The EMBO Journal vol.13 no.24 pp.5944–5957, 1994

Immunophilins interact with calcineurin in the absence of exogenous immunosuppressive ligands

Maria E.Cardenas[1], Charles Hemenway[2], R.Scott Muir[3], Ruby Ye[4], David Fiorentino[5] and Joseph Heitman[1,3,6,7]

Departments of [1]Genetics, [6]Pharmacology, [2]Pediatrics and Pathology, and [3]Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC 27710, [4]Memorial Sloan Kettering Cancer Center, New York, NY 10021 and [5]Howard Hughes Medical Institute, Stanford University, Palo Alto, CA, USA

[7]Corresponding author

Communicated by S.M.Gasser

The peptidyl-prolyl isomerases FKBP12 and cyclophilin A (immunophilins) form complexes with the immunosuppressants FK506 and cyclosporin A that inhibit the phosphatase calcineurin. With the yeast two hybrid system, we detect complexes between FKBP12 and the calcineurin A catalytic subunit in both the presence and absence of FK506. Mutations in FKBP12 surface residues or the absence of the calcineurin B regulatory subunit perturb the FK506-dependent, but not the ligand-independent, FKBP12–calcineurin complex. By affinity chromatography, both FKBP12 and cyclophilin A bind calcineurin A in the absence of ligand, and FK506 and cyclosporin A respectively potentiate these interactions. Both *in vivo* and *in vitro*, the peptidyl-prolyl isomerase active sites are dispensable for ligand-independent immunophilin–calcineurin complexes. Lastly, by genetic analyses we demonstrate that FKBP12 modulates calcineurin functions *in vivo*. These findings reveal that immunophilins interact with calcineurin in the absence of exogenous ligands and suggest that immunosuppressants may take advantage of the inherent ability of immunophilins to interact with calcineurin.

Key words: cyclosporin A/FK506/FKBP12/signal transduction/yeast

Introduction

Cyclosporin A (CsA), FK506 and rapamycin are immunosuppressive and antifungal natural products (Schreiber, 1991, 1992; Heitman *et al.*, 1992; Schreiber and Crabtree, 1992; Sigal and Dumont, 1992; Kunz and Hall, 1993; Liu, 1993; Cardenas *et al.*, 1994). CsA is a cyclic peptide, whereas FK506 and rapamycin are macrolide antibiotics. These three compounds suppress the immune system by preventing intermediate steps in signal transduction cascades required for T lymphocyte activation. CsA and FK506 impair T cell responses to antigen presentation and block G_0 to G_1 phase cell cycle progression. In contrast, rapamycin interferes with a later step and blocks responses to interleukin (IL)-2 that normally drive T cell proliferation by promoting progression from G_1 to S phase.

CsA binds to a ubiquitous, highly conserved family of abundant proteins, termed cyclophilins, that are expressed in different intracellular compartments in most, if not all, organisms (Handschumacher *et al.*, 1984; Fischer *et al.*, 1989; Takahashi *et al.*, 1989). The macrolides FK506 and rapamycin associate with a distinct family of ubiquitous proteins termed FK506 binding proteins (FKBPs; Harding *et al.*, 1989; Siekierka *et al.*, 1989; Standaert *et al.*, 1990). These two protein families (the immunophilins) share no primary or tertiary homology, yet both are enzymes that catalyze *cis–trans* peptidyl-prolyl isomerization and may participate in protein folding *in vivo* (Gething and Sambrook, 1992). Because drug binding inhibits this enzymatic activity, early models speculated that T cell activation required proline isomerization. However, several observations, such as the description of drug analogs that inhibit enzyme activity *in vitro* but are not immunosuppressive *in vivo*, argue against this model (Bierer *et al.*, 1990; Sigal *et al.*, 1991; Liu *et al.*, 1992).

Three different approaches reveal that CsA, FK506 and rapamycin associate with cyclophilins and FKBPs to form immunophilin–drug complexes that inhibit signal transduction. First, FK506 and rapamycin competitively bind to and inhibit FKBP12 *in vitro*, but block different signaling pathways and reciprocally antagonize each other's actions in T cells (Bierer *et al.*, 1990; Dumont *et al.*, 1990a,b). Second, cyclophilin A and FKBP12 are dispensable for viability but are required for sensitivity of yeast to CsA and FK506/rapamycin, respectively (Tropschug *et al.*, 1989; Heitman *et al.*, 1991a,b; Koltin *et al.*, 1991; Breuder *et al.*, 1994). Third, the cyclophilin–CsA and FKBP12–FK506 complexes inhibit the serine–threonine Ca^{2+}-dependent phosphatase calcineurin *in vitro* (Friedman and Weissman, 1991; J.Liu *et al.*, 1991, 1992) and *in vivo* (Clipstone and Crabtree, 1992; Foor *et al.*, 1992; Fruman *et al.*, 1992; O'Keefe *et al.*, 1992; Breuder *et al.*, 1994). Calcineurin is a heterodimer of catalytic (A) and regulatory (B) subunits that is activated by calmodulin in response to increased intracellular calcium (Klee *et al.*, 1988). During T cell activation, calcineurin dephosphorylates the NF-AT transcription factor cytoplasmic subunit, and thereby allows NF-AT to enter the nucleus, associate with Fos and Jun, and activate gene transcription (Flanagan *et al.*, 1991; Jain *et al.*, 1993; McCaffrey *et al.*, 1993; Northrop *et al.*, 1993).

Two models can be invoked to explain the ability of CsA and FK506 to form inhibitory complexes with immunophilins and their targets. In the first, the immunosuppressant–immunophilin complexes gain the ability to interact with a target protein to which immunophilins or immunosuppressants alone do not bind. In the second, the immunophilins normally interact with and modulate calcineurin; immunophilin–calcineurin interactions could be mediated by endogenous ligands, but no such ligand has yet been found.

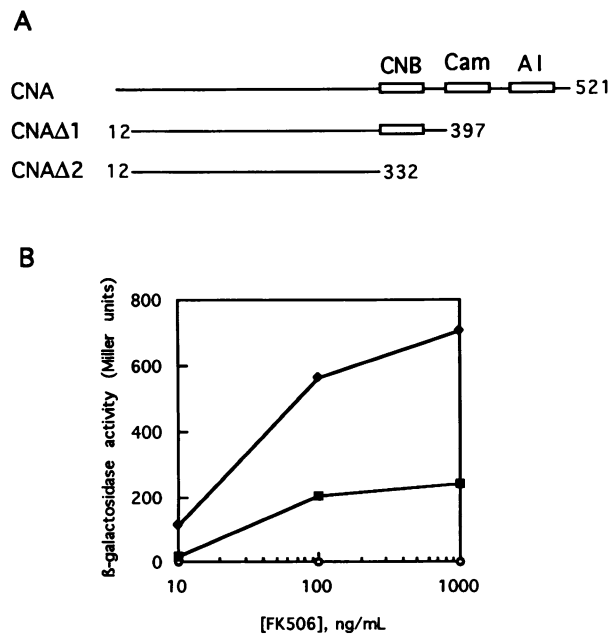

Fig. 1. FK506 promotes an FKBP12–calcineurin A interaction in the two hybrid system. (**A**) Structures of calcineurin A and calcineurin A deletions employed in the two hybrid system. CNB, Cam and AI indicate calcineurin B binding, calmodulin binding and auto-inhibitory domains, respectively. (**B**) Cultures were inoculated in duplicate in synthetic medium lacking tryptophan and leucine and containing FK506 at the indicated concentrations. Following growth for 24–36 h at 30°C, samples were removed and β-galactosidase expression was measured by CPRG assay (see Materials and methods) and averaged for duplicate samples. The GAL4(BD)–yeast FKBP12 fusion protein was co-expressed with the GAL4(AD) fused to the mouse calcineurin Aα1 catalytic subunit lacking the calmodulin binding and auto-inhibitory domains [GAL4(AD)–CNAΔ1] in isogenic two hybrid host strains expressing (■, strain RR1; Table II) or lacking the endogenous yeast FKBP12 protein (◆, strain SMY4; Table II). In control reactions, the GAL4(BD)–FKBP12 protein failed to interact when co-expressed with the GAL4(AD) fused to mouse calcineurin A lacking not only the calmodulin binding and auto-inhibitory domains, but also the calcineurin B binding domain [GAL4(AD)–CNAΔ2] (○).

Although previous biochemical studies failed to detect interactions between immunophilins and calcineurin in the absence of immunosuppressants *in vitro* (Friedman and Weissman, 1991; J.Liu *et al.*, 1991, 1992), several observations suggest that they may interact *in vivo*. (i) FKBP12 and calcineurin are abundant and colocalized in the vertebrate central nervous system (Steiner *et al.*, 1992). (ii) A variety of FKBPs have been identified in different cellular compartments (FKBP12, FKBP13, FKBP25 and FKBP59), but only the FKBP12–FK506 complex potently inhibits calcineurin *in vitro* (Bram *et al.*, 1993; Rosen *et al.*, 1993; Yang *et al.*, 1993). Calcineurin and FKBP12 may have evolved to interact with each other in the cytoplasm, whereas calcineurin and FKBPs from the endoplasmic reticulum (ER) or nucleus could not have done so. (iii) FK506 and CsA promote the formation of FKBP12–FK506–calcineurin and cyclophilin A–CsA–calcineurin complexes in yeast (Foor *et al.*, 1992; Breuder *et al.*, 1994), in the plant *Vicia faba* (Luan *et al.*, 1993) and in vertebrates (J.Liu *et al.*, 1991). This suggests that the interacting surfaces of immunophilins and calcineurin have been conserved over roughly a billion years of evolution. It seemed implausible that these surfaces are so highly conserved in several disparate organisms simply to permit bacterial or fungal toxins to bridge two unrelated proteins.

Table I. FKBP12–calcineurin ligand-independent interactions

Host strain	GAL4(BD)–	GAL4(AD)–	β-Gal (U)
Y190	FKBP12	CNAΔ1	4.00
	FKBP12	CNAΔ2	0.10
	FKBP12	–	0.13
	–	CNAΔ1	0.05
	–	CNAΔ2	0.04
	FKBP13	CNAΔ1	0.09
	FKBP13	CNAΔ2	0.04
Host strain	**lexA(BD)–**	**GAL4(AD)–**	**β-Gal (U)**
CTY10-5D	FKBP12	CNAΔ1	52.10
	FKBP12	CNAΔ2	8.70
	FKBP12	–	4.40
	–	CNAΔ1	6.40
	FKBP12	SRP1	6.00
	NUP1	CNAΔ1	9.30
	NUP1	SRP1	123.50
	–	SRP1	2.90
	NUP1	–	13.40

The GAL4(AD)–CNAΔ1 and GAL4(AD)–CNAΔ2 fusion proteins express truncated murine calcineurin A catalytic subunits retaining residues 12–397 and 12–332, respectively (Figure 1A). Calcineurin truncation CNAΔ1 interacts with FKBP12; truncation CNAΔ2 does not. β-Galactosidase activity was assayed by CPRG assay (see Materials and methods) and the values listed are the means of several independent measurements.

In our studies we have tested whether immunophilins and calcineurin interact with each other *in vivo* in the absence of immunosuppressive ligands. Both *in vivo*, using the yeast two hybrid system (Fields and Song, 1989), and *in vitro*, employing FKBP12 affinity chromatography, we find that FKBP12 and calcineurin indeed interact in the absence of exogenous ligands. FK506 strongly potentiates this interaction *in vivo* and *in vitro*. *In vitro*, cyclophilin A also interacts with calcineurin in the absence of ligand, and CsA potentiates this interaction. We also present genetic evidence that immunophilins modulate calcineurin function. Calcineurin mutant strains have a defect in recovering from pheromone cell cycle arrest and are LiCl-hypersensitive. In contrast, FKBP12 mutations promote recovery from pheromone arrest and increase LiCl resistance. Moreover, cyclophilin A enhances and FKBP12 inhibits the ability of a partially active calcineurin to promote recovery from pheromone arrest. Based on these observations, we propose that immunophilins and calcineurin, either alone or in complex with endogenous ligands, functionally interact in the absence of immunosuppressive ligands. In this model, CsA and FK506 no longer serve to bring two completely unrelated proteins together, but rather may capitalize upon the inherent ability of immunophilins to interact with calcineurin.

