©Copyright 1999 by Humana Press Inc.
All rights of any nature whatsoever reserved.
1085-9195/99/30/115–151/$19.25

Calcineurin

Structure, Function, and Inhibition

Charles S. Hemenway[1,2] and Joseph Heitman*[2]

[1]Department of Pediatrics, Tulane University School of Medicine, New Orleans, LA; and [2]Departments of Genetics, Pharmacology and Cancer Biology and Medicine, and the Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC 27710

ABSTRACT

Calcineurin is a serine-threonine specific Ca^{2+}-calmodulin-activated protein phosphatase that is conserved from yeast to humans. Remarkably, this enzyme is the common target for two novel and structurally unrelated immunosuppressive antifungal drugs, cyclosporin A and FK506. Both drugs form complexes with abundant intracellular binding proteins, cyclosporin A with cyclophilin A and FK506 with FKBP 12, which bind to and inhibit calcineurin. The X-ray structure of an FKPB12-FK506-calcineurin AB ternary complex reveals that FKBP12-FK506 binds in a hydophobic groove between the calcineurin A catalytic and the regulatory B subunit, in accord with biochemical and genetic studies on inhibitor action. Calcineurin plays a key role in regulating the transcription factor NF-AT during T-cell activation, and in mediating responses of microorganisms to cation stress. These findings highlight the potential of yeast genetic studies to define novel drug targets and elucidate conserved elements of signal transduction cascades.

*Author to whom all correspondence and reprint requests should be addressed.

Index Entries: Phosphatases; immunosuppression; T-cell activation; yeast genetics; drug targets.

INTRODUCTION

The power of the scientific method is to place seemingly unrelated and disordered phenomena into a cohesive context. This is the case with our current understanding of the mechanism of action of the immunosuppressive drugs cyclosporin A (CsA) and FK506 (Fig. 1). Twenty years ago, Borel of Sandoz Pharmaceuticals first described properties of CsA, a cyclic peptide product of the soil fungus *Tolypocladium inflatum (1)*. Concurrently, research in the field of an exciting new calcium "modulator" protein—calmodulin—led to the identification of a Ca^{2+}/calmodulin binding protein that on the basis of its abundance in brain tissue, was termed calcineurin *(2)*. Also, several years later, interest in mechanisms of protein folding resulted in the isolation of a peptidyl-prolyl *cis-trans* isomerase (PPI), which catalyzed folding of ribonuclease *(3,4)*.

A priori, these molecules share little in common, and were studied and characterized within the context of independent scientific niches. CsA was approved as an immunosuppressive drug, and because of its clinical effectiveness, quickly revolutionized solid organ and bone marrow transplantation. Calcineurin, a substrate-specific phosphatase, was extensively investigated by biochemists and neurobiologists *(5)*. Somewhat less interest was directed at the prolyl isomerase until 1989, when it was discovered that prolyl isomerase and a previously identified CsA binding protein, cyclophilin A, were identical *(6–8)*.

The finding that CsA binds cyclophilin A and inhibits prolyl isomerase activity led to the proposal that loss of this enzymatic activity resulted in immunosuppression. This model was strength-ened when FK506, a macrolide antibiotic produced by *Streptomyces tsukubaensis*, was also found to bind and inhibit a protein with prolyl isomerase activity. This 12-kDa immunophilin is designated FKBP12 and is conserved from yeast to humans *(9,10)*. However, based on several important observations, it soon became apparent that a model of immunosuppression invoking the inhibition of a PPI was not correct. In vivo, immunosuppressive levels of CsA and FK506 are well below those required to completely inhibit the PPI activity of cyclophilin A

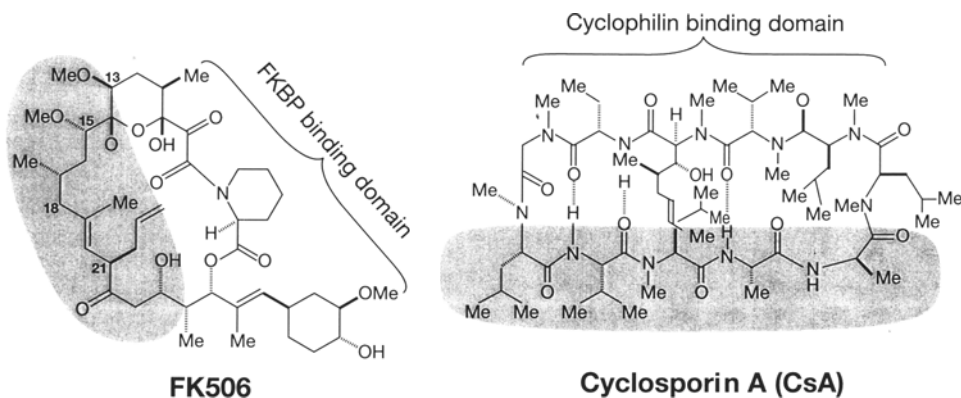

Fig. 1. Structures of FK506 and cyclosporin A. The structures of the macrolide FK506 and the cyclic peptide cyclosporin A (CsA) are shown. The FKBP binding region of FK506 and the cyclophilin binding region of CsA are indicated. The regions of FK506 and CsA that interact with calcineurin are shaded. Although FK506 and CsA share no structural similarity, both are potent antimicrobial and immunosuppressive natural products that inhibit the enzyme calcineurin.

and FKBP12, respectively. Nonimmunosuppressive drug analogs have potent PPI binding and inhibitory effects. Finally, cyclophilin A and FKBP12 mutants of *Saccharomyces cerevisiae* are viable and drug-resistant *(11–15)*. In addition, rapamycin, an immunosuppressant with structural similarities to FK506, inhibits the PPI activity of FKBP12, but is a pharmacologic antagonist of FK506 (reviewed in refs *16–18*).

With these observations in mind and based on the strikingly similar pharmacologic properties of CsA and FK506, Liu et al. *(19)* reasoned that the drug–immunophilin complexes share a common cellular target. Using cyclophilin A-CsA and FKBP12-FK506 affinity chromatography, this group identified calcineurin as the target that is specifically bound to and inhibited by the drug–immunophilin complexes (Fig. 2). In the presence of drug, both cyclophilin A–CsA and FKBP12–FK506 bind to calcineurin with high affinity (~30 nm) *(20,21)*. FKBP12 is also able to bind calcineurin weakly in the absence of FK506, but a K_d has not been measured *(20,22)*. The immunophilin–immunosuppressant complexes are potent inhibitors of calcineurin, and it is important to stress that the calcineurin inhibitors FK506 and cyclosporin A have an extremely unusual mecha-

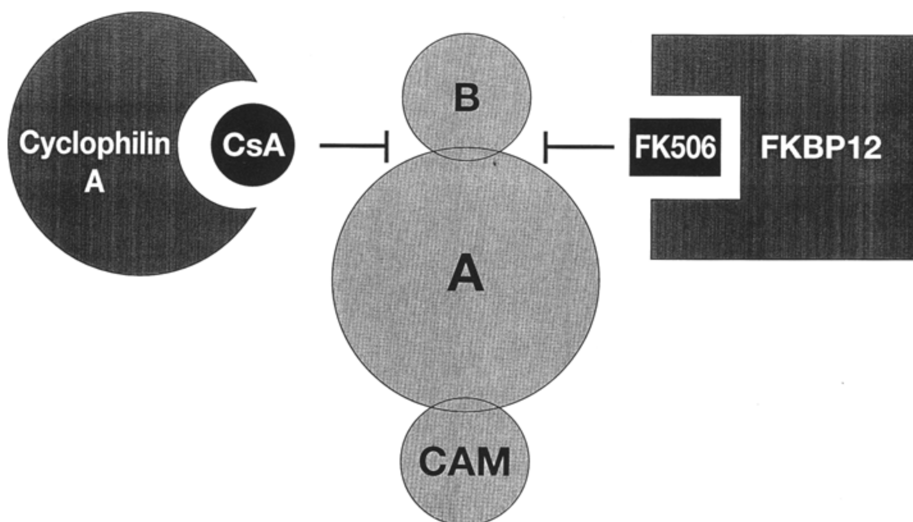

Fig. 2. Immunophilin–immunosuppressant complexes inhibit calcineurin. The natural products CsA and FK506 associate with highly conserved intracellular binding proteins, the immunophilins cyclophilin A and FKBP12, to form the active protein–drug complexes that bind to and inhibit the protein phosphatase calcineurin. Calcineurin is a heterotrimer composed of catalytic **(A)**, regulatory **(B)**, and calmodulin (CAM) subunits. The cyclophilin A–CsA and FKBP12–FK506 complexes bind to a groove between the calcineurin A and B subunits. Calcineurin, cyclophilin A, and FKBP12 are highly conserved from unicellular eukaryotic microorganisms to humans, and recent studies demonstrate that calcineurin is the common target of the immunophilin–immunosuppressant complexes in organisms as diverse as the yeast *S. cerevisiae*, the pathogenic basidiomycetous fungus *C. neoformans*, and humans.

nism of action and must first bind to a normal cellular protein to form the active protein–drug complexes that then bind to and inhibit calcineurin.

Soon after the demonstration that calcineurin is the target of CsA and FK506, direct evidence was presented that calcineurin is a key component in T-cell-signaling pathways *(21,23,24)*. The important connection among the immunosuppressants, immunophilins, and calcineurin was established, and a plethora of investigations were launched in the fields of neurobiology, immunology, and pharmacology. Neurobiologists, long familiar with calcineurin, took advantage of CsA and FK506 as pharmacologic inhibitors to define

neural pathways regulated by calcineurin. Immunologists who had studied the effects of CsA and FK506 on T-cell activation focused on the link between calcineurin and transcriptional regulation of the interleukin 2 (IL-2) gene. With a model that explains how these immunosuppressive drugs act, pharmacologists set about to define the structure of the ternary drug–protein complex to facilitate drug design. Taken together, these studies have greatly furthered our understanding of calcineurin structure, function, and inhibition by an important and lucrative class of drugs.

CALCINEURIN: BIOCHEMICAL PROPERTIES AND STRUCTURE

The biochemical purification and characterization of calcineurin preceded its identification as a key signaling molecule by some 15 yr. Calcineurin was first described as a protein-like activity that inhibited bovine brain cAMP phosphodiesterase *(25,26)*. Interest in a protein variously termed Ca^{2+}-dependent activator protein, regulator protein, modulator protein, and, later, calmodulin prompted the isolation of other proteins that could be purified by calmodulin-sepharose affinity chromatography. In the course of purifying cAMP phosphodiesterase by this method, Klee and Krinks isolated another more abundant Ca^{2+}/calmodulin binding protein with cAMP phosphodiesterase inhibitory activity *(27)*. This protein, which they termed cyclic 3', 5'-nucleotide phosphodiesterase inhibitory protein, was found to be composed of two subunits of M_r 61,000 and 15,000 (later estimated to be M_r 19,000). Using a similar purification scheme, Wallace et al. isolated a protein with virtually identical properties *(28)*. This group concluded that the ability of the protein to inhibit cAMP phosphodiesterase was attributable to a high affinity for the activator protein (calmodulin) rather than direct or specific inhibition of cAMP phosphodiesterase.

The inhibitor protein was later designated "calcineurin" by Klee et al. *(2)* when it was found that the protein, rich in bovine brain tissue, bound Ca^{2+} even in the absence of calmodulin. Moreover, purified calcineurin, like calmodulin, binds four Ca^{2+}/mole protein and with high affinity ($K_d < 10^{-6}$ *M*). The Ca^{2+} binding activity was determined to be a property of the smaller "B" subunit (CnB). On the basis of the Ca^{2+}-binding properties of calcineurin, these authors originally proposed that the protein served as an important regulator of intracellular [Ca^{2+}] in the nervous system.

An important advance in the understanding of the biochemical properties of calcineurin followed shortly thereafter. Studies of protein phosphatase-2 (PP2) revealed the presence of two distinct enzymatic activities that were further subclassified as PP2A and PP2B *(29)* (For a description of the protein phosphatase classification criteria, *see* Ingebritsen and Cohen *[30]*.) PP2B from rabbit skeletal muscle was purified to homogeneity and characterized. Based on its subunit composition and its copurification with calcineurin from bovine brain, it was correctly hypothesized that PP2B and calcineurin are identical proteins.

Several features of the enzyme were noted that continue to distinguish calcineurin/PP2B from other known phosphatases. An obvious but unique property of calcineurin is its dependence on Ca^{2+} for catalytic activity. Although millimolar concentrations of Mn^{2+} support its phosphatase activity in the absence of Ca^{2+} and calmodulin, Ca^{2+} in the micromolar concentration range eliminates the enzyme's Mn^{2+} requirement. Furthermore, in the presence of Ca^{2+}, the addition of calmodulin to calcineurin stimulates enzyme activity an additional 10-fold *(29)*. The activation constant of the calcineurin-calmodulin holoenzyme by Ca^{2+} is in the range of 0.35–0.50 μM *(31,32)*.

Additional work has further clarified the interactions of the three subunits and Ca^{2+} with respect to the catalytic activity of the holoenzyme. At high Ca^{2+} concentrations (100 μM), the phosphatase activity of calcineurin (A- and B-subunit complex) increases in a simple hyperbolic fashion as a function of calmodulin concentration. At fixed concentrations of calmodulin, the addition of Ca^{2+} causes a steep, sigmoidal rise in the phosphatase activity consistent with a high degree of cooperativity. This increase in activity results from a 10- to 25-fold increase in V_{max} with little change in K_m *(29,31,32)*. Importantly, in the absence of CnB, the phosphatase activity of reconstituted catalytic subunits and calmodulin remains low even with the addition of Ca^{2+} *(33)*.

These observations can now be considered in light of the structure of the holoenzyme. The heterotrimeric composition of the catalytically active enzyme was recognized in early studies *(2,27)*. CnA exhibits some, although minimal, phosphatase activity even in the absence of other subunits and, thus, has been recognized as the catalytic subunit *(33,34)*. CnA possesses distinct and independent binding sites for CnB and calmodulin, and can associate with either CnB

or calmodulin alone *(34)*. Genes encoding CnA have been cloned from many species, including mouse, rat, humans, *Drosophila melanogaster, Neurospora crassa, Aspergillus nidulans, S. cerevisiae,* and *Cryptococcus neoformans (35–43)*. The predicted amino acid sequence of the gene products is highly conserved; for example, the yeast and mouse proteins are 54% identical *(35)*. The protein varies in size among isoforms and species, but is composed of approx 520–600 residues. The yeast CnA subunits are somewhat larger than the mammalian homologs because of insertions at the amino-terminus and adjacent to the amino-terminal portion of the calmodulin binding domain *(44)*. Significantly, the proposed functional domains are conserved across species. Despite significant differences in mechanism of regulation and substrate specificity, CnA also shares significant homology with protein phosphatases 1 and 2A in the amino-terminal portion of the enzyme, a region that encompasses the active site *(39)*.

Limited proteolysis of the holoenzyme yields a product that is no longer dependent on Ca^{2+} or calmodulin for activity *(45,46)*. This constitutively activated enzyme is composed of intact CnB and a 45-kDa fragment of CnA. Calmodulin no longer binds this trypsinized fragment. Based on their experimental findings and by analogy to other calmodulin-regulated enzymes, Klee and coworkers *(45,47)* concluded that CnA is composed of both regulatory and catalytic domains. The regulatory domain can itself be subdivided into a calmodulin binding site and an autoinhibitory domain. On Ca^{2+}/calmodulin binding, the autoinhibitory domain is displaced, and the enzyme is activated.

Utilizing synthetic peptides encompassing the CnA carboxy-terminus, a peptide fragment was found that specifically inhibits the activity of the holoenzyme *(48)*. This 25 amino acid fragment is located 30 residues from the protein's carboxy-terminus and approx 60 residues carboxy-terminal to the calmodulin binding domain *(41)*. In experiments that helped establish the relevance of calcineurin in T-cell activation, O'Keefe et al. *(24)* showed that transfection of Jurkat cells with CnA lacking the calmodulin binding and autoinhibitory domains abolishes most of the requirement for Ca^{2+} ionophore (ionomycin) for in vitro activation. Additionally, mutation of a conserved aspartic acid residue within the autoinhibitory domain has been identified in the EL4 mutant T-lymphoma cell line. These mutant cells are hypersensitive to ionomycin, and transfection of the mutant CnA gene confers this phenotype to wild-

type Jurkat cells *(49)*. These data serve to strengthen the model that calcineurin is physiologically regulated by Ca^{2+}/calmodulin by a mechanism dependent on the CnA regulatory domain.

The location of the CnB binding site was originally inferred from regions of CnA that are most highly conserved across species *(37)*. Results of both site directed mutagenesis and synthetic peptide competition experiments map the CnB binding site to a region spanning residues 328–390 of rat CnA *(50,51)*. This site corresponds to a portion of CnA situated between the catalytic site and the calmodulin binding site.

A calmodulin binding site was predicted from the sequence of CnA by Kincaid et al. *(41)* based on the topology of clustered positively charged residues found in other calmodulin binding proteins. This sequence is located beween residues 391 and 414 of rat brain CnA *(39)*. As described earlier, limited proteolysis in the absence of calmodulin destroys this site as well as the more carboxy-terminal autoinhibitory domain.

The B subunit of calcineurin is a Ca^{2+} binding protein. The gene encoding CnB has also been cloned from several divergent species, and comparison of predicted amino acid sequences indicates that it too has been highly conserved *(37,52–54)*. Like calmodulin, CnB posesses four EF hand motifs *(55,56)*. This structural feature is also consistent with the original observations that the CnB protein binds four Ca^{2+} atoms. CnB associates tightly with CnA even in the presence of Ca^{2+} chelators, and the subunits have customarily been seperated by treatment with SDS or urea *(2,57)*. Despite the structural similarities of CnB and calmodulin, reconstitution of the holoenzyme from purified subunits demonstrates that CnB cannot substitute for the activity of calmodulin nor can calmodulin functionally substitute CnB *(34)*.

CnB isolated from bovine brain is myristoylated, as is the *S. cerevisiae CNB1* gene product *(52,57,58)*. However, this posttranslational modification does not appear to affect the in vivo functions or membrane localization of the enzyme in yeast *(58)*. As is the case with cAMP-dependent protein kinase, Rusnak and colleagues found that nonmyristoylated calcineurin B is modestly destabilized to thermal denaturation, indicative of a structural role for this modification *(59)*.

The crystal structure of the complete human and carboxy-terminal truncated bovine enzymes are generally in good agreement with biochemical data *(60,61)*. The high resolution X-ray crystal

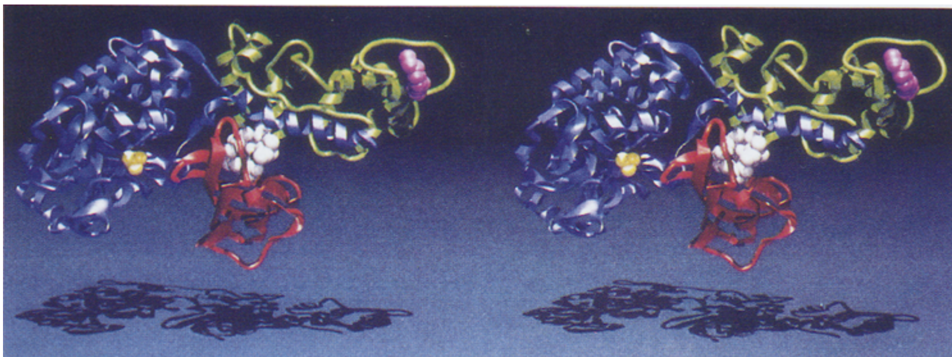

Fig. 3. Structure of calcineurin inhibited complex. The X-ray structure of the calcineurin AB–FK506–FKBP12 ternary complex has been solved and is depicted here as a stereoimage. The calcineurin catalytic A subunit is in blue (with a phosphate molecule bound at the active site shown in yellow), the calcineurin B regulatory subunit is in green (with the N-terminal myristoyl moiety shown in purple), FK506 is in gray, and FKBP12 in red. Note that functional groups on both FKBP12 and FK506 contact calcineurin, and the FKBP12–FK506 complex binds in a hydrophobic groove comprised of an extended α-helical arm of the calcineurin A catalytic subunit and the surface of the calcineurin B regulatory subunit. Adapted with permission from Griffith et al. *(60)*. (Copyright Cell Press.)

structures for calcineurin alone and in a complex with FKBP12–FK506 have been solved by two groups *(60,61)*, and a stereoview of the calcineurin AB–FK506–FKBP12 ternary complex is depicted in Fig. 3. Immediately adjacent to the catalytic site, the CnB binding site occupies residues 343–373 of human CnA (521 total amino acids). However, an amino-terminal segment of CnA (residues 14–23) also appears to contribute significantly to a CnB binding cleft. The catalytic domain is a globular structure that is comprised of residues 14–342. Crystallographic data point to the presence of two metal ions, Zn^{2+} and Fe^{3+}, in the active site and to a catalytic mechanism involving direct hydrolysis of the phosphoester bond by an SN2 mechanism. Residues within the CsA active site have also recently been identified by site-directed mutagenesis *(62)*. The substantial variability in k_{cat} observed for calcineurin substrates is consistent with the proposed absence of an enzyme intermediate *(5)*. The autoinhibitory domain is an 18-residue structure (residues 469–486) that covers the substrate binding cleft precluding substrate access to the site of catalysis. The calmodulin binding site and the extreme

amino- and carboxy-terminal portions of CnA are not visible in the electron density map of human calcineurin.

CnB, as expected, shares much structural homology with calmodulin. It consists of two globular Ca^{2+} binding domains each composed of two EF hands with Ca^{2+} coordinated by five ligands at each binding site. This implies that all four Ca^{2+} binding sites are high-affinity, although Stemmer and Klee have presented biochemical evidence that two high- and two low-affinity sites exist *(63)*. Unlike well-described calmodulin–protein complexes in which the two Ca^{2+} binding domains lie on opposite sides of an α-helical structure of the interacting protein, the Ca^{2+} binding domains of CnB are arranged linearly and fit into a hydrophobic linear groove termed the BBH (for CnB binding α-helix). The amino-terminal myristate group lies parallel to an amino-terminal α-helix of CnB, and may help stabilize the protein or the complex, consistent with the findings of Rusnak and colleagues *(59)*. Therefore, the overall structure of the holoenzyme can be conceptualized as a globular catalytic domain comprising the amino two-thirds of the protein, an adjacent CnB binding domain, and a carboxy-terminal regulatory domain, itself subdivided into a calmodulin binding site and a carboxy-terminal autoinhibitory tail.

REGULATION OF CALCINEURIN

Although the calcineurin crystal structures have provided invaluable data, particularly with regard to the mode of calcineurin inhibition by immunosuppressants, the mechanisms of activation by CnB, calmodulin, and Ca^{2+} remain more speculative. Ca^{2+}/calmodulin binding likely displaces the autoinhibitory domain from its position adjacent to the catalytic site rendering it more accessible to substrate entry. On the other hand, conformational changes induced by Ca^{2+} binding to CnB are probably more subtle and more likely mediated by alterations in the adjacent catalytic domain.

Despite the limitations in the available crystal structures, the dynamic interactions between the three protein subunits and divalent cations are functionally important and have been biochemically characterized. The two regulatory proteins, CnB and calmodulin, are critical to the function of the enzyme. Reconstitution of the holoenzyme from individual subunits resolved in the presence of SDS

reveals a marked stimulation of activity when CnB is added to the catalytic subunit *(33)*. However, this effect is observed only in the presence of Mn^{2+}.

In reconstitution experiments utilizing subunits separated by urea, Merat et al. *(34)* have shown that CnB stimulates the phosphatase activity of the catalytic subunit alone as well as the catalytic subunit plus calmodulin. These experiments were conducted in the presence of 1.5 m*M* Ca^{2+} and demonstrated a clear synergistic effect of CnB and calmodulin on the activity of the enzyme. With the apparently unique exception of the recombinant *Neurospora* CnA subunit that retains significant activity alone *(38)* in the absence of CnB, the activity of reconstituted CnA and calmodulin remains low even with the addition of Ca^{2+}.

Additionally, Perrino et al. *(64)* have shown that the phosphatase activity of recombinant rat brain CnA in the presence of Mn^{2+} is stimulated three- to fivefold and 10- to 15-fold by calmodulin and CnB, respectively. The combination of CnA, CnB, and calmodulin leads to a 100- to 200-fold increase in activity. These experiments also showed that the primary effect of CnB on enzyme kinetics is a reduction in the K_m from 111 to 20 m*M*. Although calmodulin had no effect on K_m, V_{max} was increased fourfold in the absence of CnB and 18-fold in its presence. Similar results have been presented by Stemmer and Klee *(63)*. Implicit in these kinetic data was that CnB likely alters the substrate binding pocket of CnA to favor substrate binding. These findings also suggested that the autoinhibitory domain (regulated by Ca^{2+}/calmodulin) might function as a noncompetitive inhibitor of the enzyme *(48)*. However, studies performed with recombinant peptides suggested instead that the autoinhibitory domain does act as a competitive pseudo–substrate *(65)*. Finally, the calcineurin crystal structure *(61)* clearly reveals that the autoinhibitory domain probably does block access of large protein substrates to the catalytic site, which may be consistent with a role as a competitive pseudosubstrate model.

Although CnB is essential to calcineurin function it is less clear what role Ca^{2+} plays in binding CnB. CnB associates with CnA even in the presence of EGTA *(2)*. Nevertheless, proteolytically activated CnA (lacking the calmodulin binding site) can be further activated by Ca^{2+} through CnB binding *(63,66)*. This may not be physiologically relevant, however, since expression of truncated CnA renders T-cell activation Ca^{2+}-independent *(24)*.

The in vitro importance of CnB and calmodulin is consistent with the phenotypes of strains of *S. cerevisiae* that bear mutations in the genes encoding CnB (*CNB1*) or calmodulin (*CMD1*). *cnb1Δ* mutant strains exhibit the same sensitivity to cations and inability to recover from pheromone-induced growth arrest as strains lacking the functional catalytic subunit *(11,52,67,68)*. *CMD1* is an essential gene in yeast. However, mutant alleles have been produced that fail to bind Ca^{2+}, but continue to support normal vegetative growth *(69,70)*. These *cmd1* mutant strains, like *cnb1Δ* mutants, are sensitive to specific cations and do not recover from pheromone-induced arrest *(71)*. Thus, the weight of biochemical and genetic evidence indicates that in vivo, calcineurin function is completely dependent on the interaction of CnA, CnB, calmodulin, and Ca^{2+}.

In addition to the interplay of CnB, calmodulin, and Ca^{2+}, several other regulatory mechanisms may be physiologically relevant to calcineurin function. Purified calcineurin is a phosphoprotein, although the phosphate content is relatively low (72). Both the autophosphorylated (activated) form of CaM–kinase II as well as protein kinase C (PKC) have been found to phosphorylate calcineurin in vitro efficiently. Phosphorylation is blocked by Ca^{2+}/calmodulin, and, consistent with this observation, phosphopeptide mapping studies have shown that the site of phosphorylation lies adjacent to the calmodulin binding site of CnA *(73,74)*.

Phosphorylation of CnA appears to inhibit catalytic activity by increasing K_m twofold when myosin light chain is used as a substrate or by decreasing V_{max} 50% when p-nitrophenyl phosphate is the substrate *(73)*. Despite these findings, it remains unclear to what extent protein kinases regulate calcineurin activity in vivo.

Another potentially important modulator of calcineurin activity is H^+. Within the pH range of 6.9–7.5, Huang and Cheung *(75)* have shown that H^+ facilitates calcineurin activation at suboptimal Ca^{2+} concentrations. The H^+ stimulation of calcineurin is not attributable to indirect effects on the actual concentration of ionized Ca^{2+}, but results from enhanced CnA/calmodulin binding, perhaps through H^+-induced conformational changes in the calmodulin binding domain of CnA. Again, the physiologic relevance of these in vitro observations has not yet been determined, but given the changes in intracellular pH that occur under differing metabolic conditions, it may be productive to examine further the activity of calcineurin as a function of intracellular pH.

In fungi, regulation of calcineurin gene expression has begun to be explored. The regulatory subunit of calcineurin encoded by the *CNB1* gene of *S. cerevisiae* may be affected by α-factor pheromone treatment at the level of gene expression. Specifically, *CNB1* mRNA levels increase twofold in response to α-factor treatment *(52)*. However, by Western blot CNB1 protein was not increased in response to α-factor *(58)*. Furthermore, the genes encoding CnA, *CMP1*, and *CMP2*, contain the consensus pheromone response element in the 5' UTR *(76)*. Since calcineurin has been strongly implicated to play a role in the mating response of both budding and fission yeast, these observations may be of particular importance. However, conclusive evidence that calcineurin in *S. cerevisiae* is regulated at the level of transcription and/or message stability is lacking.

The *Schizosaccharomyces pombe* gene *ppb1*$^+$ encodes calcineurin A. In this species of yeast, *ppb1*$^+$ mRNA fluctuates slightly with the cell cycle with highest levels occurring during S phase *(77)*. A similar pattern of cell-cycle-dependent gene expression has also been observed in *A. nidulans*, but with a peak in G1 *(43)*. More striking is an eightfold increase in *ppb1*$^+$ mRNA that occurs in response to nitrogen starvation in *S. pombe*. Interestingly, nitrogen starvation induces mating in *S. pombe*, and induction of *ppb1*$^+$ mRNA is dependent on the *ste11*$^+$ transcription factor, a protein important in both mating and nitrogen responses *(77)*. These findings suggest that calcineurin may be involved in the mating responses in both budding and fission yeast, and that calcineurin gene expression may be regulated as part of this process. It is not yet known whether calcineurin is similarly regulated during mating or nutrient starvation in other species.

Calcineurin is required for virulence in the pathogenic basidiomycetous fungus *C. neoformans*, and is required for growth at elevated temperature (37°C) and other stress conditions found in the infected host *(42)*.

INHIBITION OF CALCINEURIN BY CSA AND FK506

As indicated earlier, the immunosuppressant CsA was in widespread clinical use long before the mechanism of action was established. The status of our understanding of the molecular pharmacology of both CsA and FK506, as well as important aspects of T-cell function, was significantly advanced by the simultaneous publications of Fried-

man and Weissman and Liu et al. demonstating that immunophilin–immunosuppressant complexes bind to and inhibit calcineurin *(19,78)*. The nature of calcineurin inhibition has been further elucidated in a large number of experiments.

Mutational analysis and crystallographic data characterizing the structures of cyclophilin A, FKBP12, cyclophilin A–CsA, and FKBP12–FK506 complexes are quite detailed (reviewed by Braun et al. *[78a]*). Interestingly, cyclophilin A (an eight-stranded β-barrel) and FKBP12 (a five-stranded b-sheet) possess few common structural features. CsA, a cyclic peptide, and FK506, a macrolide, are likewise structurally dissimilar compounds (Fig. 1). In each case, drug binding occurs within the hydrophobic catalytic site. The conformation of the protein-bound immunosuppressant molecule may deviate from that of the unbound structure, whereas the binding protein retains its overall conformation. Despite the striking structural dissimilarities among the immunosuppressants and the immunophilins, the resulting cyclophilin A–CsA and FKBP12–FK506 complexes present calcineurin binding surfaces that may share at least some common features. First, four strands of β-sheet structure coincide. Second, each of two solvent-exposed flexible loops found in both complexes positionally overlap. Third, an exposed loop contributed by each drug assumes a similar position in the two complexes. Nevertheless, it remains remarkable that these two complexes share a common molecular target.

These data complement the recently solved crystal structure of the FKBP12–FK506-calcineurin complex (*60,61*, depicted in Fig. 3). The FKBP12–FK506 complex undergoes only slight conformational changes on calcineurin binding, although FK506 rotates slightly with respect to FKBP12 to optimize two points of contact with calcineurin. Significantly, the FKBP12–FK506 complex interacts with both the B-subunit binding helix (BBH) and an adjacent region on the CnA catalytic subunit as well as with CnB. The importance of CnB for the binding of the complex had been predicted from both physical crosslinking studies as well as mutational analysis of calcineurin and the immunophilins *(20,22,79–82)*. On the other hand, yeast genetic studies in which CsA or FK506-resistant calcineurin mutants were isolated revealed that all resulted from single amino acid substitutions in the calcineurin A catalytic subunits encoded by the *CMP1* or *CMP2* genes *(83)*. This finding led to a model that the cyclophilin–CsA and FKBP12–FK506 complexes bind to both the calcineurin A

and B subunits. This model was subsequently confirmed by the solution of the calcineurin AB–FK506–FKBP12 ternary complex in which the FKBP12–FK506 complex binds to a hydrophobic cleft formed from the surfaces of both calcineurin A and calcineurin B (Fig. 3). In addition to establishing the critical nature of CsA and CnB binding to ternary complex formation, these genetic and biochemical studies provide data not available from the crystal structure. They show that neither the regulatory domain nor calmodulin is required for binding of the immunophilin–drug complex, but that the regulatory domain nevertheless is able to modulate binding of the complex. These studies, which also examine the cyclophilin A–CsA complex, indicate that both cyclophilin A–CsA and FKBP12–FK506 bind calcineurin at approximately the same site.

The crystal structure reveals that important van der Waals contacts within a hydrophobic cleft created by the BBH and CnB result from interactions with the C15-C21 portions of the FK506 molecule (Fig. 4). This feature of the structure explains the absence of binding among calcineurin, FKBP12, and nonimmunosuppressive analogs of FK506 and accounts for the FK506 resistance of W380C mutant yeast calcineurin *(83)*, since W380 forms two critical hydrogen bonds with the C13 and C15 methoxy groups of FK506 and is conserved in all known calcineurins (Fig. 4). Finally, the C15-C21 portion of the FK506 molecule differs substantially from rapamycin, providing a molecular explanation of why the FKBP12–rapamycin complex does not bind calcineurin.

The FKBP12–FK506 complex neither blocks nor induces an observable conformational change in the active site. Kissinger et al. *(61)* have presented evidence that FKBP12–FK506 inhibits calcineurin in a "classical," noncompetitive fashion. This has been shown to be the case for cyclophilin A–CsA inhibition as well *(84)*. Consistent with a mechanism of noncompetitive inhibition and an alteration in the enzyme active site is the observation that the immunosuppressant–immunophilin complexes slightly increase calcineurin phosphatase activity toward *p*-nitrophenyl phosphate *(19)*. Given a model of noncompetitive inhibition, it follows that an enzyme–substrate–inhibitor (ESI) complex may be formed *(85)*. This possibility needs to be considered in interpreting experiments that make use of FKBP12–FK506 in an attempt to disrupt the interaction between calcineurin and a substrate *(86)*. For example, FKBP12–FK506 can inhibit NFAT binding to calcineurin, but is much less effective at

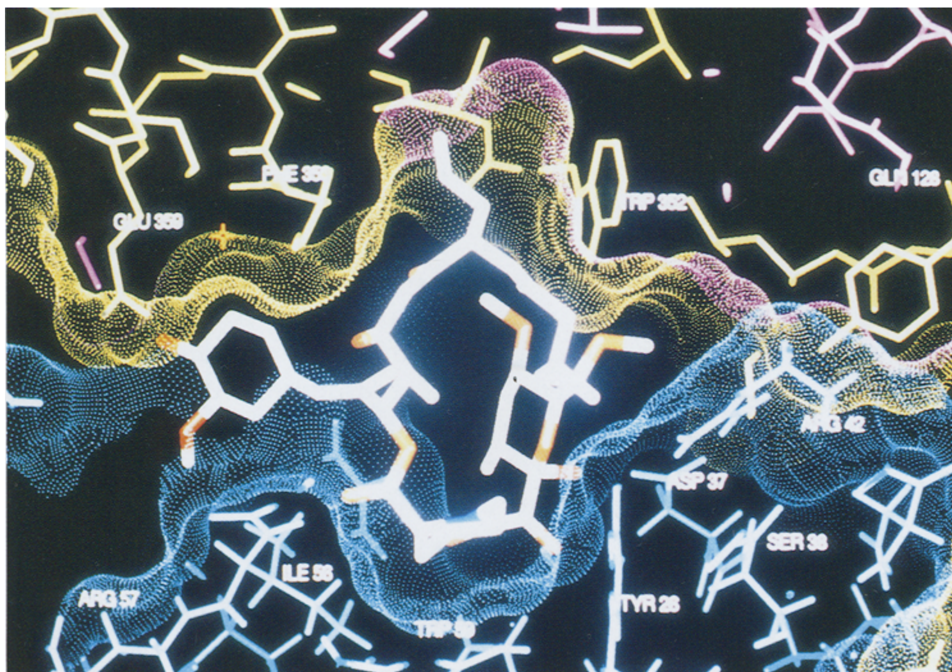

Fig. 4. Interface of the FKBP12–FK506–calcineurin complex. The detailed structure of FK506 (white) bound to the enveloping surfaces of FKBP12 (blue), calcineurin A (yellow), and calcineurin B (pink) in the high-resolution structure of the calcineurin AB-FK506-FKBP12 complex is illustrated *(61)*. FKBP12-FK506 contacts both the A and the B subunits of calcineurin, and a central feature of this interaction surface involves an unusual bifurcated hydrogen bond formed between the Nε1 nitrogen of residue tryptophan 352 on the surface of calcineurin A and the oxygens of the C13 and C15 methoxy groups on FK506. This tryptophan is conserved in all calcineurins and, when altered, confers dominant resistance to FK506 action in yeast by preventing binding of the FKBP12–FK506 complex to calcineurin without impairing the catalytic activity and physiological functions of calcineurin *(83)*.

disrupting a preformed NFAT–calcineurin complex in vitro *(86)*. Nevertheless, the fact that the immunosuppressant–immunophilin complex does not bind the active site of calcineurin, a domain that is highly conserved among all serine/threonine phosphatases *(60)*, remains an important distinction and may, at least in part, account for the specificity of these immunosuppressants for calcineurin.

CALCINEURIN AND ITS IN VIVO SUBSTRATES

In addition to regulation by Ca^{2+}, another distinguishing feature of calcineurin is narrow substrate specificity. Ingebritsen and Cohen *(30)* have classified the protein phosphatases on the basis of sensitivity to the protein inhibitor-1 and rate of dephosphorylation of the α- vs. the β-subunit of phosphorylase kinase. Protein phosphatase subclass 2B (PP2B), or calcineurin, efficiently dephosphorylated only 3 of 13 substrates tested (α-subunit of phosphorylase kinase [a requisite of type-2 phosphatases], inhibitor-1, and the myosin light chain). In contrast, the other subclasses of protein phosphatases dephosphorylated the majority of substrates tested in vitro. The potential for calcineurin participation in specific Ca^{2+}-regulated signaling pathways was recognized on the basis of these unique properties *(87)*.

A PANOPLY OF REGULATORY MOLECULES

Much of the original work on possible in vivo targets of calcineurin focused on inhibitor-1 and a functionally and structurally related protein, dopamine and cAMP-regulated phosphoprotein (DARPP) (reviewed by Cohen *[87]*). Inhibitor-1 and DARPP are phosphoproteins that when activated by cAMP dependent protein kinase (PKA), inhibit protein phosphatase-1 (PP1) at nanomolar concentrations. Inhibition of PP1 by inhibitor-1 thus may serve to amplify signals transmitted by PKA. In skeletal muscle and liver extracts, dephosphorylation of inhibitor-1 is effected by calcineurin in a Ca^{2+}-dependent fashion *(30)*. Notably, inactivation of inhibitor-1 by calcineurin leading to the activation of PP1 provides a mechanism for the activation of a broad-specificity phosphatase by a Ca^{2+} signal.

In isolated hippocampal slices, the application of specific calcineurin inhibitors CsA, FK506, or autoinhibitory peptide blocks the long-term depression (LTD) of neurons *(88)*. LTD is thought to be mediated by a NMDA-induced Ca^{2+} signal and PP1 activation. Thus, calcineurin may link a Ca^{2+} signal and PP1 activation by dephosphorylating and inactivating DARPP.

In a similar fashion, Ca^{2+}-mediated signals acting through calcineurin may antagonize cAMP second messenger systems by activating PP1 and, in turn, dephosphorylating targets of PKA. Given the history of calcineurin isolation (as a cAMP phosphodi-

esterase inhibitor), it is ironic that there is now evidence that calcineurin also dephosphorylates and activates calmodulin-dependent cAMP phosphodiesterase *(89,90)*, an event that would further attenuate a cAMP-mediated signal. It is intriguing that PKA and calcineurin are physically associated by a common anchoring protein, AKAP79, in hippocampal neurons possibly as a means of compartmentalizing a regulatory network within post-synaptic densities *(91)*. This complex interplay of two second messenger systems has been proposed as one mechanism of learning and memory *(92)*.

Several other potential calcineurin substrates have been implicated, largely through work in the field of neurobiology. Activation of nitric oxide synthase (NOS) via the NMDA receptor contributes to ischemia-induced neurotoxicity. Dawson et al. *(93)* found that NMDA-mediated neurotoxicity can be blocked by CsA and FK506. Interestingly, an earlier brief report had shown that CsA diminishes NO-dependent macrophage cytotoxicity due to reduced production of NO *(94)*. Dawson et al., however, were able to extend these findings and show that FK506 inhibits NOS activity while increasing its in vivo phosphorylation state. These findings imply that calcineurin, directly or indirectly, activates NOS through dephosphorylation. These investigators found that NOS is dephosphorylated by calcineurin in vitro and proposed that NOS is a calcineurin substrate. However, NMDA stimulation, probably through activation of calcineurin and dephosphorylation of DARPP, activates a phosphatase cascade *(95)*, and thus PP1, and not calcineurin, may therefore be responsible for the direct dephosphorylation of NOS in vivo.

Calcineurin may also play a role in neuronal development. Axonal elongation and establishment of cell polarity is a process that depends, in part, on the cytoskeleton and the microtubule-associated protein, τ. The specific dephosphorylation that normally occurs with neuronal development is blocked both by CsA and CnA autoinhibitory peptide. At the ultrastructural level, CsA-treated neurons show little axonal elongation *(96)*. Additional data based on the use of inhibitors support the finding that calcineurin regulates the phosphorylation state of τ *(97)*. As yet, however, it is not certain whether τ is a direct substrate of calcineurin or, perhaps, of another calcineurin-regulated phosphatase.

Like τ, calponin is a component of a dynamic cell structure that may be regulated by calcineurin. Calponin participates in the regulation of smooth muscle contraction, and it has been shown that

phosphorylation of calponin blocks actin binding and inhibits actin-activated myosin ATPase. Calponin has been shown to be dephosphorylated by calcineurin in vitro, thereby restoring actin binding *(98)*. Given the importance of Ca^{2+} in the excitation/contraction of muscle, calcineurin could play a physiologic role in this process, but there are currently few additional data to support such a model.

Another area of active and fruitful investigation is the participation of calcineurin in the regulation of ion channels, both in cells of the mammalian nervous system and in other cell types and organisms. Study of the NMDA receptor provides a model of negative feedback in which calcineurin acts to dampen Ca^{2+} entry via the stimulated channel *(99)*. Effects of specific and nonspecific phosphatase inhibitors, including CsA, FK506, and okadaic acid, suggest that calcineurin (and not PP1) dephosphorylates the NMDA receptor and shortens the duration of channel opening. As a consequence, activation of calcineurin by NMDA-mediated Ca^{2+} entry serves to close the channel and limit further Ca^{2+} influx. A prediction of this model is that calcineurin inhibition may exacerbate ischemia-induced cell damage by amplifying NMDA responses in contrast to the expected beneficial effects of calcineurin inhibition with respect to NOS.

A comparable regulatory mechanism modulates activity of the inositol 1,4,5-trisphosphate receptor (IP_3R) and ryanodine receptor (RyR). Cameron et al. *(100)* have demonstrated a physical interaction between calcineurin and these receptors by copurification. In a series of well-controlled reconstitution experiments, these investigators show that the phosphorylation of IP_3R by PKC is substantially augmented by inhibition of receptor-bound calcineurin. Furthermore, calcineurin alone has no effect on PKC activity, indicating that the observed changes in receptor phosphate content are directly attributable to calcineurin phosphatase inhibition. Like the NMDA receptor, the Ca^{2+} conductance of the IP_3R is decreased by dephosphorylation. Similarly, Ca^{2+} influx via the channel activates calcineurin, dephosphorylates the channel, and dampens Ca^{2+} conductance. Although these observations are interesting in their own right, earlier work had shown that the IP_3R and RyR form a complex with FKBP12, and that dissociation of the receptor–FKBP12 complex increases Ca^{2+} flux *(101–103)*. Although it was initially speculated that FKBP12 was directly responsible for stabilization of the channel, an alternative hypothesis that has been presented is that FKBP12

serves to anchor calcineurin to the receptor (*see* ref. *100*), analogous to previous studies indicating that FKBP12 and calcineurin can form a complex in the absence of FK506 *(22)*. Thus, FKBP12 may serve to localize calcineurin to specific macromolecular complexes in a manner analagous to AKAP79.

Ion channels outside the CNS are also regulated by calcineurin. The ubiquitous Na^+,K^+-ATPase of mammalian cells is a target of calcineurin that has particular pharmacologic relevance with respect to the toxicity of calcineurin-inhibiting immunosuppressive drugs. Studies of microdissected renal tubules clearly implicate calcineurin as participating in the regulation of the Na^+,K^+-ATPase. In isolated tubules, CsA, FK506, and inhibitor peptide block both α-adrenergic/Ca^{2+}-mediated stimulation and basal activity of the ion pump *(104,105)*. Similar effects are observed for the Na^+,K^+-ATPase activity of cultured cerebellar neurons when stimulated by the NMDA/Ca^{2+} pathway *(106)*. Therefore, tissue specific receptors may act through a common downstream mechanism involving Ca^{2+} and calcineurin to activate the Na^+,K^+-ATPase in response to different stimuli. It is not clear, however, whether or not calcineurin directly dephosphorylates the ion pump.

As an example of the evolutionary conservation of calcineurin-regulated processes, there is evidence to suggest that a calcineurin-like protein regulates the K^+ channel of guard cells in the plant *Vicia faba (107)*. The conductance of the K^+ channel is inhibited by cytosolic Ca^{2+}. This Ca^{2+}-induced inhibition can be specifically blocked by CsA and FK506, whereas expression of a constitutively active calcineurin fragment inhibits the K^+ channel even in the absence of Ca^{2+}. Thus, these findings suggest calcineurin also regulates ion channels in plants as well.

Similarly, there are a growing number of reports that point to the participation of calcineurin in cellular processes based on the effects of CsA, FK506, inhibitory peptides, and/or the expression of constitutively activated calcineurin. These include processes as diverse as exocytosis and degranulation *(108–110)*, neutrophil migration *(111)*, and even programmed cell death *(112–115)*. Much work remains to be done in defining the precise role of calcineurin in these events, but these observations suggest that despite its narrow substrate specificity—or perhaps because of it—calcineurin participates in a wide range of tightly regulated processes.

THE SPECIAL CASE OF NFAT1

Possible substrates of calcineurin that have thus far been cited include regulatory proteins and enzymes comprising components of signaling pathways (e.g., inhibitor-1, NOS), dynamic structural proteins (e.g., τ, calponin), and ion channels (e.g., IP_3R, Na^+,K^+-ATPase). The excitement generated by the identification of calcineurin as a signalling molecule in the immune system has revealed yet another important regulatory mechanism that is employed through the action of calcineurin, namely transcriptional regulation. Originally, CsA and FK506 were known to block the transcription of genes involved in T-cell activation *(116)*. The IL-2 gene is an important participant in this response and has served as an experimental indicator of T-cell activation. It had further been observed that IL-2 transcription requires the intact function of a transcription factor, nuclear factor of activated T-cells (NF-AT) and that NF-AT activity is blocked by CsA *(116,117)*. In a series of reconstitution experiments utilizing cytoplasmic and nuclear extracts of Jurkat cells, Flanagan et al. *(118)* showed that NF-AT consists of at least two components, one cytoplasmic and one nuclear. Transcriptional activation requires the association of these two components. Thus, the cytoplasmic component must translocate to the nucleus on T-cell activation. Moreover, CsA and FK506 appear to prevent the nuclear translocation of the cytoplasmic component. This model was further extended by the experimental work of Jain et al. *(119)* demonstrating that the nuclear component of NF-AT consists of *fos* and *jun*.

With the identification of calcineurin as the target of CsA and FK506 came the rapid recognition that calcineurin participates in T-cell activation (*see above*). To complete this important line of investigation, the cytoplasmic component of NF-AT, termed NFAT1 (NFATp), was isolated and cloned *(120,121)*. NFAT1 is a phosphoprotein that is an in vitro substrate of calcineurin *(120,121)*. In experiments exploiting a calcineurin/calmodulin affinity matrix, NFAT1 forms a physical complex with calcineurin *(86,122)*, a finding consistent with the in vitro dephosphorylation data. Perhaps more exciting is the observation based on immunolocalization data that NFAT1 translocates from the cytoplasm to the nucleus. This event occurs within minutes of T-cell activation and is blocked by CsA and FK506 *(123,124)*. A related T-cell factor, NFAT4, first associates with calcineurin in the cytoplasm and then is translocated into the nucleus

as a complex with calcineurin *(125)* where calcineurin may continue to act to counter the effects of nuclear NF-AT kinases. There is now evidence that several kinases phosphorylate NF-ATs, including PKA and glycogen synthase kinase-3 (GSK-3). Overexpression of GSK-3 blocks Ca^{2+}-induced calcineurin-dependent nuclear import of $NFAT_c$ *(126)*. The phosphorylation state of NFAT1, by determining its subcellular localization, regulates its interaction with *fos* and *jun*. Additionally, the in vitro behavior of NFAT1 indicates that the phosphorylated form binds DNA poorly even in the presence of *fos* and *jun*. This may be particularly relevant to some cell types in which NFAT1 is constitutively localized to the nucleus *(124,127)*. Activation of NF-AT therefore is controlled by both subcellular localization and DNA binding through the phosphorylation state of NFATs.

In summary, a large body of literature now supports a model in which T-cell activation occurs as follows: stimulation via the T-cell receptor simultaneously activates Ca^{2+}- and PKC-mediated signaling cascades. The PKC pathway results in the *de novo* synthesis of *fos* and *jun*, whereas the Ca^{2+} pathway activates calcineurin. Calcineurin, in turn, dephosphorylates NFAT1. The hypophosphorylated form of NFAT1 translocates to the nucleus and is competent to oligomerize with *fos* and *jun* to create a functional (DNA binding) NF-AT complex. By inhibiting calcineurin, CsA and FK506 block NFAT1 translocation to the nucleus as well as prevent the binding of any phosphorylated nuclear NFAT1 to DNA. Although it has not been formally excluded that calcineurin activates a phosphatase cascade to dephosphorylate NFAT1, there are much in vitro data to suggest that NFAT1 is a direct substrate of calcineurin.

Consequently, in mammalian T-cells, calcineurin appears to exert an important regulatory influence by directly controlling the activity of a transcription factor. Furthermore, several NFAT-like proteins exist in T-cells, and there is accumulating evidence that calcineurin may regulate these proteins in a similar fashion *(128–130)*. The induction of several lymphokines may be under the transcriptional control of calcineurin. The regulation of expression of genes in diverse species by calcineurin may indicate that this is a fundamental feature of calcineurin function.

CALCINEURIN IN FUNGI

The degree of evolutionary conservation of calcineurin is remarkable. This conservation supports the notion that calcineurin-

regulated pathways are of fundamental physiological importance in eukaryotes. As previously described, genes encoding CnA and CnB have been cloned from diverse species. The proteins share the same distinctive structural characteristics that contribute to its Ca^{2+}-dependent catalytic activity *(35,38,42,43)*. The immunosuppressant binding proteins cyclophilin A and FKBP12 are also conserved from yeast to mammals, and calcineurin is potently inhibited in yeast by CsA and FK506 (reviewed in ref. *16*).

There are two isozymes of CnA in *S. cerevisiae* encoded by the genes *CMP1/CNA1* and *CMP2/CNA2 (35,76)*. The proteins are 64% identical to each other and 52–54% identical to murine and rat brain enzymes. The *CNB1* gene of *S. cerevisiae* encodes the CnB regulatory subunit and is 56% identical to human CnB *(52)*. Genes encoding CnA have been cloned in several other fungal species including *S. pombe, A. nidulans, N. crassa*, and *C. neoformans (38,42,43,131)*. All share approx 55% sequence identity compared to the mammalian counterparts.

Given the evolutionary conservation of these proteins, it is of considerable scientific interest to explore the signaling pathways mediated by calcineurin. In mammals, the T-cell activation pathway involving calcineurin and NFAT1 has been fairly well characterized, but a myriad of other calcineurin-dependent pathways are less well understood. Elucidation of calcineurin functions in other species should define important and general biological themes.

In *A. nidulans*, calcineurin function is essential and disruption of the $cnaA^+$ gene is lethal *(43)*. Inspection of the abortive growth of germinating conidia of *cnaA1* disruption mutants reveals a nuclear morphology consistent with G1 arrest. That calcineurin may play an important role in cell-cycle progression in *A. nidulans* is also suggested by a peak in $cnaA^+$ mRNA in early G1 (43). The gene encoding calcineurin A, *CNA1*, has also been cloned from the pathogenic basidiomycete *C. neoformans*, a common cause of opportunistic infections and the leading cause of fungal meningitis *(42)*. The calcineurin A gene in this organism has been disrupted by homologous recombination, revealing that calcineurin is not essential for viability, but is required for survival at 37°C and for virulence in an animal model of cryptococcal meningitis *(42)*. Growth at 37°C is a well-established virulence trait in this pathogen, and calcineurin is the first molecular determinant identified involved in growth at high temperature. Finally, a nonimmunosuppressive FK506 analog (L-

685, 818) has been identified that retains antifungal activity, likely by taking advantage of inherent structural differences between host and fungal calcineurin and FKBP12, and which could represent a starting point in the design of novel antifungal drugs *(132)*.

Calcineurin null mutants of *S. pombe* (*ppb1* null) are also viable but display a distinctly abnormal phenotype *(131)*. They are sterile and grow slowly at 22°C. The cell morphology of *ppb1* null mutants is grossly abnormal when grown at 22°C with numerous multiseptate cells and branching filaments. These features are consistent with abnormalities in cytokinesis and determination of cell polarity, respectively, and point to a possible defect in microtubule function. Likewise, overexpression of $ppb1^+$ results in abnormalities in spindle pole body positioning and microtubule morphology. The connection between calcineurin and microtubule structure is reminiscent of the interaction between calcineurin and the neuronal τ protein of mammals.

Calcineurin null mutants of *S. cerevisiae,* in contrast to other fungal species thus far studied, exhibit relatively few phenotypes. Specifically, calcineurin null mutants fail to recover from α-factor pheromone-induced G1 cell-cycle arrest and are sensitive to high concentrations of Na^+, Li^+, or Mn^{2+} ions *(11–13,35,67,68,133–135)*. This is not to imply that calcineurin does not play an important role in *S. cerevisiae,* but that the pathways that it regulates are partly redundant. Therefore, perturbations in overlapping or parallel pathways may unmask a more severe phenotype in calcineurin null mutants. For example, a synthetic lethal phenotype has been observed in mutants of the *FKS1* gene encoding a subunit of 1,3-β-D-glucan synthase. This observation led to the findings that a homologous subunit encoded by the *FKS2* gene is dependent on calcineurin for expression and that 1,3-β-D-glucan synthase activity is essential in *S. cerevisiae (136–138)*. Similarly, both calcineurin and Ca^{2+}-calmodulin-dependent protein kinase (CaMK) pathways function independently to mediate recovery from pheromone *(139)*.

Inhibition or mutation of calcineurin is also lethal in yeast mutants that are unable to acidify the vacuole, likely as a consequence of broad derangements in cation homeostasis in the absence of proper vacuole function *(13,140,141)*. Finally, calcineurin plays a central role in regulating Ca^{2+} homeostasis in *S. cerevisiae* via transcriptional regulation of the vacuolar Ca^{2+} ATPase pump PMC1 and post translational regulation of the vacuolar H^+/Ca^{2+} exchanger VCX1/HUM1 *(142–145)*.

In summary, calcineurin plays a diverse role in regulating cellular responses to physiological and toxic cations in *S. cerevisiae,* and likely in other organisms as well (*see,* for example, ref. 42).

SUMMARY

Our studies are based on the premise that small natural products serve as useful probes of signal transduction cascades. We began by analyzing the effects of the immunosuppressants cyclosporin A and FK506, which although specific for T-cells in animals, are both natural products of soil microorganisms. Our hypothesis is that the role of CsA and FK506 in nature might be as toxins to inhibit growth of competing microorganisms, which we have studied in yeast and a pathogenic fungus. Our studies reveal that the mechanisms of CsA and FK506 antimicrobial and immunosuppressive action have been conserved from yeast and pathogenic fungi to humans over a billion years of evolution. Drug action involves an initial binding to a highly conserved enzyme, CsA to cyclophilin A and FK506 to FKBP12, to form protein–drug complexes that inhibit calcineurin, a Ca^{2+}-regulated phosphatase also conserved from yeast to fungi to man. Much of experimental biology has been based on the premise that studies of model organisms, such as bacteria, yeast, insects, and worms, will reveal conserved principles that govern how all organisms function. The studies reviewed have support this view and suggest further studies of model organisms can contribute much to our understanding of the molecular basis of life. Moreover, the remarkable conservation in mechanisms of drug action suggest that targets for many other important natural products can be identified by similar genetic approaches in yeast and other genetically tractable microorganisms. Now that the yeast genome has been sequenced, and a set of viable gene disruption mutant strains will soon be available, the importance of genetic approaches to study natural product action should be greatly simplified in this model microorganism.

ACKNOWLEDGMENTS

We thank Sandra Bowling for diligent and patient assistance with manuscript preparation and Ernie Villafranca for providing the image shown in Fig. 4. This work was supported, in part, by a Rhône

Poulenc Rorer American Society of Hematology Scholar Award (C. H.) and NIAID R01 grants AI39115, AI41937, (J. H.). Joseph Heitman is a Burroughs Wellcome Scholar in Molecular Patholgenic Mycology and an associate investigator of the Howard Hughes Medical Institute.

REFERENCES

1. Borel, J. F. (1976) Comparative study of *in vitro* and *in vivo* drug effects on cell-mediated cytotoxicity. *Immunology* **31,** 631–641.
2. Klee, C. B., Crouch, T. H., and Krinks, M. H. (1979) Calcineurin: a calcium- and calmodulin-binding protein of the nervous system. *Proc. Natl. Acad. Sci. USA* **76,** 6270–6273.
3. Fischer, G. and Bang, H. (1985) The refolding of urea-denatured ribonuclease A is catalyzed by peptidyl-prolyl *cis*-trans isomerase. *Biochimica et Biophysica Acta* **828,** 39–42.
4. Fischer, G., Bang, H., and Mech, C. (1984) Nachweis einer Enzymkatalyse für die cis-trans- isomerisierung der peptidbindung in prolinhaltigen peptiden. *Biomed. Biochim. Acta.* **43,** 1101–1111.
5. Klee, C. B., Draetta, G. F., and M. J. Hubbard. (1988) Calcineurin, in *Advances in Enzymology and Related Areas of Molecular Biology, Vol. 61* (A. Meister, ed.), Wiley, New York, pp. 149–209.
6. Fischer, G., Wittmann-Liebold, K., Lang, K., Kiefhaber, T., and Schmid, F. X. (1989) Cyclophilin and peptidyl-prolyl cis-trans isomerase are probably identical proteins. *Nature* **337,** 476–478.
7. Handschumacher, R. E., Harding, M. W., Rice, J., and Drugge, R. J. (1984) Cyclophilin: A specific cytosolic binding protein for cyclosporin A. *Science* **226,** 544–547.
8. Takahashi, N., Hayano, T., and Suzuki, M. (1989) Peptidyl-prolyl *cis*-trans isomerase is the cyclosporin A-binding protein cyclophilin. *Nature* **337,** 473–475.
9. Harding, M. W., Galat, A., Uehling, D. E., and Schreiber, S. L. (1989) A receptor for the immunosuppressant FK506 is a cis-trans peptidyl-prolyl isomerase. *Nature* **341,** 758–760.
10. Heitman, J., Movva, N. R., Hiestand, P. C., and Hall, R. N. (1991) FK506-binding protein proline rotamase is a target for the immunosuppressive agent FK506 in *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA* **88,** 1948–1952.
11. Breuder, T., Hemenway, C. S., Movva, N. R., Cardenas, M. E., and Heitman, J. (1994) Calcineurin is essential in cyclosporin A- and FK506-sensitive yeast strains. *Proc. Natl. Acad. Sci. USA* **91,** 5372–5376.
12. Foor, F., Parent, S. A., Morin, N., Dahl, A. M., Ramadan, N., Chrebet, G., Bostian, K. A., and Nielsen, J. B. (1992) Calcineurin mediates

inhibition by FK506 and cyclosporin of recovery from α-factor arrest in yeast. *Nature* **360,** 682–684.

12a. Heitman, J., Movva, N. R., and Hall, M. N. (1991) Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science* **253,** 905–909.

13. Hemenway, C. S., Dolinski, K., Cardenas, M. E., Hiller, M. A., Jones, E. W., and Heitman, J. (1995) *vph6* mutants of *Saccharomyces cerevisiae* require calcineurin for growth and are defective in vacuolar H^+-ATPase assembly. *Genetics* **141,** 833–844.

14. Koltin, Y., Faucette, L., Bergsma, D. J., Levy, M. A., Cafferkey, R., Koser, P. L., Johnson, P. K., and Livi, G. P. (1991) Rapamycin sensitivity in *Saccharomyces cerevisiae* is mediated by a peptidyl-prolyl cis-trans isomerase related to human FK506-binding protein. *Mol. Cell. Biol.* **11,** 1718–1723.

15. Tropschug, M., Barthelmess, I. B., and Neupert, W. (1989) Sensitivity to cyclosporin A is mediated by cyclophilin in *Neurospora crassa* and *Saccharomyces cerevisiae. Nature* **342,** 953–955.

16. Cardenas, M. E., Lorenz, M., Hemenway, C., and Heitman, J. (1994) Yeast as model-T cells. *Perspectives in Drug Discovery and Design.* **2,** 103–126.

17. Cardenas, M. E., Zhu, D., and Heitman, J. (1995) Molecular mechanisms of immunosuppression by cyclosporine, FK506, and rapamycin. *Curr. Opin. Nephrol. Hyperten.* **4,** 472–477.

18. Heitman, J., Movva, N. R., and Hall, M. N. (1992) Proline isomerases at the crossroads of protein folding, signal transduction, and immunosuppression. *New Biologist* **4,** 448–460.

19. Liu, J., Farmer J. D., Lane, W. S., Friedman, J., Weissman, I., and Schreiber, S. L. (1991) Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66,** 807–815.

20. Husi, H., Luyten, M. A., and Zurini, M. G. M. (1994) Mapping of the immunophilin-immunosuppressant site of interaction of calcineurin. *J. Biol. Chem.* **269,** 14,199–14,204.

21. Liu, J., Albers, M. W., Wandless, T. J., Luan, S., Alberg, D. G., Belshaw, P. J., Cohen, MacKintosh, C., Klee, C. B., and Schreiber, S. L. (1992) Inhibition of T cell signaling by immunophilin-ligand complexes correlates with loss of calcineurin phosphatase activity. *Biochemistry* **31,** 3896–3901.

22. Cardenas, M., Hemenway, C., Muir, R. S., Ye, R., Fiorentino, D., and Heitman, J. (1994) Immunophilins interact with calcineurin in the absence of exogenous immunosuppressive ligands. *EMBO J.* **13,** 5944–5957.

23. Clipstone, N. A. and Crabtree, G. R. (1992) Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature* **357,** 695–697.

24. O'Keefe, S. J., Tamura, J. I., Kincaid, R. L., Tocci, M. J., and O'Neill, E. A. (1992) FK-506- and CsA-sensitive activation of the interleukin-2 promoter by calcineurin. *Nature* **357,** 692–694.
25. Wang, J. H. and Desai, R. (1976) A brain protein and its effect on the Ca^{2+} - and protein modulator-activated cyclic nucleotide phosphodiesterase. *Biochem. Biophys. Res. Comm.* **72,** 926–932.
26. Wang, J. H. and Desai, R. (1977) Modulator binding protein. *J. Biol. Chem.* **252,** 4175–4184.
27. Klee, C. B. and Krinks, M. H. (1978) Purification of cyclic 3', 5'-nucleotide phosphodiesterase inhibitory protein by affinity chromatography on activator protein coupled to sepharose. *Biochemistry* **17,** 120–126.
28. Wallace, R. W., Lynch, T. J., Tallant, E. A., and Cheung, W. Y. (1978) Purification and characterization of an inhibitor protein of brain adenylate cyclase and cyclic nucleotide phosphodiesterase. *J. Biol. Chem.* **254,** 377–382.
29. Stewart, A. A., Ingebritsen, T. S., Manalan, A., Klee, C. B., and Cohen, P. (1982) Discovery of a Ca^{2+} - and calmodulin-dependent protein phosphatase. *FEBS Lett.* **137,** 80–84.
30. Ingebritsen, T. S. and Cohen, P. (1983) The protein phosphatases involved in cellular regulation I. Classification and substrate specificities. *Eur. J. Biochem.* **132,** 255–261.
31. Stewart, A. A., Ingebritsen, T. S., and Cohen, P. (1983) The protein phosphatases involved in cellular regulation. *Eur. J. Biochem.* **132,** 289–295.
32. Tallant, E. A. and Cheung, W. Y. (1984) Chacterization of bovine brain calmodulin-dependent protein phosphatase. *Arch. Biochem. Biophys.* **232,** 269–279.
33. Gupta, R. C., Khandelwal, R. H., and Sulakhe, P. V. (1985) Resolution of bovine brain calcineurin subunits: stimulatory effect of subunit B on subunit A phosphatase activity. *FEBS.* **190,** 104–108.
34. Merat, D. L., Hu, Z. Y, Carter, T. E., and Cheung, W. Y. (1985) Bovine brain calmodulin-dependent protein phosphatase. *J. Biol. Chem.* **260,** 11,053–11,059.
35. Cyert, M. S., Kunisawa, R., Kaim, D., and Thorner, J. (1991) Yeast has homologs (*CNA1* and *CNA2* gene products) of mammalian calcineurin, a calmodulin-regulated phosphoprotein phosphatase. *Proc. Natl. Acad. Sci. USA* **88,** 7376–7380.
36. Guerini, D. and Klee, C. B. (1989) Cloning of human calcineurin A: Evidence for two isozymes and identification of a polyproline structural domain. *Proc. Natl. Acad. Sci. USA* **86,** 9183–9187.
37. Guerini, D., Montell, C., and Klee, C. B. (1992) Molecular cloning and characterization of the genes encoding the two subunits of *Drosophila melanogaster* calcineurin. *J. Biol. Chem.* **267,** 22,542–22,549.

38. Higuchi, S., Tamura, J. I., Giri, P. R. , Polli, J. W., and Kincaid, R. L. (1991) Calmodulin-dependent protein phosphatase from *Neurospora crassa*. *J. Biol. Chem.* **266,** 18,104–18,112.
39. Ito, A., Hashimoto, T., Hirai, M., Takeda, T., Shuntoh, H., Kuno, T., and Tanaka, C. (1989) The complete primary structure of calcineurin A, a calmodulin binding protein homologous with protein phosphatases 1 and 2A. *Biochem. Biophys. Res. Comm.* **163,** 1492–1497.
40. Kincaid, R. L., Giri, P. R., Higuchi, S., Tamura, J. I., Dixon, S. C., Marietta, C. A., Amorese, D. A., and Martin, B. M. (1990) Cloning and characterization of molecular isoforms of the catalytic subunit of calcineurin using nonisotopic methods. *J. Biol. Chem.* **265,** 11,312–11,319.
41. Kincaid, R. L., Nightingale, M. S., and Martin, B. M. (1988) Characterization of a cDNA clone encoding the calmodulin-binding domain of mouse brain calcineurin. *Proc. Natl. Acad. Sci. USA* **85,** 8983–8987.
42. Odom, A., Muir, S., Lim, E., Toffaletti, D. L., Perfect, J., and Heitman, J. (1997) Calcineurin is required for virulence of *Cryptococcus neoformans*. *EMBO J.* **16,** 2576–2589.
43. Rasmussen, C., Garen, C., Brining, S., Kincaid, R. L., Means, R. L., and Means, A. R. (1994) The calmodulin-dependent protein phosphatase catalytic subunit (calcineurin A) is an essential gene in *Aspergillus nidulans*. *EMBO J.* **13,** 2545–2552.
44. Cyert, M. S. (1993) The function of Ca^{2+}/calmodulin-regulated phosphatases in yeast. *Adv. Prot. Phosphatases.* **7,** 429–443.
45. Manalan, A. S. and Klee, C. B. (1983) Activation of calcineurin by limited proteolysis. *Proc. Natl. Acad. Sci. USA* **80,** 4291–4295.
46. Winkler, M. A., Merat, D. L., Tallant, E. A., Hawkins, S., and Cheung, W. Y. (1984) Catalytic site of calmodulin-dependent protein phosphatase from bovine brain residues in subunit A. *Proc. Natl. Acad. Sci. USA* **81,** 3054–3058.
47. Hubbard, M. J. and Klee, C. B. (1989) Functional domain structure of calcineurin A: mapping by limited proteolysis. *Biochemistry* **28,** 1868–1874.
48. Hashimoto, Y., Perrino, B. A., and Soderling, T. R. (1990) Identification of an autoinhibitory domain in calcineurin. *J. Biol. Chem.* **265,** 1924–1927.
49. Fruman, D. A., Pai, S.-Y., Burakoff, S. J., and Bierer, B. E. (1995) Characterization of a mutant calcineurin Aα gene expressed by EL4 lymphoma cells. *Mol. Cell. Biol.* **15,** 3857–3863.
50. Sikkink, R., Haddy, A., MacKelvie, S., Mertz, P., Litwiller, R., and Rusnak, F. (1995) Calcineurin subunit interactions: mapping the calcineurin B binding domain on calcineurin A. *Biochemistry* **34,** 8348–8356.

51. Watanabe, Y., Perrino, B. A., Chang, A. H., and Soderling, T. R. (1995) Identification in the calcineurin A subunit of the domain that binds the regulatory B subunit. *J. Biol. Chem.* **270,** 456–460.
52. Cyert, M. S. and Thorner, J. (1992) Regulatory subunit (*CNB1* gene product) of yeast Ca^{2+}/calmodulin-dependent phosphoprotein phosphatases is required for adaptation to pheromone. *Mol. Cell. Biol.* **12,** 3460–3469.
53. Guerini, D., Krinks, M. H., Sikela, M., Hahn, W. E., and Klee, C. B. (1989) Isolation and sequence of a cDNA clone for human calcineurin B, the Ca^{2+}- binding subunit of the Ca^{2+}/calmodulin-stimulated protein phosphatase. *DNA* **8,** 675–682.
54. Ueki, K., Muramatsu, T., and Kincaid, R. L. (1992) Structure and expression of two isoforms of the murine calmodulin-dependent protein phosphatase regulatory subunit. *Biochem. Biophys. Res. Comm.* **187,** 537–543.
55. Aitken, A., Klee, C. B., and Cohen, P. (1984) The structure of the B subunit of calcineurin. *Eur. J. Biochem.* **139,** 663–671.
56. Anglister, J., Grzesiek, S., Wang, A. C., Ren, H., Klee, C. B., and Bax, A. (1994) ^{1}H, ^{13}C, ^{15}N nuclear magnetic resonance backbone assignments and secondary structure of human calcineurin B. *Biochemistry* **33,** 3540–3547.
57. Aitken, A., Cohen, P., Santikarn, S., Williams, D. H., Calder, A. G., Smith, A. G., and Klee, C. B. (1982) Identification of the NH_2-terminal blocking group of calcineurin B as myristic acid. *FEBS Lett.* **150,** 314–318.
58. Zhu, D., Cardenas, M. E., and Heitman, J. (1995) Myristoylation of calcineurin B is not required for function or interaction with immunophilin-immunosuppressant complexes in the yeast *Saccharomyces cerevisiae. J. Biol. Chem.* **270,** 24,831–24,838.
59. Kennedy, M. T., Brockman, H., and Rusnak, F. (1996) Contributions of myristoylation to calcineurin structure/ function. *J. Biol. Chem.* **271,** 26,517–26,521.
60. Griffith, J. P., Kim, J. L., Kim, E. E., Sintchak, M. D., Thomson, J. A., Fitzgibbon, M. J., Felming, M. A., Caron, P. R., Hsiao, K., and Navia, M. A. (1995) X-ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP12-FK506 complex. *Cell* **82,** 507–522.
61. Kissinger, C. R., Parge, H. E., Knighton, D. R, Lewis, C. T., Pelletier, L. A., Tempczyk, A., Kalish, V. J., Tucker, K. D., Showalter, R. E., Moomaw, R. E., Gastinel, L. N., Habuka, N., Chen, X., Maldonado, F., Barker, J. E., Bacquet, R., and Villafranca, J. E. (1995) Crystal structures of human calcineurin and the human FKBP12-FK506-calcineurin complex. *Nature* **378,** 641–644.

62. Mondragon, A., Griffith, E. C., Sun, L., Xiong, F., Armstrong, C., and Liu, J. O. (1997) Overexpression and purification of human calcineurin α from *Escherichia coli* and assessment of catalytic functions of residues surrounding the binuclear metal center. *Bichemistry* **36,** 4934–4942.
63. Stemmer, P. M., and Klee, C. B. (1994) Dual calcium ion regulation of calcineurin by calmodulin and calcineurin B. *Biochemistry* **33,** 6859–6866.
64. Perrino, B. A., Fong, Y.-A., Brickey, D. A., Saitoh, Y., Ushio, Y., Fukunaga, K., Miyamoto, E., and Soderling, T. R. (1992) Characterization of the phosphatase activity of a baculovirus-expressed calcineurin A isoform. *J. Biol. Chem.* **267,** 15,965–15,969.
65. Sagoo, J. K., Furman, D. A., Wesselborg, S., Walsh, C. T., and Bierer, B. E. (1996) Competitive inhibition of calcineurin phosphatase activity by its autoinhibitory domain. *Biochem. J.* **320,** 879–884.
66. Perrino, B. A., Ng, L. Y., and Soderling, T. R. (1995) Calcium regulation of calcineurin phosphatase activity by its B subunit and calmodulin. *J. Biol. Chem.* **270,** 340–346.
67. Mendoza, I., Rubio, F., Rodriquez-Navarro, A., and Pardo, J. M. (1994) The protein phosphatase calcineurin is essential for NaCl tolerance of *Saccharomyces cerevisiae. J. Biol. Chem.* **269,** 8792–8796.
68. Nakamura, T., Liu, Y., Hirata, D., Namba, H., Harada, S.-I., Hirokawa, T., and Miyakawa, T. (1993) Protein phosphatase type 2B (calcineurin)-mediated, FK506-sensitive regulation of intracellular ions in yeast is an important determinant for adaptation to high salt stress conditions. *EMBO J.* **12,** 4063–4071.
69. Davis, T. N., Urdea, M. S., Masiarz, F. R., and Thorner, J. (1986) Isolation of the yeast calmodulin gene: Calmodulin is an essential protein. *Cell* **47,** 423–431.
70. Geiser, J. R., VanTuinen, D., Brockerhoff, S. E., Neff, M. M., and Davis, T. N. (1991) Can calmodulin function without binding calcium? *Cell* **65,** 949–959.
71. Davis, T. N. (1995) Calcium in *Saccharomyces cerevisiae. Advances in Second Messenger and Phosphoprotein Res.* **30,** 339–358.
72. King, M. M. and Huang, C. Y. (1984) The calmodulin-dependent activation and deactivation of the phosphoprotein phosphatase, calcineurin, and the effect of nucleotides, pyrophosphate, and divalent metal ions. *J. Biol. Chem.* **259,** 8847–8856.
73. Hashimoto, Y., King, M. M., and Soderling, T. R. (1988) Regulatory interactions of calmodulin-binding proteins: Phosphorylation of calcineurin by autophosphorylated Ca^{2+}/calmodulin-dependent protein kinase II. *Proc. Natl. Acad. Sci. USA* **85,** 7001–7005.

74. Hashimoto, Y. and Soderling, T. R. (1989) Regulation of calcineurin by phosphorylation. *J. Biol. Chem.* **264,** 16,524–16,529.
75. Huang, S. and Cheung, W. Y. (1994) H^+ is involved in the activation of calcineurin by calmodulin. *J. Biol. Chem.* **269,** 22,067–22,074.
76. Liu, Y., Ishii, S., Tokai, M., Tsutsumi, H., Ohki, O., Akade, R., Tanaka, K., Tsuchiya, E., Fukui, S., and Miyakawa, T. (1991) The *Saccharomyces cerevisiae* genes (*CMP1* and *CMP2*) encoding calmodulin-binding proteins homologous to the catalytic subunit of mammalian protein phosphatase 2B. *Mol. Gen. Gene.* **227,** 52–59.
77. Plochocka-Zulinska, D., Rasmussen, G., and Rasmussen, C. (1995) Regulation of calcineurin gene expression in *Schizosaccharomyces pombe. J. Biol. Chem.* **270,** 24,794–24,799.
78. Friedman, J. and Weissman, I. (1991) Two cytoplasmic candidates for immunophilin action are revealed by affinity for a new cyclophilin: one in the presence and one in the absence of CsA. *Cell* **66,** 799–806.
78a. Braun, W., Kallen, J., Mikol, V., Walkinshaw, M. D., and Wuthrich, K. (1995) Three-dimensional structure and actions of immunosuppressants and their immunophilins. *FASEB J.* **9,** 63–72.
79. Clipstone, N. A., Fiorentino, D. F., and Crabtree, D. R. (1994) Molecular analysis of the interaction of calcineurin with drug-immunophilin complexes. *J. Biol. Chem.* **269,** 26,431–26,437.
80. Haddy, A., Swanson, S. K.-H., Born, T. L., and Rusnak, F. (1992) Inhibition of calcineurin by cyclosporin A-cyclophilin requires calcineurin B. *FEBS Lett.* **314,** 37–40.
81. Li, W. and Handschumacher, R. E. (1993) Specific interaction of the cyclophilin-cyclosporin complex with the B subunit of calcineurin. *J. Biol. Chem.* **268,** 14,040–14,044.
82. Milan, D., Griffith, J., Su, M., Price, E. R., and McKeon, F. (1994) The latch region of calcineurin B is involved in both immunosuppressant-immunophilin complex docking and phosphatase activation. *Cell* **79,** 437–447.
83. Cardenas, M., Muir, R. S., Breuder, T., and Heitman, J. (1995) Targets of immunophilin-immunosuppressant complexes are distinct highly conserved regions of calcineurin A. *EMBO J.* **14,** 2772–2783.
84. Etzkorn, F. A., Chang, Z. Y., Stolz, L. A., and Walsh, C. T. (1994) Cyclophilin residues that affect noncompetitive inhibition of the protein serine phosphatase activity of calcineurin by the cyclophilin-cyclosporin A complex. *Biochemistry* **33,** 2380–2388.
85. Bell, J. E. and Bell, E. T. (1988) *Proteins and Enzymes.* Prentice-Hall, Englewood Cliffs, NJ, pp. 325–331.
86. Wesselborg, S., Furman, D. A., Sagoo, J. K., Bierer, B. E., and Burakoff, S. J. (1996) Identification of a physical interaction between

calcineurin and nuclear factor of activated T cells (NFATp). *J. Biol. Chem.* **271,** 1274–1277.

87. Cohen, P. (1988) Protein phosphorylation and hormone action. *Proc. R. Soc. Lond.* **B234,** 115–144.
88. Mulkey, R. M., Endo, S., Shenolikar, S., and Malenka, R. C. (1994) Involement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. *Nature* **369,** 486–488.
89. Sharma, R. and Wang, J. H. (1985) Differential regulation of bovine brain calmodulin- dependent cyclic nucleotide phosphodiesterase isozymes by cyclic AMP-dependent protein kinase and calmodulin-dependent phosphatase. *Proc. Natl. Acad. Sci. USA* **82,** 2603–2607.
90. Sharma, R. K. and Wang, J. H. (1986) Calmodulin and Ca^{2+}-dependent phosphorylation and dephosphorylation of 63-kDa subunit-containing bovine brain calmodulin-stimulated cyclic nucleotide phosphodiesterase isozyme. *J. Biol. Chem.* **261,** 1322–1328.
91. Coghlan, V. M., Perrino, B. A., Howard, M., Langeberg, L. K., Hicks, J. B., Gallatin, W. M., and Scott, J. D. (1995) Association of protein kinase A and protein phosphatase 2B with a common anchoring protein. *Science* **267,** 108–111.
92. Lisman, J. (1989) A mechanism for the Hebb and the anti-Hebb processes underlying learning and memory. *Proc. Natl. Acad. Sci. USA* **86,** 9574–9578.
93. Dawson, T. M., Steiner, J. P., Dawson, V. L., Dinerman, J. L., Uhl, G. R., and Snyder, S. H. (1993) Immunosuppresant FK506 enhances phosphorylation of nitric oxide synthase and protects against glutamate neurotoxicity. *Proc. Natl. Acad. Sci. USA* **90,** 9808–9812.
94. Burkart, V., Imai, Y., Kallmann, B., and Kolb, H. (1992) Cyclosporin A protects pancreatic islet cells from nitric oxide-dependent macrophage cytotoxicity. *FEBS.* **313,** 56–58.
95. Halpain, S., Girualt, J.-A., and Greengard, P. (1990) Activation of NMDA receptors induces dephosphorylation of DARPP-32 in rat striatal slices. *Nature* **343,** 369–372.
96. Ferreira, A., Kincaid, R., and Kosik, K. S. (1993) Calcineurin is associated with the cytoskeleton of cultured neurons and has a role in the acquisition of polarity. *Mol. Biol. Cell.* **4,** 1225–1238.
97. Fleming, L. M. and Johnson, G. V. W. (1995) Modulation of the phosphorylation state of tau *in situ*: the roles of calcium and cyclic AMP. *Biochem. J.* **309,** 41–47.
98. Fraser, E. D. and Walsh, M. P. (1995) Dephosphorylation of calponin by type 2B protein phosphatase. *Biochemistry* **34,** 9151–9158.
99. Lieberman, D. N. and Mody, I. (1994) Regulation of NMDA channel function by endogenous Ca^{2+} -dependent phosphatase. *Nature* **369,** 235–239.

100. Cameron, A. M., Steiner, J. P., Roskams, A. J., Ali, S. M., Ronnett, G. V., and Snyder, S. H. (1995) Calcineurin associated with the inositol 1,4,5-trisphophate receptor-FKBP12 complex modulates Ca^{2+} flux. *Cell* **83,** 463–472.
101. Brillantes, A.-M. Ondrias, B. K., Scott, A., Kobrinsky, E., Ondriasova, E., Moschella, M. C., Jayaraman, T., Landers, M., Ehrlich, B. E., and Marks, A. R. (1994) Stabilization of calcium release channel (ryanodine receptor) function by FK506-binding protein. *Cell* **77,** 513–523.
102. Cameron, A. M., Steiner, J. P., Sabatini, D., Kaplin, A. I., Walensky, J. D., and Snyder, S. H. (1995) Immunophilin FK506 binding protein associated with inositol 1,4,5-trisphosphate receptor modulates calcium flux. *Proc. Natl. Acad. Sci. USA* **92,** 1784–1788.
103. Jayaraman, T., Brillantes, A.-M., Timerman, A. P., Fleischer, S., Erdjument-Bromage, H., Tempst, P., and Marks, A. R. (1992) FK506 binding protein associated with the calcium release channel (ryanodine receptor). *J. Biol. Chem.* **267,** 9474–9477.
104. Aperia, A., Ibarra, F., Svensson, L.-B., Klee, C. B., and Greengard, P. (1992) Calcineurin mediates α-adrenergic stimulation of Na^+, K^+-ATPase activity in renal tubule cells. *Proc. Natl. Acad. Sci. USA* **89,** 7394–7397.
105. Lea, J. P., Sands, J. M., McMahon, S. J., and Tumlin, J. A. (1994) Evidence that the inhibition of Na^+/K^+-ATPase activity by FK506 involves calcineurin. *Kidney Int.* **46,** 647–652.
106. Marcaida, G., Kosenko, E., Minana, M.-D., Grisolia, S., and Felipo, V. (1996) Glutamate induces a calcineurin-mediated dephosphorylation of Na^+, K^+-ATPase that results in its activation in cerebellar neurons in culture. *J. Neurochem.* **66,** 99–104.
107. