–CNB1 mutant calcineurin complex binds cyclophilin A–CsA but fails to bind FKBP12–FK506, which is consistent with the ability of this mutant to confer resistance to FKBP12–FK506 but not to cyclophilin A–CsA.

Co-expression of CsA- and FK506-resistant calcineurin mutants confers resistance to CsA or FK506; calcineurin double mutants confer resistance to CsA plus FK506

Recombinant strains expressing both a CsA-resistant and an FK506-resistant calcineurin mutant were constructed to test if expression of both mutant proteins would confer resistance to both drugs. Yeast strains expressing a CsA-resistant CMP1 calcineurin mutant were crossed to strains expressing an FK506-resistant CMP2 calcineurin mutant (CMP2-1). In three different cases (*CMP1-1* × *CMP2-1*, *CMP1-2* × *CMP2-1* and *CMP1-3* × *CMP2-1*), the resulting heterozygous diploids grew on medium containing either CsA or FK506, indicating that both mutant proteins are functional and do not interfere with each other's ability to confer drug resistance (Figure 6A and data not shown). Furthermore, as expected, these strains were unable to grow on medium containing CsA plus FK506, since, under these conditions, each mutant calcineurin is inhibited by the drug to which it is not resistant (Figure 6A).

By site-directed PCR overlap mutagenesis, we introduced a CsA-resistant mutation originally isolated in the *CMP1* gene into the *CMP2* gene, and also the FK506-resistant *CMP2* mutation into the *CMP1* gene, resulting in single and double mutant calcineurin subunits that appropriately conferred resistance to CsA, to FK506, or to CsA plus FK506 (Figure 6B). For example, when a W388C substitution (corresponding to the FK506-resistant W430C mutation in the CMP2 subunit) was introduced into the CMP1 calcineurin subunit, cells expressing this W388C CMP1 mutant protein (CMP1-4) were resistant to FK506 but sensitive to CsA (Figure 6B). Similarly, a Y419F mutation, corresponding to the the CsA-resistant mutation Y377F in the CMP1 subunit, rendered the resulting CMP2 calcineurin catalytic subunit resistant to CsA but not to FK506 (Figure 6B). Thus, in both cases, mutations originally identified in one subunit could be introduced into the related calcineurin subunit with predictable phenotypic consequences.

We extended this analysis with two different calcineurin double mutant proteins (Materials and methods). In this case, both a Y377F, W388C CMP1 double mutant calcineurin (CMP1-3,4), and also a Y419F, W430C CMP2 double mutant (CMP2-1,3), conferred resistance to CsA, to FK506 and to CsA plus FK506 (Figure 6B). Thus, combination of the two mutations rendered calcineurin resistant to both drugs and neither mutation interfered with the ability of the other to confer drug resistance.

Discussion

Calcineurin has been identified as a common target of the immunosuppressive cyclophilin A–CsA and FKBP12–FK506 complexes (Liu,J. *et al.*, 1991). Moreover, calcineurin is a target for CsA and FK506 actions in organisms as diverse as yeast and mammals (Foor *et al.*, 1992;

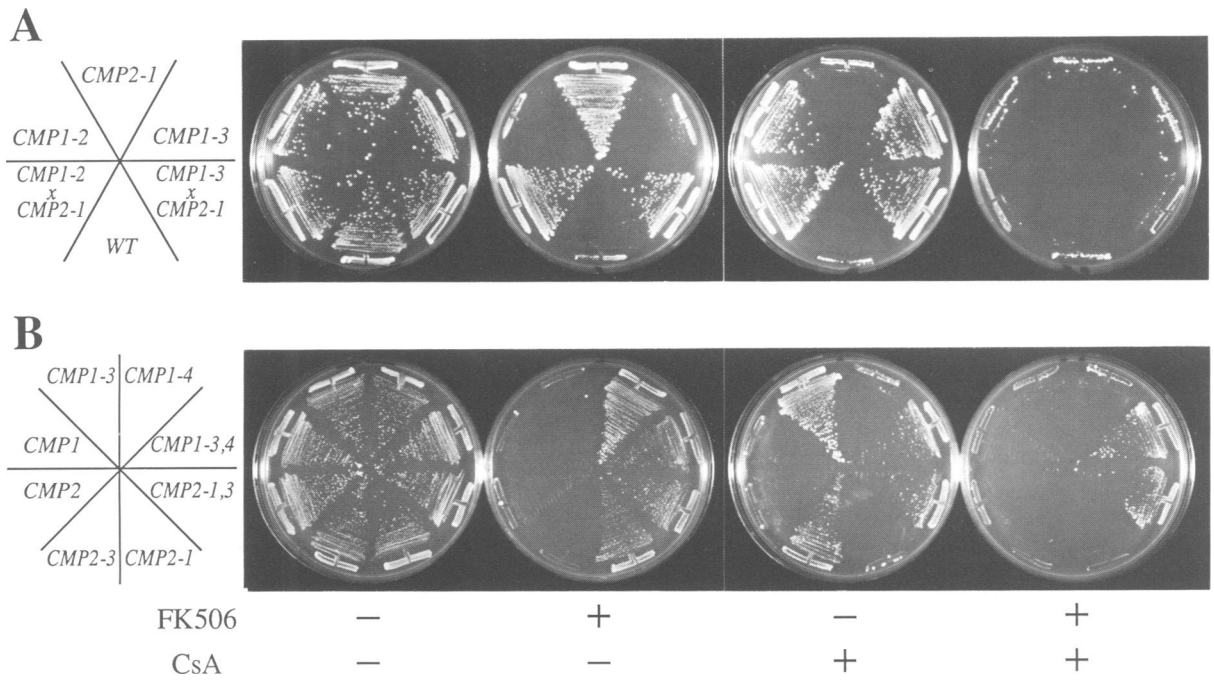

Fig. 6. Calcineurin double mutants resistant to CsA and FK506. (**A**) Isogenic strains expressing wild-type calcineurin (WT), the CsA-resistant T350R or Y377F CMP1 mutant proteins (CMP1-2 and CMP1-3, respectively), the FK506-resistant W430C CMP2 mutant protein (CMP2-1), or both a CsA-resistant CMP1 mutant and the FK506-resistant CMP2 mutant (CMP1-2×CMP2-1 and CMP1-3×CMP2-1) were grown for 72 h at 30°C on YPD medium containing drug vehicle alone, 1 μg/ml FK506, 100 μg/ml CsA or 1 μg/ml FK506 plus 100 μg/ml CsA, as indicated. Diploid strains expressing both CMP1-2 and CMP2-1, or CMP1-3 and CMP2-1, were isolated as described in Materials and methods. (**B**) CsA–FK506-sensitive strain TB23 expressing wild-type or drug-resistant calcineurin CMP1 or CMP2 mutant proteins from the *CEN LEU2* vector pRS315 was grown for 168 h at 30°C on synthetic dextrose medium lacking leucine and containing drug vehicle alone, 1 μg/ml FK506, 100 μg/ml CsA or 1 μg/ml FK506 plus 100 μg/ml CsA. Strains expressed wild-type CMP1 (CMP1), CsA-resistant Y377F CMP1 mutant (CMP1-3), FK506-resistant W388C CMP1 mutant (CMP1-4) and CsA–FK506-resistant Y377F, W388C CMP1 double mutant (CMP1-3,4), wild-type CMP2 (CMP2), CsA-resistant Y419F CMP2 mutant (CMP2-3), FK506-resistant W430C CMP2 mutant (CMP2-1) and CsA-resistant FK506-resistant Y419F, W430C CMP2 double mutant (CMP2-1,3). Plasmids expressing wild-type and mutant CMP1 and CMP2 proteins were constructed as described in Materials and methods.

Breuder *et al.*, 1994). Activated calcineurin is a heterotrimer composed of the catalytic A subunit bound to the regulatory B subunit and calmodulin. In the present study, we set out to identify where the immunophilin–drug complexes bind within the calcineurin complex.

Previous *in vitro* studies revealed that neither calmodulin nor the C-terminal regulatory domain of calcineurin A are required for calcineurin inhibition by immunophilin–drug complexes (Liu,J. *et al.*, 1991; Swanson *et al.*, 1992; Parsons *et al.*, 1994). Calcineurin B is required for calcineurin binding or inhibition by cyclophilin–CsA (Haddy *et al.*, 1992) and by FKBP12–FK506 (Cardenas *et al.*, 1994b). Moreover, cyclophilin A–CsA and FKBP12–FK506 complexes bound to the calcineurin AB holoenzyme readily cross-link to calcineurin B, but not to calcineurin A (Li and Handschumacher, 1993; Husi *et al.*, 1994). In contrast, a photoactivatable CsA analog cross-links to the calcineurin A subunit (Ryffel *et al.*, 1993; Woerly *et al.*, 1994), or to both the calcineurin A and B subunits (Husi *et al.*, 1994), in the cyclophilin–CsA–calcineurin AB complex. Furthermore, neither immunophilin–drug complex binds to the isolated calcineurin B subunit *in vitro* (Liu *et al.*, 1992). Lastly, a recent study has identified residues of calcineurin B critical for association with both immunophilin–drug complexes (Milan *et al.*, 1994). These apparently conflicting reports can be reconciled by suggesting that both the calcineurin A and B subunits participate in interactions with immunophilin–drug complexes.

To identify the target residues in calcineurin of the cyclophilin A–CsA and FKBP12–FK506 complexes, we have isolated yeast mutants resistant to the antifungal effects of CsA and FK506. Amongst such mutants, we have identified mutations that render calcineurin resistant to the toxic effects of the cyclophilin A–CsA or the FKBP12–FK506 complexes. Thus, the T350K, T350R or Y377F mutations render yeast calcineurin A CMP1 resistant to CsA, whereas a W430C mutation in calcineurin A CMP2, and similarly a corresponding W388C mutation in CMP1, confer resistance to FK506 (Figures 1, 3 and 6). These calcineurin A residues could interact with either the immunophilin or the ligand. With both the yeast two-hybrid system *in vivo* (Figure 4) and immunophilin affinity matrices *in vitro* (Figure 5), we demonstrate that the CsA-resistant FK506-sensitive T350K, T350R and Y377F CMP1 mutant proteins show reduced affinity for cyclophilin A–CsA but bind to FKBP12–FK506 with little or no reduction in affinity compared to wild-type calcineurin. The T350K CMP1-1 and T350R CMP1-2 mutant proteins do support binding, albeit weaker than with wild-type CMP1, of the calcineurin B regulatory subunit CNB1 to cyclophilin A–CsA *in vitro* (Figure 5B), suggesting that these mutations may confer CsA resistance by interfering both with binding and some event subsequent to binding required for inhibition of calcineurin.

The FK506-resistant W388C CMP1-4 mutant protein did not bind FKBP12–FK506 *in vivo* in the two-hybrid system (Figure 4A). Because this mutant does not bind

FKBP12–FK506, we could not analyze cyclophilin A–CsA binding by competition in the two-hybrid system. In our *in vitro* binding assays (Figure 5), neither the CMP1-4 mutant subunit nor its associated calcineurin B subunit CNB1 bound to the FKBP12–FK506 affinity matrix (Figure 5A). In contrast, both the CMP1-4 mutant calcineurin A subunit and the associated calcineurin B regulatory subunit CNB1 did bind to the cyclophilin A–CsA complex (Figure 5B, lane 10). We conclude that the W388C mutation confers FK506 resistance by inhibiting binding of the FKBP12–FK506 complex to calcineurin.

Taken together, our findings demonstrate that the cyclophilin A–CsA complex binds to the calcineurin A catalytic subunit and implicate a conserved region between the active site and extending to and overlapping with the calcineurin B binding site as the target of CsA action (Figure 3). Similarly, the FKBP12–FK506 complex also binds calcineurin A in the region overlapping the calcineurin B binding domain; in this case, we propose that the binding target extends further towards the C-terminus (Figure 3). Moreover, FKBP12–FK506 and cyclophilin A–CsA compete to bind to calcineurin. Taken together, these findings suggest that the cyclophilin A–CsA and FKBP12–FK506 complexes bind to distinct but partially overlapping sites on calcineurin A and explain why these two structurally dissimilar complexes competitively bind to calcineurin. An alternative explanation, that calcineurin B is the target of CsA and FK506 and that these mutations confer drug resistance by rendering calcineurin A independent of calcineurin B, is not supported by our finding that the T350K, T350R and Y377F CMP1, and the W430C CMP2 mutant proteins cannot provide calcineurin activity in strains lacking calcineurin B. Moreover, mutations which render calcineurin A independent of calcineurin B could not give rise to the two different drug resistance phenotypes we have observed (CsA-resistant, FK506-sensitive and CsA-sensitive, FK506-resistant). Both immunophilin–drug complexes may have evolved to target this region of calcineurin A because it is highly conserved, interacts with the regulatory B subunit and is at least partially solvent exposed.

Although it has been proposed that calcineurin A might allosterically alter calcineurin B, allowing immunophilin–drug complexes to bind to the B subunit alone (Li and Handschumacher, 1993), based on our genetic evidence, we propose that cyclophilin A–CsA and FKBP12–FK506 complexes make direct protein–protein or drug–protein contacts with the calcineurin A subunit. Because no one has observed direct binding of either immunophilin–drug complex with isolated calcineurin A, or with calcineurin B (Liu *et al.*, 1992), the immunophilin–drug complexes may contact both subunits in the calcineurin AB complex. In summary, our finding that the immunophilin–drug binding targets lie adjacent to and overlap the calcineurin B binding site on calcineurin A, and previous observations that calcineurin B plays a pivotal role in calcineurin interactions with immunophilin–drug complexes (Haddy *et al.*, 1992; Li and Handschumacher, 1993; Cardenas *et al.*, 1994b; Milan *et al.*, 1994), strongly suggest that the protein–drug complexes contact both calcineurin A and B. Further structural studies will be required to determine the structures of the immunophilin–drug–calcineurin complexes, and to resolve if cyclophilin–CsA and FKBP12–FK506 compete for calcineurin by occluding each other's binding to nearby distinct sites or by interacting with different residues within a common binding pocket.

All of the spontaneous mutations conferring CsA resistance occurred in the CMP1 calcineurin A catalytic subunit, whereas all of those conferring FK506 resistance were mutations in the CMP2 subunit. Given that these two gene products are functionally redundant and 67% identical, why is this the case? By site-directed mutagenesis, we found that mutations originally isolated in one calcineurin A subunit were capable of rendering the other calcineurin A subunit drug resistant (Figure 6B). However, other factors might contribute to the bias in mutants obtained from genetic selections. For example, one subunit might be inherently more drug resistant. The MIC required for growth inhibition by FK506 was decreased ~2-fold (from 0.5 μg/ml to 0.25 μg/ml) in strains lacking either CMP1 or CMP2 (not shown), consistent with the interpretations that the two subunits are equally abundant and sensitive to FKBP12–FK506, and that strains expressing half the normal level of calcineurin are inhibited by half the drug required in wild-type. In contrast, the MIC for CsA was decreased ~2-fold in a *cmp1* mutant strain (MIC = 10 μg/ml), but was not decreased in a *cmp2* mutant strain (MIC = 25 μg/ml) compared to the wild-type *CMP1 CMP2* strain (MIC = 25 μg/ml) (not shown). Thus, the CMP1 subunit is somewhat less sensitive than CMP2 to inhibition by cyclophilin–CsA. Mutations that render CMP1 CsA resistant may do so by increasing this inherent CsA resistance. Some of the bias in mutation distribution is also likely to be attributable to codon usage. For example, the T350K and T350R mutations in CMP1 can arise via single nucleotide substitutions (ACA→AAA or AGA), whereas the corresponding changes in CMP2 (T392K or T392R) would require a minimum of two base changes (ACC→AAG or AAA, or ACC→CGC, AGA or AGG). Thus, the isolation of spontaneous mutations was biased towards identifying this change in CMP1 rather than in CMP2.

Our *in vitro* analysis revealed that, while the affinity of the T350K and T350R CMP1 CsA-resistant mutant proteins for cyclophilin A–CsA is reduced, both supported calcineurin binding to cyclophilin A–CsA to some extent, as detected by association of both the calcineurin A and B subunits (Figure 5B). Thus, these mutations are likely to confer CsA resistance by reducing, but not abolishing, cyclophilin A–CsA binding and, possibly, also by inhibiting some step subsequent to binding required for inhibition. Previous findings are in accord with such a model. For example, because immunophilin–drug complexes inhibit calcineurin activity towards phosphopeptides but stimulate activity with a small organic phosphotyrosine mimetic, PNPP, it has been proposed that calcineurin inhibition might involve conformational changes indirectly modulating the active site (Liu,J. *et al.*, 1991). In addition, kinetic studies reveal that cyclophilin A–CsA non-competitively inhibits calcineurin (Etzkorn *et al.*, 1994). Taken together, these findings reveal that the cyclophilin A–CsA complex inhibits calcineurin by an indirect non-competitive mechanism.

Two final points deserve comment. First, because these mutations occur in highly conserved regions of calcineurin

(Figure 3), it should be possible to engineer drug-resistant mutants of mammalian calcineurins. Expression of such mutants in transgenic mice should provide a means to test if calcineurin is the target for the nephrotoxic side effects of CsA and FK506. If so, human kidney grafts could be genetically engineered prior to transplantation to express drug-resistant forms of calcineurin to prevent nephrotoxic side effects in the recipient. Alternatively, one could generate transgenic animals expressing drug-resistant forms of calcineurin to provide donor kidneys that would be immune to nephrotoxic side effects in human xeno-transplant recipients. A second point of interest is the description of transplant recipients who suffer rejection episodes which do not respond to CsA but which do respond to FK506 (Starzl *et al.*, 1989; Armitage *et al.*, 1991; Fung *et al.*, 1991). Our findings suggest that these patients may harbor somatic or germline cyclophilin A or calcineurin A mutations that confer resistance to CsA but not to FK506. Identification of such mutations might further our understanding of immunosuppressant action and improve transplant therapies.

Materials and methods

Media preparation and strain constructions

Standard yeast medium and immunosuppressant-containing medium were prepared as described (Sherman, 1991; Heitman *et al.*, 1993). Strains employed were isogenic derivatives of the CsA–FK506-sensitive yeast strain IL993/5c (Tropschug *et al.*, 1989), some of which have been described previously (Breuder *et al.*, 1994). Genotypes of derivatives were: TB24 (*MAT***a** ρ° *ura3*), TOC6 (*MAT***a** ρ° *ura3 CMP1-1*), TB53 (*MAT***a** ρ° *ura3 CMP1-1 leu2::hisG-URA3-hisG*), TB54 (*MAT***a** ρ° *ura3 CMP1-1 leu2::hisG*), TB70 (*MAT*α ρ° *ura3 cmp1::LEU2 leu2::hisG ilv5*), TB71 (*MAT*α ρ° *ura3 cmp2::URA3 leu2::hisG ilv5*), TB72 (*MAT***a** ρ° *ura3 CMP1-1 leu2::hisG cmp1::LEU2*), TB73 (*MAT***a** ρ° *ura3 CMP1-1 leu2::hisG cmp2::URA3*), C1 (*MAT***a** ρ° *ura3 CMP1-2*), TB101 (*MAT***a** ρ° *ura3 CMP1-2 cmp1Δ1::hisG-URA3-hisG*), TB118 (*MAT***a** ρ° *ura3 CMP1-2 cmp2::URA3*), F1 (*MAT***a** ρ° *ura3 CMP1-3*), TB102 (*MAT***a** ρ° *ura3 CMP1-3 cmp1Δ1::hisG-URA3-hisG*), TB119 (*MAT***a** ρ° *ura3 CMP1-3 cmp2::URA3*), TB23 (*MAT*α ρ° *ura3 ilv5 leu2::hisG*), 6A (*MAT*α ρ° *ura3 ilv5 leu2::hisG CMP2-1*), TB127 (*MAT*α ρ° *ura3 ilv5 leu2::hisG CMP2-1 cmp1::LEU2*), TB128 (*MAT*α ρ° *ura3 ilv5 leu2::hisG CMP2-1 cmp2::URA3*), 11A (*MAT*α ρ° *ura3 ilv5 leu2::hisG CMP2-2*), TB129 (*MAT*α ρ° *ura3 ilv5 leu2::hisG CMP2-2 cmp1::LEU2*), TB130 (*MAT*α ρ° *ura3 ilv5 leu2::hisG CMP2-2 cmp2::URA3*), and TB62 (*MAT***a** ρ° *ura3 fpr1::URA3-3*). The genotype of strain TB174 is: *MAT***a** *HMLa leu2-3,112 ura3-52 trp1 his4 rme1 cmp1::hisG cmp2::URA3/pCN1F.*

The C1 and F1 CsA-resistant mutants were isolated from strain TB24 containing plasmid pTB3, which has a 2 kb *Eco*RI fragment containing the cyclophilin A-encoding gene *CPR1* cloned in the *Eco*RI site of YEplac195 (2μ *URA3*) (Gietz and Sugino, 1988). The FK506-resistant mutants 6A and 11A were isolated in strain TB23 containing the 2μ *URA3 FPR1* plasmid pYJH23 (Heitman *et al.*, 1991a). The *cmp1::LEU2*, *cmp1Δ1::hisG-URA3-hisG*, and *cmp2::URA3* disruptions have been described (Liu,Y. *et al.*, 1991; Cyert and Thorner, 1992). Note that *CMP1* and *CNA1* are alternative designations for the same gene. The *leu2::hisG-URA3-hisG* mutation was introduced with *Bgl*II-cleaved pNKY85 plasmid DNA and selection for Ura$^+$, as described (Alani *et al.*, 1987). The *leu2::hisG-URA3-hisG* allele was converted to *leu2::hisG* by selecting on 5-FOA medium for Ura$^-$ segregants arising from recombination between the *hisG* repeats.

Genetic crosses were performed between TB23 (wild-type, FK506-resistant mutants 6A and 9C) and TB24 (wild-type, and CsA-resistant mutants TOC6, C1 and F1), which are of opposite mating type. Matings were performed on YPD medium, replica plated to YNB plus uracil medium to select against the TB23 parent (which is *leu2* and *ilv5*) and, following single colony isolation, diploids were identified by failure to mate with mating type tester strains 70 (*MAT*α *trp3-10*) and 227 (*MAT***a** *crp1 lys1-1*). Diploids were scored to test if drug resistance mutations were dominant or recessive and if strains expressing two mutant calcineurins were resistant to both drugs.

Plasmids and sequence analysis

Wild-type and CsA-resistant calcineurin A *CMP1* genes were isolated by PCR amplification from genomic DNA with oligonucleotides JOHE141, 5′-CCGG**GGATCC**AGATCTACTACTGGGAAACAAAAGGG, and JOHE44, 5′-GGCCGG**GGATCC**TTATCACAGTTGTGGCTTTTTCTCCGC, as primers. The resulting ~1700 bp PCR products were cleaved with *Bam*HI and cloned in the *CEN LEU2* plasmid pRS315 (Sikorski and Hieter, 1989), resulting in plasmids pRCAN, pRCANF, pC1, pF1 and pCN1F, which express CMP1, CMP1-1 (T350K), CMP1-2 (T350R), CMP1-3 (Y377F) and CMP1-4 (W388C), respectively. The *CMP1* and *CMP1-1* genes were also cloned into the *CEN URA3* vector pRS316 (Sikorski and Hieter, 1989) as *Bam*HI fragments to yield plasmids pCAN and pCANF. Truncated forms of the *CMP1-1* CsA-resistant calcineurin A gene were PCR amplified with 5′ oligonucleotide JOHE141 and 3′ oligonucleotides: JOHE43 (Δ1), 5′-GGCCGG**GGATCC**TTATCACGTTTCATTCAAACCTTCAGTCCC; JOHE42 (Δ2), 5′-GGCCGG**GGATCC**TTATCACTTATTTCTCAAAGCC; JOHE41 (Δ3), 5′-GGCCGG**GGATCC**TTATCAGGTTTCATCTTCTAATATCGC; and JOHE52 (Δ4), 5′-CCGG**GGATCC**TTATCACATGACGTTTTCTTCATATTTTAACAC cleaved with *Bam*HI, and cloned in pRS316, yielding plasmids pCANT1, pCANT2, pCANT3 and pCANT4, which express CMP1-1Δ1 (1–508), CMP1-1Δ2 (1–461), CMP1-1Δ3 (1–452) and CMP1-1Δ4 (1–365) (see Figure 2). Hybrid genes containing portions of the wild-type *CMP1* and the mutant *CMP1-1* gene were constructed by exchanging the large and small *Pst*I fragments of pRCAN and pRCANF to yield plasmids pHYB1 and pHYB2 (Figure 2).

The wild-type and FK506-resistant *CMP2* genes were PCR amplified from genomic DNA with oligonucleotides: JOHE189, 5′-CGCG**GGATCC**CATTGTCCCCCTCTCTTGATCAATGC and JOHE94, 5′-CCTA**CTGCAG**TCTCTATTTGCTATCATTCTTTGC, cleaved with *Bam*HI–*Pst*I, and cloned in the *CEN LEU2* vector pRS315, yielding plasmids pWTC2, p6AC2 and p9AC2, which express CMP2, CMP2-1 (W430C) and CMP2-2 (W430C).

Mutations in the *CMP1* and *CMP2* genes were identified by DNA sequence analysis using synthetic primers based on the known sequences, the Sequenase kit, and PCR amplified and cloned genes as templates. At least two clones derived from independent PCR reactions were sequenced to ensure that mutations identified were not the result of PCR. In addition, the Y377F CMP1-3 and the W430C CMP2-1 mutations conferred resistance to CsA and to FK506, respectively, when introduced into the opposite calcineurin A catalytic subunit by PCR overlap mutagenesis, further confirming that these mutations confer the observed drug-resistant phenotype.

Yeast two-hybrid system

Plasmid pSBH1 expressing the GAL4(BD)–yeast FKBP12 fusion protein has been described (Cardenas *et al.*, 1994b). The yeast calcineurin A catalytic subunit CMP1 was fused to the transcriptional activation domain of GAL4 [GAL4(AD)] as follows. First, a fragment of the *CMP1* gene encoding residues 1–508 was PCR amplified from the cloned *CMP1* gene (Ye and Bretscher, 1992) with oligonucleotides JOHE40, 5′-CGCGTA**GGATCC**CACTCTCAACGCCA**ATG**TCG, and JOHE43, 5′-GGCCGG**GGATCC**TTATCACGTTTCATTCAAACCTTCAGTCCC, as primers. The resulting ~1500 bp PCR fragment was purified, cleaved with *Bam*HI, cloned into the *Bam*HI site of the GAL4(AD) vector pGAD424, and the entire insert and junction were confirmed by DNA sequence analysis. The resulting plasmid, pCH113, expresses the fusion protein GAL4(AD)–CMP1Δ, in which the C-terminal autoinhibitory domain of calcineurin A has been deleted to render calcineurin calmodulin-independent. The CsA-resistant calcineurin A mutants CMP1-1 (T350K), CMP1-2 and CMP1-3, and the FK506-resistant calcineurin A mutant CMP1-4 (W388C), were fused to the GAL4(AD) in a similar fashion, with the same oligonucleotides and PCR amplification using the cloned *CMP1-1*, *CMP1-2*, *CMP1-3* and *CMP1-4* mutant genes as templates. The resulting plasmids, pTCA1, pGAC1, pGAF1 and pGACN1F express fusion proteins GAL4(AD)–CMP1-1Δ, GAL4(AD)–CMP1-2Δ, GAL4(AD)–CMP1-3Δ and GAL4(AD)–CMP1-4Δ, respectively. Host strains for the two-hybrid system, Y190 (*MAT***a** *trp1-901 his3 leu2-3,112 ura3-52 ade2 gal4 gal80 URA3::GAL-lacZ LYS2::GAL-HIS3*) and the isogenic derivative CHY1 lacking cyclophilin A (Y190 *cpr1::ADE2*), have been described (Harper *et al.*, 1993; Breuder *et al.*, 1994). β-Galactosidase activity was assayed with the chromogenic substrate CPRG as described (Cardenas *et al.*, 1994b).

Western analyses and immunophilin affinity chromatography

Procedures for affinity purification of the His6–FKBP12 and His6–cyclophilin A proteins, preparation of yeast protein extracts, immunophilin affinity chromatography, Western blot and detection with affinity-purified antibodies against the yeast calcineurin A catalytic subunit CMP1 were performed as described previously (Heitman *et al.*, 1993; Cardenas *et al.*, 1994b). Production of antisera directed against the yeast calcineurin B regulatory subunit CNB1 will be described elsewhere (Zhu, Cardenas and Heitman, submitted).

PCR overlap mutagenesis

PCR overlap mutagenesis (Ho *et al.*, 1989) was used to introduce mutations conferring CsA or FK506 resistance, either alone or in combination, into the calcineurin catalytic subunit genes *CMP1* and *CMP2*. Outer primers for mutagenesis of the *CMP1* gene were JOHE141 and JOHE44. Inner mutagenic primers were: JOHE226, 5′-GTTTTCACCTGTTCACTACCTTTTG, and JOHE227, 5′-GGTAGTGAACAGGTGAAAACATCC, for the W388C FK506-resistant mutation, and JOHE228, 5′-CCTTTCTGGTTGCCTGATTTTATGGATGTTTTCACCTGTTCAC, and JOHE229, 5′-GTGAACAGGTGAAAACATCCATAAAATCAGGCAACCAGAAAGG, for the Y377F, W388C double mutation. Outer primers for the *CMP2* gene were JOHE189 and JOHE94. Inner mutagenic primers were: JOHE230, 5′-CTCCACACCCCTTCTGGTTACCAG, and JOHE231, 5′-CTGGTAACCAGAAGGGGTGTGGAG, for the Y419F mutation, and JOHE232, 5′-CCCCTTCTGGTTACCAGATTTCATGGACGTTTTCACGTGTTCCTTG, and JOHE233, 5′-CAAGGAACACGTGAAAACGTCCATGAAATCTGGTAACCAGAAGGGG for the Y419F, W430C double mutation. First round PCR products were purified, combined and amplified with outer primers, cleaved with *Bam*HI (*CMP1*) or *Bam*HI–*Pst*I (*CMP2*) and the resulting fragments were cloned in the *CEN LEU2* vector pRS315. Introduction of the desired mutations was confirmed by DNA sequence analysis. Plasmids expressing the CMP1 W388C FK506-resistant mutant (pCN1F, *CMP1-4* allele), the CMP1 Y377F, W388C CsA–FK506-resistant double mutant (pCN1CF, *CMP1-3,4* allele), the CMP2 Y419F CsA-resistant mutant (pCN2C, *CMP2-3* allele) and the CMP2 Y419F, W430C CsA–FK506-resistant double mutant (pCN2CF, *CMP2-1,3* allele) were obtained. Upon DNA sequence analysis, one Y377F, W388C CMP1 mutant isolate was found with an additional mutation, M341I (ATG→ATA mutation), resulting in a M341I, Y377F, W388C triple mutant CMP1 protein that was functional and conferred resistance to CsA, FK506 and CsA plus FK506. Interestingly, M341 is replaced by leucine in the yeast CMP2 calcineurin A and by isoleucine in the murine calcineurin Aα1 isoform.

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Note added in proof

We note that at the junction at which protein phosphatases PP1, PP2A and calcineurin (PP2B) diverge lie two residues N-terminal to the site of mutation in two of our CsA-resistant calcineurin mutants (T350K, T350R). The findings that an okadaic acid-resistant PP2A mutant is altered in the residue adjacent to T350 of calcineurin (Shima,H., Tohda,H., Aonuma,S., Nakayasu,M., DePaoli-Roach,A.A., Sugimura,T. and Nagao,M. (1994) Characterization of the PP2Aα gene mutation in okadaic acid-resistant variants of CHO-K1 cells. *Proc. Natl Acad. Sci. USA*, **91**, 9267–9271), and that substitution of a tetrapeptide spanning this residue from PP2A into PP1 renders PP1 okadaic acid sensitive (Zhang,Z., Zhao,S., Long,F., Zhang,L., Bai,G., Shima,H., Nagao,M. and Lee,E.Y.C. (1994) A mutant of protein phosphatase-1 that exhibits altered toxin sensitivity. *J. Biol. Chem.*, **269**, 16997–17000), suggests that structurally dissimilar phosphatase inhibitors may target the same regions of different phosphatases and that their mechanisms of action may be more similar than previously suspected.