Results

FKBP12 interacts with calcineurin in the absence of an immunosuppressive ligand

We employed the yeast two hybrid system (Fields and Song, 1989) to test if FKBP12 and calcineurin interact under normal cellular conditions. FKBP12 was fused to the GAL4 DNA binding domain [GAL4(BD)]; this fusion

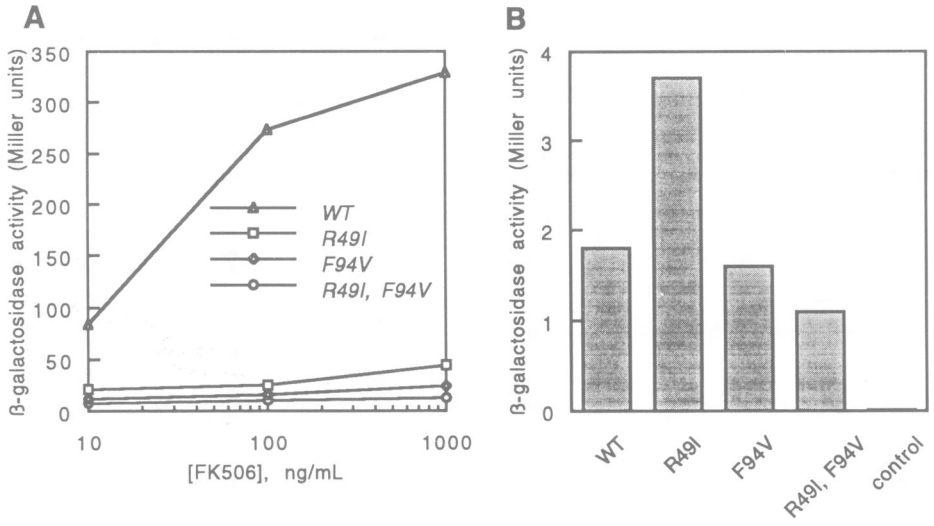

Fig. 2. Surface residues flanking the FKBP12 ligand binding pocket are required for the FKBP12–FK506–calcineurin complex but are dispensable for the FKBP12–calcineurin complex. The GAL4(AD)–calcineurin A (CNAΔ1) fusion protein was co-expressed with the GAL4(BD)–FKBP12 wild-type (WT) or mutant (R49I, F94V and R49I/F94V) fusion proteins in the RR1 two hybrid host strain in the presence of 10, 100 or 1000 ng/ml FK506 (**A**) or with no ligand (**B**). β-Galactosidase activity was measured by CPRG assay.

protein did not activate reporter gene expression on its own, either in the absence or presence of FK506 (Figure 1 and Table I). Calcineurin was fused to the GAL4 transcriptional activation domain [GAL4(AD)]. Activated calcineurin is a heterotrimer composed of catalytic (A) and regulatory (B) subunits and calmodulin-Ca^{2+} (Klee *et al.*, 1988). To overcome the calmodulin requirement, we used truncated forms of the calcineurin A catalytic subunit lacking the C-terminal calmodulin binding and auto-inhibitory domains (Figure 1A, CNAΔ1). This renders calcineurin calmodulin-independent and constitutively active, but does not prevent inhibition by immunophilin–drug complexes (Hubbard and Klee, 1989; J.Liu *et al.*, 1991, 1992; O'Keefe *et al.*, 1992; Swanson *et al.*, 1992).

FK506 promotes a strong interaction between FKBP12 and calcineurin in the two hybrid system (Figure 1B). FK506 alone did not activate transcription (data not shown). The FKBP12–FK506–calcineurin interaction increased 2- to 3-fold in a host strain lacking yeast FKBP12, suggesting that endogenous yeast FKBP12 competes for FK506 and calcineurin (Figure 1B). This *in vivo* assay exhibits several hallmarks of the FKBP12–FK506–calcineurin complex that have been observed previously *in vitro* and *in vivo*. First, the interaction is inhibited by rapamycin, which is known to compete with FK506 for FKBP12 but does not inhibit calcineurin (data not shown). Second, the interaction is inhibited by cyclophilin A–CsA, which competitively inhibits FKBP12–FK506 binding to calcineurin (data not shown). Third, the interaction requires FKBP12 residues that contact calcineurin (Figure 2A; Aldape *et al.*, 1992).

Remarkably, we found that FKBP12 and calcineurin also interact in the two hybrid system in the absence of any exogenous ligand (Table I). Although not as robust as the FK506-dependent interaction, this ligand-independent interaction results in β-galactosidase expression at 30- to 100-fold higher levels than the background level of expression with either of the vectors alone, or with GAL4(BD)–FKBP12 and a non-interacting GAL4(AD)–calcineurin fusion protein [GAL4(AD)–CNAΔ2, Table I and Figure 1A]. The FKBP12–calcineurin interaction did not increase in a host strain lacking yeast FKBP12 (data not shown). To exclude the possibility that the GAL4 DNA binding domain contributes to this ligand-independent FKBP12–calcineurin interaction, yeast FKBP12 was fused to the DNA binding domain of the bacterial repressor lexA. Again, an interaction between FKBP12 and calcineurin was readily detectable without added ligand, resulting in β-galactosidase expression at 8- to 12-fold higher levels compared with the background level of expression in strains lacking one or the other fusion protein (Table I). Fusions to two other interacting yeast proteins (NUP1 and SRP1; Belanger *et al.*, 1994; Table I) did not interact with GAL4(AD)–calcineurin or the lexA(BD)–FKBP12 fusion proteins (Table I), further demonstrating specificity of the ligand-independent FKBP12–calcineurin interaction. Lastly, no complex was detected between a GAL4(AD)–FKBP13 fusion protein and calcineurin (Table I), indicating that the observed interaction is specific for FKBP12.

FKBP12–calcineurin and FKBP12–FK506–calcineurin complexes have different structures

The calcineurin binding surface of the FKBP12–FK506 complex is composed of both drug and protein functional groups (Aldape *et al.*, 1992; Liu *et al.*, 1992; Rosen *et al.*, 1993; Yang *et al.*, 1993). To test if the same FKBP12 residues contact calcineurin in the ligand-independent and FK506-dependent complexes, we introduced mutations adjacent to the FKBP12 ligand binding pocket which are known to spare FK506 binding but impair binding of FKBP12–FK506 to calcineurin (Aldape *et al.*, 1992). As shown in Figure 2A, the R49I and F94V mutations severely impair binding of the mutant GAL4(BD)–FKBP12–FK506 complex to calcineurin. Combining the two mutations exacerbated this defect (Figure 2A). In contrast, these mutations did not impair the ligand-independent FKBP12–calcineurin interaction (Figure 2B). In fact, the R49I mutation increased the interaction by

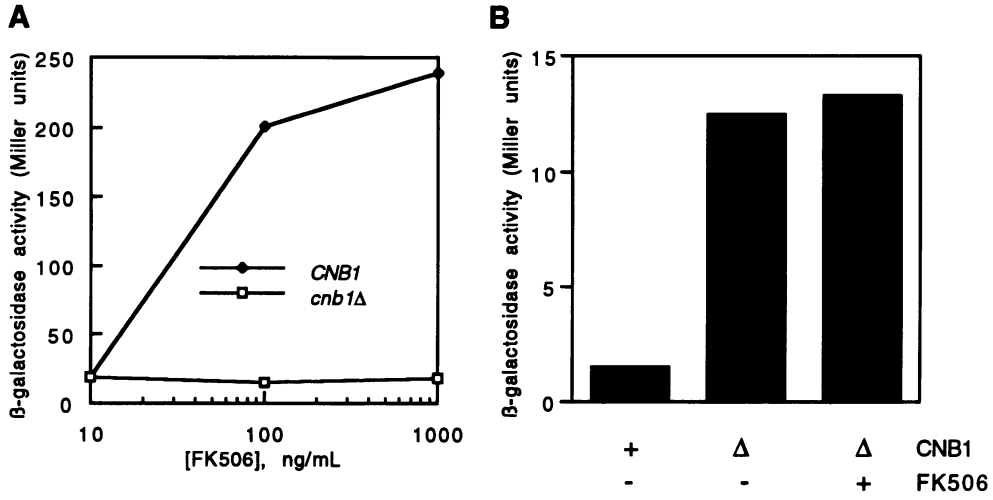

Fig. 3. Calcineurin B is required for the FKBP12–FK506–calcineurin complex but not the FKBP12–calcineurin complex. The GAL4(BD)–FKBP12 and GAL4(AD)–calcineurin A (CNAΔ1) fusion proteins were co-expressed in isogenic two hybrid host strains which express (◇, strain RR1, *CNB1*) or lack calcineurin B (□, SMY7, *cnb1Δ*). FK506 at 1000 ng/ml was added in (**B**) as indicated. FK506 at 100 μg/ml also failed to compete the ligand-independent FKBP12–calcineurin A complex observed in the absence of calcineurin B (data not shown). β-Galactosidase activity was measured by CPRG assay and the values presented are the mean of duplicate cultures.

~2-fold. Thus, different FKBP12 surface residues participate in the ligand-independent and FK506-dependent complexes.

In vitro, FKBP12 can be cross-linked to the calcineurin B regulatory subunit in the FKBP12–FK506–calcineurin complex (Li and Handschumacher, 1993). We therefore tested the role of calcineurin B in both FKBP12–calcineurin complexes. For these experiments we constructed a mutant strain in which the calcineurin B gene was precisely deleted (see Materials and methods). The FK506-dependent FKBP12–calcineurin complex was abolished in this calcineurin B mutant strain (Figure 3A). Thus, calcineurin B is required for the FK506-dependent FKBP12–calcineurin A complex. In contrast, the ligand-independent FKBP12–calcineurin interaction increased 10-fold in the absence of calcineurin B (Figure 3B). Thus, calcineurin B is dispensable for the ligand-independent complex, and calcineurin B and FKBP12 may compete for binding to calcineurin A. Lastly, FK506 did not inhibit formation of the ligand-independent FKBP12–calcineurin A complex observed in the absence of calcineurin B (Figure 3B), suggesting that the FKBP12 ligand binding pocket is accessible in the complex.

Immunophilin–calcineurin interactions in vitro

To test if the ligand-independent FKBP12–calcineurin complex is detectable *in vitro*, yeast FKBP12 was affinity tagged on its N-terminus with a six histidine stretch, overexpressed in bacteria and purified on Ni^{2+} affinity columns (Van Dyke *et al.*, 1992; see Materials and methods). By this procedure, highly purified H6-FKBP12 was obtained (Figure 4A). The recombinant H6-FKBP12 protein has proline isomerase activity, using succ-Ala-Ala-Pro-Phe-*p*-nitroanilide as substrate (see Materials and methods; Figure 4B), and is inhibited by FK506 or rapamycin (Figure 4B). From these results, the recombinant yeast H6-FKBP12 protein is fully active and proficient at drug binding, and we proceeded to test interactions with calcineurin.

Because FKBP12–FK506 binding inhibits calcineurin activity towards a phosphopeptide substrate (Liu *et al.*, 1991), we tested if FKBP12 alone would inhibit calcineurin activity. Neither free H6-FKBP12 nor the H6-FKBP12–rapamycin complex profoundly inhibited the phosphatase activity of bovine calcineurin towards the synthetic phosphopeptide DLDVPIPGRFDRRVS(PO_4)-VAAE (derived from the RII subunit of cAMP-dependent protein kinase). However, we note that when present at a 100-fold excess, both FKBP12 and FKBP12–rapamycin weakly inhibited calcineurin (Figure 4C). In accordance with previous reports, the FKBP12–FK506 complex at a 2.5-fold molar excess effectively inhibited bovine calcineurin activity (Figure 4C).

We next directly tested if calcineurin binds to an FKBP12 affinity matrix in the absence of ligand. For this purpose, the H6-FKBP12 protein was coupled to affigel 10 beads to produce the affinity matrix. This cross-linked enzyme retains proline isomerase activity that is inhibited by FK506 (data not shown). For these FKBP12 binding experiments we employed cell extracts from strain JK9-3d (wild-type strain) and an isogenic set of mutants lacking calcineurin B (*cnb1::ADE2*), the calcineurin A catalytic subunit CMP1 (*cmp1*), CMP2 (*cmp2*) or both CMP1 and CMP2 (*cmp1 cmp2*), or FKBP12 (*fpr1*) (Table II). Following elution from the matrix and PAGE, calcineurin bound to FKBP12 was detected by Western blot with affinity purified antibodies directed against the calcineurin A catalytic subunit CMP1 (Ye and Bretscher, 1992). With extracts from wild-type cells, we detect binding of calcineurin (visualized as a doublet of ~61 kDa) to the FKBP12–affigel beads in the absence of an immunosuppressive ligand (Figure 5A, lane 2). This binding is specific because no calcineurin binding was observed with affigel beads alone (Figure 5A, lane 1) or with extracts from cells lacking CMP1 (Figure 5A, lanes 8 and 14). Similar to our findings in the two hybrid system, this *in vitro* ligand-independent FKBP12–calcineurin interaction was modestly increased in the calcineurin B mutant extracts (Figure 5A, lane 5), but not to the magnitude observed in the two hybrid system. The ligand-independent

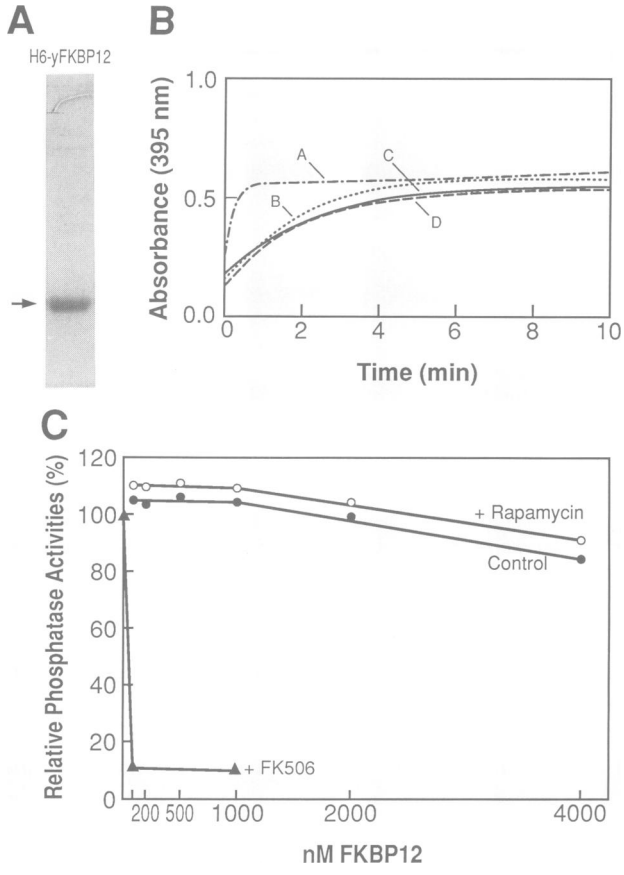

Fig. 4. Hexahistidine-tagged recombinant yeast FKBP12 is fully functional. (**A**) Purity of H6-yeast FKBP12. Yeast FKBP12 was tagged with six histidines at the N-terminus and overexpressed from the *lac* promoter in *Escherichia coli*. Following affinity purification by Ni^{2+} affinity chromatography, the H6-yFKBP12 (y = yeast) protein was analyzed by SDS–PAGE (15%) and Coomassie blue staining. The arrow marks the position at which the H6-yFKBP12 protein migrates. (**B**) H6-yFKBP12 exhibits FK506 and rapamycin-sensitive proline isomerase activity. The assay was performed as described in Materials and methods using the peptide substrate *N*-succ-Ala-Ala-Pro-Phe-*p*-nitroanilide with H6-yFKBP12 in the absence (A) or presence of 1 μM rapamycin (B) or 1 μM FK506 (C), or a control reaction without H6-yFKBP12 (D). The plots presented are from one experiment that is representative of several independent experiments. (**C**) H6-yFKBP12–FK506 complex efficiently inhibits bovine calcineurin phosphatase activity but H6-yFKBP12–rapamycin or H6-yFKBP12 do not. Calcineurin phosphatase activity towards a synthetic phosphopeptide was assayed as described in Materials and methods. Reactions were conducted in the absence (●) or presence of 300 nM FK506 (▲) or 3 μM rapamycin (○) with increasing amounts of FKBP12 as indicated. Results are expressed as a percentage of the control reaction in the absence of FKBP12 and immunosuppressive ligands. The results presented in (B) and (C) are representative of at least six independent experiments.

FKBP12–calcineurin A complex was not increased with extracts lacking FKBP12 (data not shown). In addition, neither FK506 nor rapamycin inhibited formation of the ligand-independent complex in extracts lacking calcineurin B (Figure 5A, lanes 6 and 7), in agreement with our *in vivo* findings suggesting that the FKBP12 active site is not required to form this complex.

When these *in vitro* binding experiments were performed in the presence of FK506, a dramatic enhancement of the FKBP12–calcineurin interaction was observed (Figure 5A, lanes 3 and 12). In agreement with our two hybrid system results, the ability of FK506 to promote this interaction required calcineurin B (Figure 5A, lane 6). The FK506-mediated FKBP12–calcineurin interaction was not detected in cell extract from a strain lacking the CMP1 and CMP2 gene products (Figure 5A, lane 15). With extracts from cells lacking the CMP1 catalytic subunit, FK506 did afford the binding of a polypeptide with a slightly slower electrophoretic mobility than the two CMP1 polypeptides (Figure 5A, lane 9). We conclude that this polypeptide is the CMP2 protein because it is not detected in the *cmp1 cmp2* mutant cell extracts (Figure 5A, lane 15), the CMP2 protein is known to migrate slightly slower than CMP1 in SDS–PAGE (Cyert and Thorner, 1992) and the antibodies raised against CMP1 would be expected to cross-react with CMP2 (see legend to Figure 5A). When binding to the FKBP12 beads was performed in the presence of rapamycin, the level of calcineurin binding detected was comparable with that bound in the absence of ligand (Figure 5A, compare lanes 2, 5, 8, 11 and 14 with lanes 4, 7, 10, 13 and 16). These results confirm those in the two hybrid system and demonstrate that calcineurin and FKBP12 interact in the absence of an immunosuppressant *in vitro*.

We performed similar experiments to test cyclophilin A–calcineurin interactions employing a his6-cyclophilin A affinity matrix. As shown in Figure 5B, cyclophilin A also binds the calcineurin A CMP1 subunit in the absence of an immunosuppressive ligand. No binding occurred with extracts lacking CMP1. The addition of CsA potentiated binding of cyclophilin to both calcineurin A catalytic subunits CMP1 (Figure 5B, lane 18) and CMP2 (Figure 5B, lane 20). Compared with the FKBP12–calcineurin complex, the ligand-independent cyclophilin A–calcineurin A complex is more robust and is potentiated to a lesser degree by ligand binding.

Calcineurin and immunophilin mutations confer related phenotypes

Immunophilin roles in recovery from pheromone arrest. To test for a biological function of the ligand-independent immunophilin–calcineurin interaction, we tested if strains lacking immunophilins have phenotypes related to the known phenotypes of yeast calcineurin mutants. Calcineurin is known to perform two functions in yeast. First, calcineurin is required for recovery from cell cycle arrest imposed by the mating pheromone α-factor (Cyert *et al.*, 1991; Cyert and Thorner, 1992; Foor *et al.*, 1992). Because the yeast pheromone response pathway is a well-studied signal transduction pathway that shares many features with signaling pathways of multicellular eukaryotes, including adaptation, it provides an important link between calcineurin functions in yeast and in T cells (Cardenas *et al.*, 1994). Calcineurin also renders yeast cells resistant to lithium and sodium cations through an unknown mechanism (Nakamura *et al.*, 1993; Breuder *et al.*, 1994).

An isogenic series of strains lacking calcineurin subunits and immunophilins was constructed (Table II) and tested for physiological responses to pheromone. All strains tested, including calcineurin mutants, responded to pheromone by growth arrest in the G_1 phase of the cell cycle, undergoing morphological changes (shmooing) and inducing a *FUS1–lacZ* reporter gene (M.Lorenz, M.E.Cardenas and J.Heitman, unpublished results). In

Table II. Yeast strains

Strain	Genotype	Reference/source
JK9-3da	*MATa trp1 his4 leu2-3,112 ura3-52 rme1 GAL+ HMLa*	Heitman *et al.* (1991a)
MH250-2C	JK9-3da *cpr1::LEU2*	this study
JHY2-1B	JK9-3dα *fpr1::ADE2-2*	Heitman *et al.* (1991b)
JHY2-1C	JK9-3da *fpr1::ADE2-2*	this study
JHY3-3D	JK9-3da *fpr1::URA3-3*	this study
JHY8-2C	JK9-3da *fpr1::URA3-3 cpr1::LEU2*	this study
TB64	JK9-3da *cmp1::LEU2*	this study
TB66	JK9-3da *cmp2::URA3*	this study
TB68	JK9-3da *cmp1::LEU2 cmp2::URA3*	this study
TB85	JK9-3da *cnb1Δ1::LEU2*	this study
TB123	JK9-3da *cnb1Δ1::LEU2 fpr1::URA3-3*	this study
JHY58	JK9-3da *Δade2 cnb1::ADE2*	this study
JHY61	JK9-3da *Δade2 cnb1::ADE2 cpr1::LEU2*	this study
JHY62	JK9-3da *Δade2 cnb1::ADE2 fpr1::URA3-3*	this study
JHY63	JK9-3da *Δade2 cnb1::ADE2 cpr1::LEU2 fpr1::URA3-3*	this study
EL1	JK9-3da *Δade2 cnb1::ADE2 cmp1::LEU2*	this study
EL2	JK9-3da *Δade2 cnb1::ADE2 cmp2::URA3*	this study
EL3	JK9-3da *Δade2 cnb1::ADE2 cmp1::LEU2 fpr1::URA3-3*	this study
EL4	JK9-3da *Δade2 cnb1::ADE2 cmp1::LEU2 cmp2::URA3*	this study
EL5	JK9-3da *Δade2 cnb1::ADE2 cmp2::URA3 fpr1::URA3-3*	this study
EL6	JK9-3da *Δade2 cnb1::ADE2 cmp1::LEU2 cmp2::URA3 fpr1::URA3-3*	this study
EL7	JK9-3da *Δade2 cnb1::ADE2 cmp2::URA3 cpr1::LEU2*	this study
EL8	JK9-3da *Δade2 cnb1::ADE2 cmp2::URA3 fpr1::URA3-3 cpr1::LEU2*	this study
Two hybrid strains		
Y190	*MATa trp1-901 his3 leu2-3,112 ura3-52 ade2 gal4 gal80 URA3::GAL-lacZ LYS2::GAL-HIS3*	Harper *et al.* (1993)
RR1	Y190 *TOR1-3*	this study
SMY3	Y190 *TOR1-3 cnb1::ADE2*	this study
SMY4	Y190 *TOR1-3 fpr1::ADE2-2*	this study
SMY7	Y190 *cnb1Δ2::ADE2*	this study
CTY10-5D	*MATa trp1-901 his3-200 leu2-3,112 ade2 gal4 gal80 URA3::lexAop-lacZ*	S.Fields (SUNY-Stony Brook)

Strain constructions are described in Materials and methods.

addition, calcineurin mutants exhibited no mating defect with isogenic wild-type or calcineurin mutant strains. By halo assays, sensitivity to α-factor (at 48 h exposure to α-factor) and recovery from α-factor promoted cell cycle arrest (at 144 h exposure to α-factor) were assessed. All strains were sensitive to α-factor growth arrest (data not shown). In agreement with a previous report (Cyert and Thorner, 1992), strains lacking both calcineurin catalytic subunits (*cmp1 cmp2*) or the calcineurin B regulatory subunit (*cnb1Δ1::LEU2*) failed to recover from α-factor arrest (Figure 6).

Because yeast cells deprived of Ca^{2+} are killed by α-factor under some conditions (Iida *et al.*, 1990) and calcineurin is calcium-dependent, we tested whether calcineurin mutants fail to recover or are killed by α-factor. Isogenic wild-type and calcineurin mutant cultures were exposed to 2, 5 or 100 μg/ml α-factor. Following 1, 2, 4 or 24 h of incubation, pheromone was removed by washing and portions of the cultures were serially diluted and plated. Viability of calcineurin mutant strains was not reduced by α-factor in our strain background under these conditions. We conclude that calcineurin is required for recovery but not for viability after pheromone arrest.

We tested for a role of immunophilins in pheromone response with isogenic strains lacking FKBP12, cyclophilin A, or both. In contrast to calcineurin-deficient strains, recovery from α-factor arrest was promoted in strains lacking FKBP12. By 144 h of incubation, recovered cells within the zone of inhibition were more pronounced compared with the isogenic wild-type strain, especially near the edge of the α-factor disc, and were increased in abundance by 2.3-fold compared with wild-type (Figure 6 and Table III). Thus, an FKBP12 mutation (*fpr1*) promotes recovery from α-factor arrest. In contrast, a cyclophilin A mutation (*cpr1*) had no effect on recovery in *FPR1* strains, or on the enhanced recovery observed in *fpr1* mutant strains lacking FKBP12 (data not shown). Enhanced recovery of *fpr1* mutants is not attributable to slower growth because slowing growth at lower temperatures (12 or 22°C) or with an *ade2* mutation inhibited recovery.

If FKBP12 inhibits calcineurin, then FKBP12 mutations might enhance recovery from α-factor-imposed cell cycle arrest by increasing calcineurin activity. This predicts that an FKBP12 mutation would require calcineurin to have an effect. In fact, isogenic calcineurin mutant strains expressing (Table II, strain EL4) or lacking FKBP12 (strain EL6) both exhibited an absolute recovery defect (data not shown), consistent with our model that FKBP12 acts on calcineurin. In addition, halo assays performed at 37°C revealed that calcineurin is not absolutely required for recovery in that a significant proportion of calcineurin mutant cells recovered. Under these conditions, an FKBP12 mutation again had no phenotype in the absence of calcineurin. These epistasis findings support our model that FKBP12 acts on calcineurin.

Immunophilins regulate calcineurin in calcineurin B mutant strain

To further test the effects of immunophilin mutations on recovery from pheromone arrest, we sought conditions

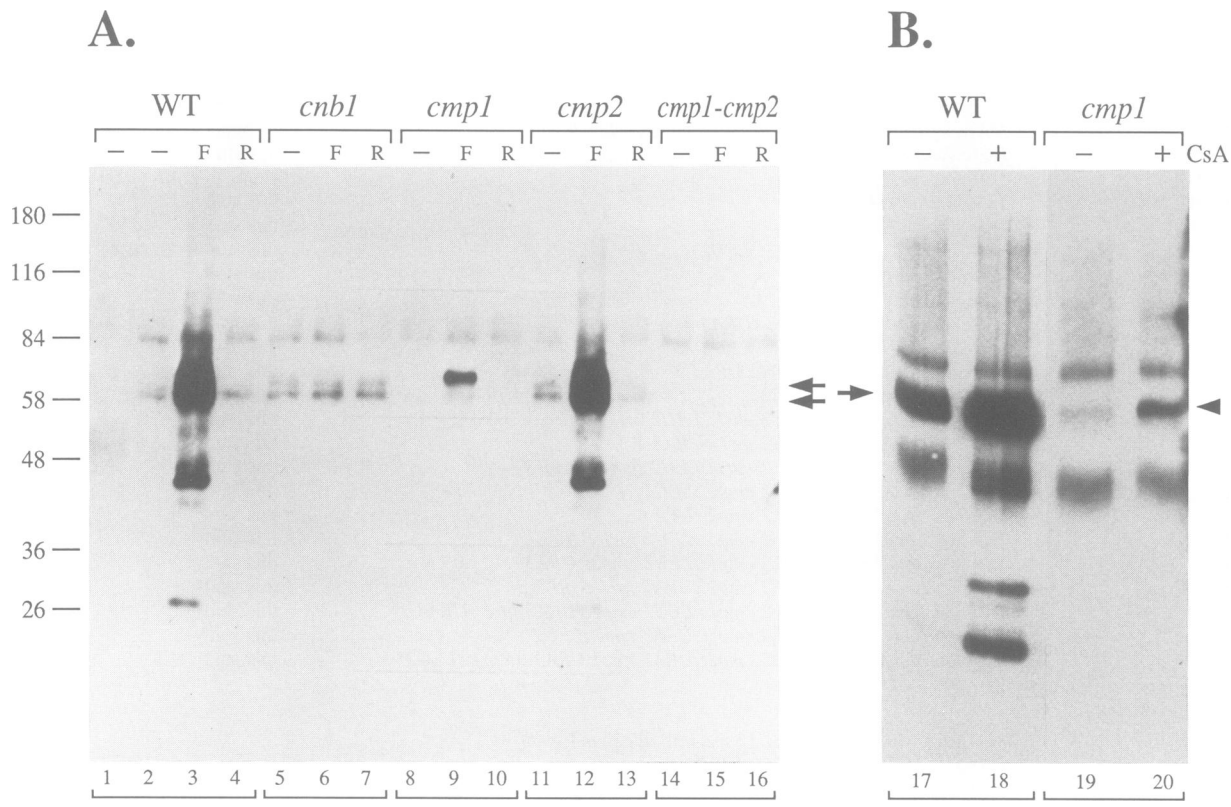

Fig. 5. Yeast FKBP12 and cyclophilin A specifically bind yeast calcineurin A CMP1 catalytic subunit in the absence of immunosuppressive ligands. (**A**) Binding assays with the H6-yFKBP12–affigel beads and cell extracts from wild-type (WT, strain JK9-3da, lanes 2–4) and isogenic derivatives lacking the C-terminal half of the calcineurin B regulatory subunit (*cnb1::ADE2*, strain JHY58, lanes 5–7), and either or both calcineurin A catalytic subunits CMP1 and CMP2 (*cmp1*, strain TB64, lanes 8–10; *cmp2*, strain TB66, lanes 11–13; *cmp1 cmp2*, strain TB68, lanes 14–16), were performed in the absence (–, lanes 1, 2, 5, 8, 11 and 14) or presence of 20 μM FK506 (F, lanes 3, 6, 9, 12 and 15) or 20 μM rapamycin (R, lanes 4, 7, 10, 13 and 16). Lane 1 is a control binding assay in which affigel beads without FKBP12 were incubated with the wild-type (WT) extract. The figure shows the Western blot of the material eluted from the beads and probed with an antibody against the yeast CMP1 calcineurin subunit. Because the antibody employed here was raised against a trpE–CMP1 fusion protein encompassing the CMP1 calmodulin binding domain, which is conserved between CMP1 and CMP2 (Ye and Bretscher, 1992), this antibody weakly cross-reacts with CMP2. Additional details are described in Materials and methods. Numbers to the left indicate the size and position of migration of molecular weight standards. Arrows to the right indicate the position of migration of the CMP1 protein. (**B**) His6-cyclophilin A was purified by Ni^{2+} chromatography and shown to have CsA-sensitive proline isomerase activity and to potently inhibit calcineurin when bound to CsA (data not shown). Purified H6-cyclophilin A was coupled to affigel 10 beads and the resulting affinity matrix incubated with extracts from wild-type strain JK9-3da (WT, lanes 17 and 18) or an isogenic *cmp1* mutant strain (*cmp1*, strain TB64); bound proteins were analyzed as described above for H6-FKBP12. Binding was in the absence (–, lanes 17 and 19) or presence of 100 μM CsA (+, lanes 18 and 20). The figure shows a Western blot of the material eluted from the beads probed with an antibody against CMP1, as above. The arrowhead to the right indicates the position of migration of the CMP2 calcineurin A catalytic subunit.

under which calcineurin is limiting to permit detection of increases or decreases in calcineurin activity. The calcineurin B regulatory subunit is a calmodulin homolog that also has four calcium binding sites. Previous studies have demonstrated that the N- or C-terminal halves of calmodulin are partially functional when over-expressed in yeast (Sun *et al.*, 1991, 1992). By analogy with the half-calmodulin mutants, we reasoned that one-half of calcineurin B might be partially functional. To test this, we constructed an isogenic strain expressing only the N-terminal half of calcineurin B encompassing the first two calcium binding domains (residues 1–92). This half-calcineurin B mutant strain (half-CnB, encoded by *half-cnb1* or *cnb1::ADE2*) exhibited a recovery defect; however, with prolonged incubation some cells escaped arrest to produce colonies within the halo (Figure 7A). Following purification, these colonies were indistinguishable from the parental half-CnB mutant strain, and thus were not sterile mutants, revertants or the result of secondary site suppressors.

We next tested the effects of cyclophilin A and FKBP12 on recovery from α-factor arrest promoted by the half-CnB protein. In contrast to the partial recovery of the half-CnB mutant, a double mutant expressing half-CnB but lacking cyclophilin A failed to recover from α-factor arrest (Figure 7B). Thus, cyclophilin A is required for recovery in the half-CnB mutant, suggesting that cyclophilin A may normally activate calcineurin; however, we cannot exclude that cyclophilin A may be required for function of mutant, but not of wild-type calcineurin B. In contrast, when compared with the half-CnB mutant, a mutant expressing half-CnB and lacking FKBP12 exhibited an enhanced ability to recover from α-factor arrest leading to a 3-fold increase in recovered colonies (Figure 7B and Table III). This finding suggests that FKBP12 inhibits calcineurin. The FKBP12 mutation did not promote recovery in mutant strains expressing half-CnB but lacking cyclophilin A (strains JHY63 and EL8, data not shown).

The partial recovery of the half-CnB mutant strain from

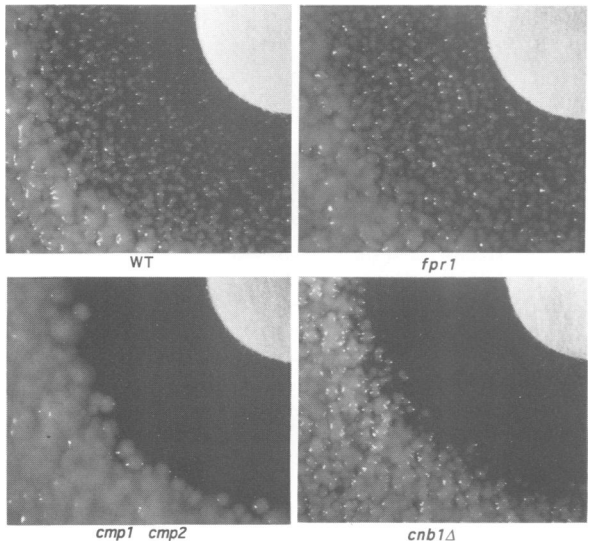

Fig. 6. Growth recovery from α-factor-induced arrest in calcineurin and FKBP12 mutants. Cells of isogenic MATa strains (Table II) were plated in top agar on agar plates. Discs soaked with 10 μg synthetic α-factor were placed on the surface. A zone of growth inhibition (halo) surrounding the disc is indicative of growth arrest imposed on the lawn of cells by the peptide pheromone. With prolonged incubation, wild-type MATa cells recover to produce colonies within the zone of growth inhibition, leading to effacement of the halo. Strains that produce clear halos, even with prolonged incubation, are unable to recover from pheromone cell cycle arrest. Halos were photographed after 144 h of incubation at 30°C. Recovery was quantified as described in Materials and methods (Table III). Calcineurin mutations prevent recovery from α-factor arrest, whereas an FKBP12 mutation promotes recovery by 2.3-fold (Table III) compared with the isogenic wild-type strain. Portions of halo were photographed with a Zeiss Stemi SV6 stereomicroscope with 10× magnification. Isogenic strains are: JK9-3da (WT), JHY3-3D (*fpr1*), TB68 (*cmp1 cmp2*) and TB85 (*cnb1*Δ).

Table III. Recovery from pheromone arrest

Genotype	Recovery (%)
WT	100.00
fpr1	233.00
cmp1 cmp2	0.00
*cnb1*Δ	0.10
half-cnb1	3.90
cpr1 half-cnb1	0.00
fpr1 half-cnb1	12.00
cmp1 half-cnb1	0.22
cmp2 half-cnb1	7.50

Recovered colonies within several α-factor halos were counted (see Materials and methods) and averaged. These values were then divided by the number of recovered wild-type colonies and multiplied by 100 to obtain the percentage of cells recovering from α-factor arrest compared with wild-type.

α-factor arrest might result from activity of either or both calcineurin A catalytic subunits. Mutant strains expressing the half-CnB protein but lacking the calcineurin A CMP2 subunit exhibited the partial recovery phenotype of the half-CnB mutant, whereas those lacking the CMP1 subunit failed to recover (Figure 7C). Thus, the calcineurin A CMP1 subunit mediates recovery in the half-CnB mutant.

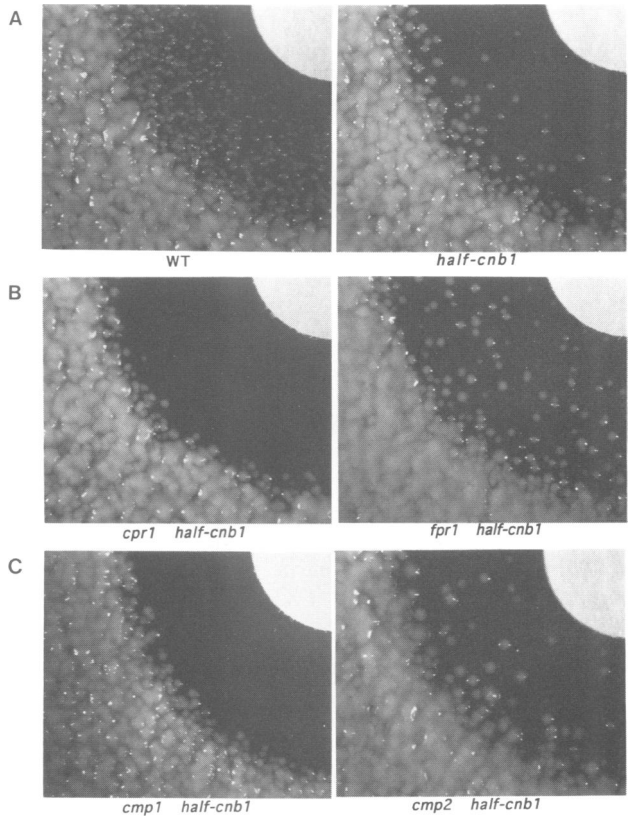

Fig. 7. Effect of calcineurin and immunophilin mutations on recovery from α-factor arrest promoted by half-calcineurin B mutant. Halo assays were performed as described in the legend to Figure 6. (**A**) Isogenic strains expressing the wild-type (WT, JK9-3da) or the half-calcineurin B mutant protein (*half-cnb1*, JHY58) were tested for their ability to recover from α-factor cell cycle arrest, revealing that the half-calcineurin B mutant protein promotes partial recovery from α-factor arrest. (**B**) Isogenic strains expressing the half-calcineurin B mutant protein but lacking cyclophilin A (*cpr1 half-cnb1*, JHY61) or FKBP12 (*fpr1 half-cnb1*, JHY62) were tested for their ability to recover from α-factor cell cycle arrest. The cyclophilin mutation completely blocked recovery from cell cycle arrest, whereas the FKBP12 mutation enhanced recovery 3-fold (Table III). These findings indicate that cyclophilin A activates and FKBP12 inhibits recovery promoted by the half-calcineurin B mutant protein. (**C**) Isogenic strains expressing the half-calcineurin B mutant protein and lacking calcineurin A subunit CMP2 (*cmp2 half-cnb1*, EL2) or CMP1 (*cmp1 half-cnb1*, EL1) were tested for recovery from α-factor arrest. The strain lacking CMP1 failed to recover, whereas the CMP2 mutation had no effect, indicating that the calcineurin A CMP1 catalytic subunit mediates recovery from α-factor arrest in the half-calcineurin B mutant.

Calcineurin and FKBP12 roles in LiCl toxicity

Calcineurin mutant strains are also hypersensitive to growth inhibition by LiCl (Nakamura *et al.*, 1993; Breuder *et al.*, 1994). Strains lacking FKBP12 (*fpr1*), cyclophilin A (*cpr1*), or both (*fpr1 cpr1*), were tested for altered responses to LiCl. As shown in Figure 8, compared with an isogenic strain expressing FKBP12, mutant strains lacking FKBP12 are more resistant to the growth inhibitory effects of 500 mM LiCl. Enhanced resistance to LiCl in strains lacking FKBP12 was not attributable to slower growth because slowing growth at lower temperatures (22°C) or with an *ade2* mutation did not alter LiCl sensitivity (data not shown). Cyclophilin A mutant strains were as LiCl-sensitive as wild-type (Figure 8), and isogenic

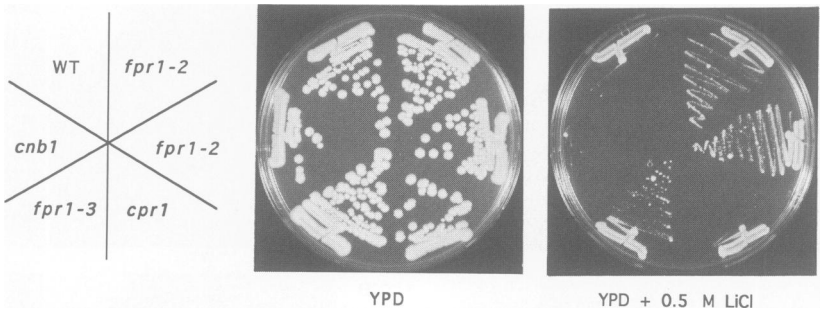

Fig. 8. Calcineurin mutants confer LiCl hypersensitivity and FKBP12 mutations confer increased LiCl resistance. Isogenic strains (Table III) lacking FKBP12 (*fpr1*), cyclophilin A (*cpr1*) or the C-terminal half of calcineurin B (*cnb1::ADE2*) were grown for 144 h at 30°C on YPD medium and YPD medium containing 500 mM LiCl. Strains were, clockwise from the top, *fpr1-2* (JHY2-1B), *fpr1-2* (JHY2-1C), *cpr1* (MH250-2C), *fpr1-3* (JHY3-3D), *cnb1* (JHY58, *cnb1::ADE2*) and WT (JK9-3da).

double mutants lacking both FKBP12 and cyclophilin A were as resistant as an FKBP12 mutant alone (data not shown). Thus, calcineurin mutations confer LiCl hypersensitivity, whereas FKBP12 mutations confer increased LiCl resistance.

Discussion

FKBP12 and cyclophilin A bind calcineurin in the absence of exogenous ligands

The immunosuppressants CsA and FK506 form complexes with the immunophilins cyclophilin A or FKBP12 that inhibit calcineurin (Liu *et al.*, 1991), thereby preventing signal transduction events required for T cell activation and recovery of yeast cells from pheromone arrest. In previous studies, neither drug nor immunophilin alone were found to bind or inhibit calcineurin *in vitro* (Liu *et al.*, 1991), leading to the model that the drugs bridge two unrelated proteins (Schreiber and Crabtree, 1992). However, circumstantial evidence exists that the two proteins might normally interact. First, FKBP12, cyclophilin and calcineurin, and the ability of the FKBP12–FK506 and cyclophilin A–CsA complexes to inhibit calcineurin, are all highly conserved in yeast, plants and vertebrates (reviewed in Heitman *et al.*, 1992; Poole, 1993). Second, FKBP12 and calcineurin colocalize in the central nervous system (Steiner *et al.*, 1992).

In this study we demonstrate directly that FKBP12 and cyclophilin interact with calcineurin in the absence of any exogenous immunosuppressive ligands (Table I and Figures 2, 3 and 5). First, we found that FKBP12 and calcineurin interacted in the two hybrid system to yield a signal that was 30- to 100-fold above background in two hybrid host strain Y190, and 8- to 12-fold above background in strain CTY10-5D (Table I). This ligand-independent FKBP12–calcineurin interaction is only reduced 2-fold compared with a robust interaction between the nucleoporin NUP1 and the SRP1 protein characterized in the two hybrid system and biochemically (Belanger *et al.*, 1994; Table I). As expected, FK506 strongly potentiates the FKBP12–calcineurin two hybrid interaction of the order of 50-fold (Figure 1). By affinity chromatography, we show that both yeast FKBP12 and cyclophilin A do not require an exogenous ligand to interact with calcineurin A (Figure 5). As expected, FK506 and CsA potentiated these *in vitro* interactions (Figure 5). *In vitro*, free FKBP12 only weakly inhibited bovine calcineurin activity, as assayed with a short synthetic phosphopeptide substrate (Figure 4C), whereas free cyclophilin A had little or no effect (data not shown). We (results not shown) and others (Cyert and Thorner, 1992) have been unable to reproducibly assay calcineurin activity in yeast cell extracts; thus, partial purification or *in vitro* reconstitution of purified components will be required to test the FKBP12 and cyclophilin A effects on yeast calcineurin activity *in vitro*.

We tested if the ligand-independent immunophilin–calcineurin interactions are physiologically relevant by a genetic approach in which we asked if immunophilin mutations confer phenotypes related to those described for calcineurin mutations. In previous studies we and others have tested FKBP12 and cyclophilin mutants for a multitude of possible phenotypes, including heat shock, induction of thermotolerance, mating, production of pheromone and barrier protease, sporulation, germination, growth on different carbon sources, temperature and cold sensitivity, survival during nitrogen starvation, production of glycogen and Ty element transposition (Heitman *et al.*, 1991a; Sykes *et al.*, 1993; unpublished results). Prior to this study, the only phenotypes that had been discovered, other than resistance to immunosuppressive drugs, were that cyclophilin A and B mutants are sensitive to extreme heat shock (Sykes *et al.*, 1993) and that FKBP12 mutants have a slight growth defect (Heitman *et al.*, 1991a). Thus, we think it highly significant that when tested for the two phenotypes associated with calcineurin mutations, we found that FKBP12 mutations confer related phenotypes in both cases. Our findings that calcineurin mutations prevent recovery from pheromone arrest and confer hypersensitivity to LiCl, whereas FKBP12 mutations increase recovery from pheromone arrest and enhance LiCl resistance (Figures 6 and 8), suggest that FKBP12 normally functions to inhibit calcineurin. In addition, in yeast strains with partial calcineurin activity, cyclophilin A mutations abolished and FKBP12 mutations enhanced the ability of calcineurin to promote recovery from pheromone arrest (Figure 7 and Table III), suggesting that cyclophilin A may normally activate calcineurin and, if so, then the two immunophilins would reciprocally modulate calcineurin. Given that few phenotypes had been associated previously with immunophilin mutations, this congruence between calcineurin and immunophilin mutant phenotypes

provides compelling support that the ligand-independent immunophilin–calcineurin complexes are physiologically relevant.

Our findings by employing three independent approaches (the two hybrid system, immunophilin affinity chromatography and genetic analysis) taken together support our model that FKBP12 and cyclophilin A functionally interact with calcineurin *in vivo* in the absence of any exogenous immunosuppressive ligand. We propose that immunophilins normally bind to and regulate calcineurin. In this model, immunosuppressants may inhibit calcineurin by taking advantage of the inherent ability of immunophilins to bind calcineurin.

Why have we detected ligand-independent immunophilin–calcineurin interactions that have not been appreciated in previous studies? First, our experiments have examined interactions between yeast immunophilins and calcineurin by highly sensitive assays, and the affinity of the yeast immunophilins for calcineurin could be higher compared with their mammalian counterparts. In fact, an FK506 analog (L-685,818) is known which when bound to mammalian FKBP12 does not bind or inhibit bovine calcineurin, but when bound to yeast FKBP12 binds and inhibits bovine calcineurin with an activity similar to FK506 (Rotonda *et al.*, 1993). Thus, yeast FKBP12 may have a higher intrinsic affinity for calcineurin. Previous studies of yeast FKBP12–FK506–calcineurin interactions detected binding by a shift in the elution position of [^{3}H]FK506 on Superose chromatography, and therefore could not have detected a ligand-independent complex (Foor *et al.*, 1992). Additional alternative possibilities are that endogenous immunophilins, which could be more abundant in some tissues or organisms, may have competed with immunophilin affinity matrices for calcineurin in previous studies. Lastly, if endogenous ligands do promote immunophilin–calcineurin interactions, these ligands could be more stable or abundant in yeast extracts compared with mammalian tissue extracts.

Structures of ligand-dependent and ligand-independent immunophilin–calcineurin complexes differ

What is the relationship between the structures of the ligand-independent FKBP12–calcineurin complex and the FKBP12–FK506–calcineurin complex? Given the highly conserved protein–protein interaction surfaces of FKBP12 and calcineurin, one might have imagined that the ligand-independent complex would be identical to the FK506-dependent complex, except that a hole would be present in the ligand binding pocket. Our results indicate that this is not the case. Based on the following observations, the two complexes must be structurally distinct. Most importantly, surface residues of FKBP12 that are required for FKBP12–FK506 to bind calcineurin are completely dispensable when FKBP12 alone binds to calcineurin (Figure 2). In fact, some of these mutant FKBP proteins bind calcineurin better than does wild-type FKBP12. This single finding demonstrates that FKBP12 and the FKBP12–FK506 complex use different surfaces of FKBP12 to interact with calcineurin. Several other observations support this conclusion: (i) rapamycin competes formation of the FKBP12–FK506–calcineurin complex, but does not compete formation of the ligand-independent complex (data not shown); (ii) the cyclophilin A–CsA complex competes with FKBP12–FK506, but not with FKBP12, for calcineurin (data not shown); and (iii) the calcineurin B regulatory subunit is required for the FK506-dependent complex but is dispensable for the FKBP12–calcineurin ligand-independent complex (Figure 3). These findings indicate that the FKBP12–calcineurin and FKBP12–FK506–calcineurin complexes are structurally distinct and that FKBP12 and the FKBP12–FK506 complex employ different surfaces to interact with calcineurin.

Our studies define a 65 residue segment of the calcineurin A catalytic subunit that is required for the ligand-independent complex with FKBP12 (Figure 1). Because this region of calcineurin A encompasses the calcineurin B binding site, and the FKBP12–calcineurin A complex detected in the two hybrid system increases in the absence of calcineurin B (Figure 3), FKBP12 and calcineurin B may compete to bind calcineurin A.

The ligand-independent immunophilin–calcineurin complexes do not appear to result from an interaction between calcineurin and the immunophilin peptidyl-prolyl isomerase active sites, as one would have expected if calcineurin were simply a substrate of FKBP12 and cyclophilin. For example, in the absence of calcineurin B, FK506 and rapamycin do not compete formation of the ligand-independent FKBP12–calcineurin A complex *in vivo* (Figure 3) or *in vitro* (Figure 5A). Because FK506 and rapamycin bind to the active site of FKBP12 and potently inhibit peptidyl-prolyl isomerase activity, these findings indicate that neither prolyl isomerase activity nor the FKBP12 active site is required for this ligand-independent FKBP12–calcineurin A complex. In addition, we find that mutations that markedly reduce FKBP12 or cyclophilin A peptidyl-prolyl isomerase activity do not hinder formation of either ligand-independent immunophilin–calcineurin complex (unpublished results).

Lastly, while the peptidyl-prolyl isomerases FKBP12 and cyclophilin A interact with calcineurin, a third prolyl isomerase, FKBP13, does not (Table I). These findings suggest that surfaces other than the active site mediate cyclophilin A and FKBP12 interactions with calcineurin. Thus, the immunophilin ligand binding pockets are accessible in immunophilin–calcineurin complexes, and FK506 and CsA binding to these pre-existing complexes enhances the interaction to potently inhibit calcineurin.

Do immunophilins assemble or regulate calcineurin?

Cyclophilins and FKBPs are known to form ligand-independent complexes with other proteins. For example, FKBP12 is a subunit of the ryanodine receptor/Ca^{2+} release channel in which one molecule of FKBP12 is bound to each subunit of the homotetrameric channel (Timerman *et al.*, 1993). The addition of FK506 dissociates FKBP12 and alters transport properties of the purified channel (Timerman *et al.*, 1993) and FKBP12 is required to fully reconstitute channel function by serving to stabilize the open conformation (Brillantes *et al.*, 1994). FKBP25 is associated with casein kinase II and nucleolin in the nucleus (Jin and Burakoff, 1993). FKBP59 and cyclophilin-40 both associate with HSP70 and HSP90 in steroid receptor complexes and FK506 augments steroid-regulated

transcription (Callebaut *et al.*, 1992; Tai *et al.*, 1992, 1993; Yem *et al.*, 1992; Ning and Sanchez, 1993). Cyclophilin C binds a 77 kDa glycoprotein; cyclophilins A and B bind to the HIV GAG protein; addition of CsA disrupts each of these cyclophilin–protein complexes (Friedman and Weissman, 1991; Friedman *et al.*, 1993; Luban *et al.*, 1993). Lastly, CsA causes secretion of cyclophilin B from its normal ER location, presumably by disrupting cyclophilin–protein interactions (Price *et al.*, 1994). The functions of cyclophilins and FKBPs in these ligand-independent immunophilin–protein complexes are, as yet, not fully understood.

In this report we show that FKBP12 modulates calcineurin activity *in vivo*. We also show that cyclophilin A is required for function of a truncated calcineurin in recovery from α-factor arrest and, based on this, suggest that cyclophilin A may also modulate calcineurin. How might FKBP12, and possibly also cyclophilin A, modulate calcineurin activity? We consider two possible models. First, the immunophilins could function solely during calcineurin assembly and, in the strictest sense, would not regulate the activity of the calcineurin holoenzyme. In this model, immunophilins would only indirectly participate in signal transduction events mediated by calcineurin. In the second model, the immunophilins could regulate the function of the calcineurin AB holoenzyme by dynamic interactions and would therefore be direct participants in signal transduction cascades.

During calcineurin assembly, FKBP12 could inhibit calcineurin by antagonizing binding of the calcineurin B regulatory subunit to the calcineurin A catalytic subunit. Our observations that in the absence of calcineurin B the FKBP12–calcineurin A complex increased 10-fold *in vivo* (Figure 2) and modestly *in vitro* (Figure 5) are in agreement with such a model. In contrast, cyclophilin A could activate calcineurin by promoting assembly of the A and B subunits. In this model, immunophilins would modulate calcineurin activity by altering the rate of assembly or the subunit composition of calcineurin. *In vitro* calcineurin reconstitution studies suggest a possible mechanism for such a model. When the purified *Neurospora crassa* calcineurin A subunit is mixed with purified mammalian calcineurin B *in vitro*, the A and B subunits rapidly associate but recovery of calcineurin activity requires longer incubation (Ueki and Kincaid, 1993). The nature of this slow reconstitution step is unknown but could involve peptidyl-prolyl isomerization, which can be the rate-limiting step during protein folding and refolding (Gething and Sambrook, 1992). Thus, immunophilins might catalyze or inhibit calcineurin assembly.

Our second model is that immunophilins regulate the function of the calcineurin AB holoenzyme via dynamic protein–protein interactions. In response to rises in intracellular calcium concentrations, calmodulin-Ca^{2+} binds to and activates the calcineurin AB holoenzyme by inducing conformational changes that release the auto-inhibitory domain from the calcineurin active site. When calcium falls to basal levels, calmodulin dissociation and conformational changes must return calcineurin to the auto-inhibited state. Peptidyl-prolyl isomerization could participate in these processes. In this model, FKBP12 and cyclophilin A peptidyl-prolyl isomerase activity would dynamically regulate the function of the calcineurin AB holoenzyme.

It has been difficult to reconcile the findings that FKBP12 and cyclophilin A are structurally unrelated, yet both share peptidyl-prolyl isomerase activity and, as protein–drug complexes, compete to inhibit calcineurin. Our findings provide the explanation that in the absence of exogenous ligands, FKBP12 and cyclophilin normally interact with and modulate calcineurin activity. The immunosuppressants CsA and FK506 now no longer need to bring two completely unrelated proteins together, but rather enhance and alter pre-existing immunophilin–calcineurin complexes. Based on the degree to which immunophilins and calcineurin are conserved, we propose that these ligand-independent immunophilin–calcineurin interactions also occur in vertebrates. Such interactions could modulate calcineurin functions in the central nervous system and during signal transduction events required for T cell activation. Our findings, and the recent observation that FKBP59 and cyclophilin-40 both associate with HSP90 in steroid receptor complexes (Callebaut *et al.*, 1992; Tai *et al.*, 1992, 1993; Yem *et al.*, 1992; Kieffer *et al.*, 1993), raise the possibility that FKBPs and cyclophilins may, in general, reciprocally modulate the functions of target proteins.

Materials and methods

Media preparation and strain constructions

Yeast media were prepared as described (Sherman, 1991; Heitman *et al.*, 1993). Isogenic derivatives of strain JK9-3da and Y190 were constructed by one-step gene disruption and are listed in Table II. *cpr1::LEU2*, *cpr1::ADE2*, *fpr1::ADE-2*, *fpr1::URA3-3*, *cnb1Δ1::LEU2*, *cmp1::LEU2* and *cmp2::URA3* disruptions have been described (Heitman *et al.*, 1991a,b; Y.Liu *et al.*, 1991; Cyert and Thorner, 1992; Breuder *et al.*, 1994). The *cnb1::ADE2* disruption was constructed by inserting the *ADE2* gene on a 2.2 kb blunt-ended *Bgl*II fragment from plasmid pASZ11 (Stotz and Linder, 1990) into a blunt-ended *Sty*I site in the middle of the *CNB1* gene, such that the *CNB1* and *ADE2* genes are transcribed in the same orientation. The resulting *cnb1::ADE2* allele expresses a truncated half-CNB1 protein retaining residues 1–92 fused to two additional amino acids from the insert (Leu-Asn-stop). The *cnb1Δ2::ADE2* precise *CNB1* gene disruption (deletes the start through to the stop codon) was isolated following transformation with a PCR product bearing the *ADE2* gene amplified from plasmid pASZ11 with adapter oligonucleotides (5′-GGCACGAGATCCATAGAAGCATTTTTATTTCTTAAAATATTAGTG-AGAAGCCGAG and 5′-CTTTTCTTAAAATATTGGCATACCATA-AATGAATGAAGTGTCCCCTAGTCGGACACCTGTAAGCGTTG) to provide 38 and 50 bp of 5′ and 3′ homology with *CNB1*; transformation with the resulting PCR product yielded the *cnb1Δ2::ADE2* strain SMY7. All disruptions were confirmed by Southern blotting or PCR and by phenotypes in medium with rapamycin, LiCl or CsA plus LiCl. FKBP12 mutations confer rapamycin resistance; calcineurin mutations (*cnb1::ADE2*, *cnb1Δ1::LEU2*, *cnb1Δ2::ADE2* or *cmp1 cmp2*) confer hypersensitivity to 100 to 200 mM LiCl; in strains with wild-type calcineurin, cyclophilin A mutations confer resistance to 100 mM LiCl plus 10 μg/ml CsA. For strain constructions requiring both *cmp2::URA3* and *fpr1::URA3-3* (EL5, EL6, EL8), the *cmp2::URA3* disruption was introduced first by selection for URA^+. *fpr1::URA3-3* disruptants were then obtained following transformation, overnight growth in YPD medium to deplete FKBP12, selection on YPD medium with 0.1 μg/ml rapamycin and confirmation by PCR. To test for rapamycin competition in the two hybrid system, a rapamycin-resistant two hybrid host strain was isolated and shown to be a *TOR1* mutant by genetic crosses with known *TOR1* and *TOR2* rapamycin-resistant mutants.

Two hybrid plasmid constructions

To fuse yeast FKBP12 to the GAL4(BD), the yeast FKBP12 gene was PCR-amplified with primers (5′-GGAAGGATCCTAATGTCTGAA-GTAATTG and 5′ GGCCGGATCCAAAATAATCACTGCTCATAT),

cleaved with *Bam*HI, cloned in the two hybrid vector pGBT9 and confirmed by sequencing the complete insert, yielding plasmid pGBT9-FPR1, which expresses GAL4(BD)–yeast FKBP12 (provided by S.Helliwell). Yeast FKBP12 was fused to the lexA(BD) by cloning an *Eco*RI–*Pst*I fragment from plasmid pGBT9-FPR1 into plasmid pBTM116, yielding plasmid pFLEX1 which expresses lexA(BD)–yeast FKBP12.

Calcineurin A was fused to the GAL4(AD) as follows. C-terminally truncated versions of the gene encoding the murine calcineurin Aα1 catalytic subunit isoform (calcineurin CNα4 in Kincaid *et al.*, 1990) were generated by PCR amplification, subcloned in pBluescript and confirmed by DNA sequencing (Figure 1A). The resulting fragments were excised with *Sal*I and *Eco*RI (which cleave in the calcineurin gene and the plasmid polylinker, respectively), blunt-ended and inserted into a blunt-ended *Xho*I site in plasmid pSE1107 (Durfee *et al.*, 1993). pYDF2 expresses a constitutive calmodulin-independent GAL4(AD)–calcineurin A fusion protein, which lacks the C-terminal calmodulin binding and auto-inhibitory domains and retains calcineurin A residues 12–397 fused in-frame and 3′ to the GAL4 activation domain [GAL4(AD)–CNAΔ1]. pYDF3 expresses a further truncated calcineurin, retaining residues 12–332 and lacking the calcineurin B binding, calmodulin binding and auto-inhibitory domains [GAL4(AD)–CNAΔ2]. Both pYDF2 and pYDF3 lack the first 11 amino acids of mouse calcineurin Aα1.

Site-directed mutagenesis

The R49I, F94V and R49I/F94V mutations were introduced into the GAL4(BD)–yeast FKBP12 fusion protein by PCR overlap mutagenesis (Ho *et al.*, 1989), with flanking primers (5′-TCGCCGGAATCCCGGGG and 5′-CGCGCTGCAGGTCGACGGATCC) and internal mutagenic primers (for R49I, 5′-GTTGACA**TT**GGCTCTCCATTCCAATG and 5′-GGAGAGCC**AA**TGACAACGGAGGAATCG; for F94V, 5′-CGTGGT-**G**TCCCAGGTTTGATTCCACC and 5′-CAAACCTGGGA**C**ACCACG-TGGGCCATAAGC). First round PCR products were gel-purified, mixed and PCR-amplified in a second round with flanking primers. The R49I/F94V double mutation was created by a second round PCR overlap with three first round PCR products. The resulting ~600 bp products were purified, cleaved with *Eco*RI and *Pst*I, phenol-extracted and precipitated, and cloned between the *Eco*RI and *Pst*I sites in plasmid pSBH1. The entire *FPR1* gene and the GAL4–FKBP12 junction were sequenced and isolates with only the desired mutations chosen for analysis.

CPRG β-galactosidase assay

Chlorophenol-β-D-galactopyranoside is a highly sensitive chromogenic substrate which, when cleaved by β-galactosidase, yields the soluble red dye CPR. Culture samples (1.5 ml) were microfuged, cells were resuspended in 0.5 ml Z buffer, vortexed for 5 min at room temperature and 25 μl $CHCl_3$ were added followed by 1 min of vortexing at room temperature. Extracts were equilibrated at 28°C, 100 μl of 24 mM CPRG were added and the reaction was incubated from 5 to 200 min, depending on activity. Reactions were transferred to 4°C, centrifuged for 5 min at 15 000 r.p.m. and the optical density of supernatants measured at 595 nm. β-Galactosidase activity was calculated as described (Miller, 1972).

Cloning, expression and purification of his6-tagged FKBP12 and cyclophilin A

The cloned yeast FKBP12 encoding gene *FPR1* was PCR-amplified with two flanking oligonucleotides (5′-CCCCAAGCTTGGATCCCGTA-ATAATGTCTGAAGTAATTGAAGG and 5′-CGCGGGATCCGAATT-CAAAATAATCACTGCTCATATATAG), cleaved with *Bam*HI, cloned into the his6 expression vector pV2a (Van Dyke *et al.*, 1992) and confirmed by DNA sequence analysis. The resulting plasmid was maintained in the $lacI^q$ host strain K561 (Davis and Model, 1985). Cells expressing his6-tagged yeast FKBP12 (H6-FKBP12) were grown at 37°C overnight in 20 ml cultures of FB medium (Zinder and Boeke, 1982) supplemented with 100 μg/ml ampicillin. These cultures were centrifuged and the pellets resuspended in 1 l FB ampicillin and grown at 37°C to OD_{600} = 0.6. At this point, 2 mM IPTG were added to the cultures and incubation continued for 4 h at 37°C. Cells were collected by centrifugation at 10 000 r.p.m. for 10 min and resuspended in 10 ml ice-cold bacterial lysis buffer [40 mM HEPES, pH 7.4, 200 mM KCl, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. Cells were lysed by sonicating 10× for 1 min, with periods of cooling in between. The cell lysate was clarified by centrifugation at 40 000 r.p.m. for 30 min in a Beckman Ti-70 rotor. 4 ml (packed volume) of Ni^{2+} nitriloacetic acid–agarose resin (Qiagen; pre-equilibrated in lysis buffer) were added to the clarified cell lysates and the suspension was stirred gently for 30 min at 4°C. The resin–cell extract mix was loaded onto an econocolumn (Bio-Rad), which was washed with 250 ml of lysis buffer, 100 ml 10 mM imidazole and 20 ml 20 mM imidazole in lysis buffer. H6-FKBP12 was eluted in 4.5 ml 200 mM imidazole in lysis buffer, and dialyzed with lysis buffer.

The cloned yeast cyclophilin A gene *CPR1* was PCR-amplified with two flanking oligonucleotides (5′-CGCGGGATCCGATGTCCCAAGT-CTATTTTGATG and 5′-GCCCATGAATTCCAAGCCTGGCAACATA-CTCCG), cleaved with *Bam*HI and *Eco*RI, cloned into the Invitrogen his6 expression vector pTrcHisB and confirmed by DNA sequence analysis. The resulting plasmid was maintained in the Invitrogen host strain TOP10. H6-cyclophilin A was induced and purified as described for H6-FKBP12.

Proline isomerase assay

This assay was performed essentially as described (Kofron *et al.*, 1991), with minor modifications (Heitman *et al.*, 1993). The final reaction mix contained 50 μM of the peptide substrate *N*-succ-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma), 1 mg chymotrypsin (Sigma), 1 μg FKBP12 or cyclophilin A in an final volume of 1 ml of assay buffer (50 mM HEPES, pH 8.0, 100 mM NaCl). Peptide substrate, dissolved at 5 mM in 470 mM LiCl in trifluoroethanol, was placed in cuvettes pre-equilibrated at 10°C in the spectrophotometer. Assay buffer, chymotrypsin and FKBP at 4°C were mixed in a tube, immediately pipetted into the substrate-containing cuvette and the OD_{395} was recorded with a Beckman DU640 spectrophotometer at 10°C. For drug inhibition, H6-FKBP12 was preincubated with 1 μM FK506 or rapamycin (added from 100 μM stocks in methanol), and H6-cyclophilin A was preincubated with 1 μM CsA for 15 min at 4°C prior to the reaction.

Calcineurin assay

This assay was performed as described (Hubbard and Klee, 1991). The peptide DLDVPIPGRFDRRVSVAAE (provided by the HHMI peptide synthesis facility at DUMC) was phosphorylated by cAMP-dependent protein kinase (Sigma) in a reaction containing 165 μM peptide, 150 μM ATP, 100 μCi [γ-^{32}P]ATP and 0.2 μM cAMP in a final volume of 200 μl in a buffer containing 20 mM MES, pH 6.5, 0.2 mM EGTA, 0.4 mM EDTA, 2 mM $MgCl_2$, 50 μM $CaCl_2$ and 50 μg/ml bovine serum albumin. The reaction was started by adding 2 μg cAMP-dependent protein kinase (Sigma) and incubated for 1 h at 30°C.

The phosphorylated peptide was purified from unincorporated label by OPC-C_{18} chromatography. For the calcineurin phosphatase assay, the reaction mixture consisted of 2 μM phosphopeptide, 40 nM bovine calcineurin (Sigma) and 80 nM calmodulin (Sigma) (where indicated) in 60 μl of reaction buffer (40 mM Tris–HCl, pH 7.5, 100 mM NaCl, 6 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.5 mM dithiothreitol, 0.1 mg/ml bovine serum albumin). For calcineurin inhibition by FKBP12, 300 nM of FK506 or 3 μM rapamycin were added from a DMSO stock, as described (J.Liu *et al.*, 1991). For calcineurin inhibition by H6-cyclophilin A, 200 nM CsA were added from a 10 μM DMSO stock.

Cell culture and preparation of cell extracts

Cells were grown in 40 ml of YPD to an OD_{600} of 0.8–1.0, harvested, resuspended in ice-cold yeast lysis buffer (50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.2% Triton X-100, 0.5 mM PMSF, 1 μg/ml pepstatin, 100 μM leupeptin, 1% trasylol and 1 μg/ml TPCK) and lysed with one volume of glass beads by shaking six times for 45 s in a minibead beater 8 (BioSpec Products) at a high setting. The cell lysates were centrifuged twice at 15 000 r.p.m. for 10 min at 4°C to obtain a clear lysate.

Immunophilin–affigel 10 binding to calcineurin in yeast cell extracts

H6-FKBP12 or H6-cyclophilin A was immobilized on affigel 10 (Bio-Rad) according to the manufacturer's instructions. For FKBP12–calcineurin and cyclophilin A–calcineurin binding assays, incubation mixtures contained 320 μl yeast cell extract (4–6 mg protein) and 60 μl FKBP–affigel 10 or cyclophilin A–affigel 10 beads (50% v/v suspension). Where indicated, 20 μM FK506 or rapamycin were added from 1 mM stocks in methanol. CsA was added to 100 μM from a 10 mM stock in methanol.

The binding mixtures were incubated at 4°C on a nutator for 1–2 h. The affigel 10 beads were collected by centrifugation for 10 s and washed four times with ice-cold yeast lysis buffer. Bound proteins were eluted from the beads by boiling for 4 min in 30 μl of SDS–PAGE sample buffer and fractionated on 12.5% SDS–PAGE.

Antibody preparation and Western blotting

FKBP12- or cyclophilin A-bound proteins were transferred onto nitrocellulose membranes using a Bio-Rad Transblot cell. The membranes were blocked by incubation for 1 h in Tris-buffered saline–Tween 20 (TBST; 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) containing 10% fat-free milk. Blots were incubated for 2–12 h at 4°C with antibodies raised against a β-gal-CMP1 fusion protein (affinity-purified by absorption onto a trpE–CMP1 fusion protein, as described (Ye and Bretscher, 1992), in TBST containing 2% milk. Following three washes with TBST, blots were reacted with the secondary antibody donkey anti-rabbit IgG coupled to horseradish peroxidase (Amersham) in TBST containing 2% milk. After 1 h of incubation at room temperature, the blots were washed three times with TBS containing 0.3% Tween 20 and three times with TBST; proteins were detected by the ECL detection system (Amersham) and exposure to X-ray film.

α-*Factor halo assays*

Strain stocks were maintained on a common set of YPD plates. All cultures for halo assays were inoculated and grown overnight in parallel in YPD medium at 30°C. Cultures were diluted 10-fold in YPD medium and, based on optical density at 600 nm, equivalent numbers of cells (~10^5 cells/plate) were inoculated into 3 ml YPD top agar (0.7% agar) and poured evenly onto YPD plates. Discs soaked with 5, 10, 20 and 40 μg synthetic α-factor (synthesized and desalted by the HHMI peptide synthesis facility) were placed on the surface of the agar. Plates were then incubated upright for 24 h at 30°C, and were inverted and incubated for up to 144 h. Halo assays shown in Figures 6 and 7 each employed a common batch of YPD medium, YPD plates and YPD top agar. The results presented are representative of at least four and as many as 12 independent experiments. To quantify halo assays (Table III), recovered colonies were counted by photomicroscopy or from enlarged photomicrographs. To avoid the effects at low pheromone concentrations at the outer edge of the halo, only those cells recovering within 3 mm of the disc were counted.

Acknowledgements

We thank N.Rao Movva, Sandoz Pharmaceuticals, Shuang Wei, Laura Davis, Stan Fields, Stephen Helliwell, Michael Hall, Hal Bogerd, Tokichi Miyakawa, Martha Cyert and Mike Vandyk for plasmids, strains and reagents, as well as Tamara Breuder and Eric Lim for excellent technical assistance, Tony Bretscher for support via NIH grant number GM39066, Joe Nevins for generous support and encouragement and Sandra Bowling for assistance with the manuscript. Ruby Ye thanks Tony Bretscher for guidance and unfailing support. Ann Marie Pendergast, Peter Model, Joe Nevins, Susan Gasser, Bryan Cullen, Robin Wharton and Laura Davis are thanked for comments on the manuscript. C.H. is supported by the Toxicology Program and NIEHS grant number NRSA ES07031. J.H. is an Investigator of the Howard Hughes Medical Institute.

References

Aldape,R.A., Futer,O., DeCenzo,M.T., Jarrett,B.P., Murcko,M.A. and Livingston,D.J. (1992) *J. Biol. Chem.*, **267**, 16029–16032.

Belanger,K.D., Kenna,M.A., Wei,S. and Davis,L.I. (1994) *J. Cell Biol.*, **126**, 619–630.

Bierer,B.E., Mattila,P.S., Standaert,R.F., Herzenberg,L.A., Burakoff,S.J., Crabtree,G. and Schreiber,S.L. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 9231–9235.

Bram,R.J., Hung,D.T., Martin,P.K., Schreiber,S.L. and Crabtree,G.R. (1993) *Mol. Cell. Biol.*, **13**, 4760–4769.

Breuder,T., Hemenway,C.S., Movva,N.R., Cardenas,M.E. and Heitman,J. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 5372–5376.

Brillantes,A.-M.B. *et al.* (1994) *Cell*, **77**, 513–523.

Callebaut,I., Renoir,J.-M., Lebeau,M.-C., Massol,N., Burny,A., Baulieu,E.-E. and Mornon,J.-P. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 6270–6274.

Cardenas,M.E., Lorenz,M., Hemenway,C. and Heitman,J. (1994) *Perspectives in Drug Discovery and Design.* Escom Science Publishers, Leiden, Netherlands, Vol. 2, pp. 103–106.

Clipstone,N.A. and Crabtree,G.R. (1992) *Nature*, **357**, 695–697.

Cyert,M.S. and Thorner,J. (1992) *Mol. Cell. Biol.*, **12**, 3460–3469.

Cyert,M.S., Kunisawa,R., Kaim,D. and Thorner,J. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 7376–7380.

Davis,N.G. and Model,P. (1985) *Cell*, **41**, 607–614.

Dumont,F.J., Melino,M.R., Staruch,M.J., Koprak,S.L., Fischer,P.A. and Sigal,N.H. (1990a) *J. Immunol.*, **144**, 1418–1424.

Dumont,F.J., Staruch,M.J., Koprak,S.L., Melino,M.R. and Sigal,N.H. (1990b) *J. Immunol.*, **144**, 251–258.

Durfee,T., Becherer,K., Chen,P.-L., Yeh,S.-H., Yang,Y., Kilburn,A.E., Lee,W.-H. and Elledge,S.J. (1993) *Genes Dev.*, **7**, 555–569.

Fields,S. and Song,O.-K. (1989) *Nature*, **340**, 245–246.

Fischer,G., Wittmann-Liebold,B., Lang,K., Kiefhaber,T. and Schmid,F.X. (1989) *Nature*, **337**, 476–478.

Flanagan,W.M., Corthésy,B., Bram,R.J. and Crabtree,G.R. (1991) *Nature*, **352**, 803–807.

Foor,F., Parent,S.A., Morin,N., Dahl,A.M., Ramadan,N., Chrebet,G., Bostian,K.A. and Nielsen,J.B. (1992) *Nature*, **360**, 682–684.

Friedman,J. and Weissman,I. (1991) *Cell*, **66**, 799–806.

Friedman,J., Trahey,M. and Weissman,I. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 6815–6819.

Fruman,D.A., Klee,C.B., Bierer,B.E. and Burakoff,S.J. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 3686–3690.

Gething,M.-J. and Sambrook,J. (1992) *Nature*, **355**, 33–45.

Handschumacher,R.E., Harding,M.W., Rice,J. and Drugge,R.J. (1984) *Science*, **226**, 544–547.

Harding,M.W., Galat,A., Uehling,D.E. and Schreiber,S.L. (1989) *Nature*, **341**, 758–760.

Harper,J.W., Adami,G.R., Wei,N., Keyomarsi,K. and Elledge,S.J. (1993) *Cell*, **75**, 805–816.

Heitman,J., Movva,N.R., Hiestand,P.C. and Hall,M.N. (1991a) *Proc. Natl Acad. Sci. USA*, **88**, 1948–1952.

Heitman,J., Movva,N.R. and Hall,M.N. (1991b) *Science*, **253**, 905–909.

Heitman,J., Movva,N.R. and Hall,M.N. (1992) *New Biol.*, **4**, 448–460.

Heitman,J., Koller,A., Cardenas,M.E. and Hall,M.N. (1993) *Methods: Compan. Methods Enzymol.*, **5**, 176–187.

Ho,S.N., Hunt,H.D., Horton,R.M., Pullen,J.K. and Pease,L.R. (1989) *Gene*, **77**, 51–59.

Hubbard,M.J. and Klee,C.B. (1989) *Biochemistry*, **28**, 1868–1874.

Hubbard,M.J. and Klee,C.B. (1991) In Chad,J. and Wheal,H. (eds), *Molecular Neurobiology.* Oxford University Press, Oxford, UK, pp. 135–157.

Iida,H., Yagawa,Y. and Anraku,Y. (1990) *J. Biol. Chem.*, **265**, 13391–13399.

Jain,J., McCaffrey,P.G., Miner,Z., Kerppola,T.K., Lambert,J.N., Verdine,G.L., Curran,T. and Rao,A. (1993) *Nature*, **365**, 352–355.

Jin,Y.J. and Burakoff,S.J. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 7769–7773.

Kieffer,L.J., Seng,T.W., Li,W., Osterman,D.G., Handschumacher,R.E. and Bayney,R.M. (1993) *J. Biol. Chem.*, **268**, 12303–12310.

Kincaid,R.L., Giri,P.R., Higuchi,S., Tamura,J., Dixon,S.C., Marietta,C.A., Amorese,D.A. and Martin,B.M. (1990) *J. Biol. Chem.*, **265**, 11312–11319.

Klee,C.B., Draetta,G.F. and Hubbard,M.J. (1988) In Meister,A. (ed.), *Advances in Enzymology and Related Areas of Molecular Biology.* John Wiley & Sons, New York, Vol. 61, pp. 149–200.

Kofron,J.L., Kuzmic,P., Kishore,V., Colon-Bonilla,E. and Rich,D.H. (1991) *Biochemistry*, **30**, 6127–6134.

Koltin,Y., Faucette,L., Bergsma,D.J., Levy,M.A., Cafferkey,R., Koser,P.L., Johnson,R.K. and Livi,G.P. (1991) *Mol. Cell. Biol.*, **11**, 1718–1723.

Kunz,J. and Hall,M.N. (1993) *Trends Biochem. Sci.*, **18**, 334–338.

Li,W. and Handschumacher,R.E. (1993) *J. Biol. Chem.*, **268**, 14040–14044.

Liu,J. (1993) *Immunol. Today*, **14**, 290–295.

Liu,J., Farmer,J.D., Lane,W.S., Friedman,J., Weissman,I. and Schreiber,S.L. (1991) *Cell*, **66**, 807–815.

Liu,J. *et al.* (1992) *Biochemistry*, **31**, 3896–3901.

Liu,Y., Ishii,S., Tokai,M., Tsutsumi,H., Ohki,O., Akade,R., Tanaka,K., Tsuchiya,E., Fukui,S. and Miyakawa,T. (1991) *Mol. Gen. Genet.*, **227**, 52–59.

Luan,S., Li,W., Rusnak,F., Assmann,S.M. and Schreiber,S.L. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 2202–2206.

Luban,J., Bossolt,K.L., Franke,E.K., Kalpana,G.V. and Goff,S.P. (1993) *Cell*, **73**, 1067–1078.

McCaffrey,P.G., Perrino,B.A., Soderling,T.R. and Rao,A. (1993) *J. Biol. Chem.*, **268**, 3747–3752.

Miller,J.H. (1972) *Experiments in Molecular Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 352–355.

Nakamura,T., Liu,Y., Hirata,D., Namba,H., Harada,S.-I., Hirokawa,T. and Miyakawa,T. (1993) *EMBO J.*, **12**, 4063–4071.

Ning,Y.-M. and Sanchez,E.R. (1993) *J. Biol. Chem.*, **268**, 6073–6076.

Northrop,J.P., Ullman,K.S. and Crabtree,G.R. (1993) *J. Biol. Chem.*, **268**, 2917–2923.
O'Keefe,S.J., Tamura,J., Kincaid,R.L., Tocci,M.J. and O'Neill,E.A. (1992) *Nature*, **357**, 692–694.
Poole,R.J. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 3125–3126.
Price,E.R., Jin,M., Lim,D., Pati,S., Walsh,C.T. and McKeon,F.D. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 3931–3935.
Rosen,M.K., Yang,D., Martin,P.K. and Schreiber,S.L. (1993) *J. Am. Chem. Soc.*, **115**, 821–822.
Rotonda,J., Burbaum,J.J., Chan,H.K., Marcy,A.I. and Becker,J.W. (1993) *J. Biol. Chem.*, **268**, 7607–7609.
Schreiber,S.L. (1991) *Science*, **251**, 283–287.
Schreiber,S.L. (1992) *Cell*, **70**, 365–368.
Schreiber,S.L. and Crabtree,G.R. (1992) *Immunol. Today*, **13**, 136–142.
Sherman,F. (1991) *Methods Enzymol.*, **194**, 3–21.
Siekierka,J.J., Hung,S.H.Y., Poe,M., Lin,C.S. and Sigal,N.H. (1989) *Nature*, **341**, 755–757.
Sigal,N.H. *et al.* (1991) *J. Exp. Med.*, **173**, 619–628.
Sigal,N.H. and Dumont,F.J. (1992) *Annu. Rev. Immunol.*, **10**, 519–560.
Standaert,R.F., Galat,A., Verdine,G.L. and Schreiber,S.L. (1990) *Nature*, **346**, 671–674.
Steiner,J.P., Dawson,T.M., Fotuhi,M., Glatt,C.E., Snowman,A.M., Cohen,N. and Snyder,S.H. (1992) *Nature*, **358**, 584–587.
Stotz,A. and Linder,P. (1990) *Gene*, **95**, 91–98.
Sun,G.-H., Ohya,Y. and Anraku,Y. (1991) *J. Biol. Chem.*, **266**, 7008–7015.
Sun,G.-H., Hirata,A., Ohya,Y. and Anraku,Y. (1992) *J. Cell Biol.*, **119**, 1625–1639.
Swanson,S.K.-H., Born,T., Zydowsky,L.D., Cho,H., Chang,H.Y., Walsh,C.T. and Rusnak,F. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 3741–3745.
Sykes,K., Gething,M.J. and Sambrook,J. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 5853–5857.
Tai,P.-K.K., Albers,M.W., Chang,H., Faber,L.E. and Schreiber,S.L. (1992) *Science*, **256**, 1315–1318.
Tai,P.-K.K., Chang,H., Albers,M.W., Schreiber,S., Toft,D.O. and Faber,L.E. (1993) *Biochemistry*, **32**, 8842–8847.
Takahashi,N., Hayano,T. and Suzuki,M. (1989) *Nature*, **337**, 473–475.
Timerman,A.P., Ogunbumni,E., Freund,E., Wiederrecht,G., Marks,A.R. and Fleischer,S. (1993) *J. Biol. Chem.*, **268**, 22992–22999.
Tropschug,M., Barthelmess,I.B. and Neupert,W. (1989) *Nature*, **342**, 953–955.
Ueki,K. and Kincaid,R.L. (1993) *J. Biol. Chem.*, **268**, 6554–6559.
Van Dyke,M.W., Sirito,M. and Sawadogo,M. (1992) *Gene*, **111**, 99–104.
Yang,D., Rosen,M.K. and Schreiber,S.L. (1993) *J. Am. Chem. Soc.*, **115**, 819–820.
Ye,R.R. and Bretscher,A. (1992) *Eur. J. Biochem.*, **204**, 713–723.
Yem,A.W., Tomasselli,A.G., Heinrikson,R.L., Zurcher-Neely,H., Ruff,V.A., Johnson,R.A. and Deibel,M.R.,Jr (1992) *J. Biol. Chem.*, **267**, 2868–2871.
Zinder,N.D. and Boeke,J.D. (1982) *Gene*, **19**, 1–10.

Received on July 14, 1994; revised on September 12, 1994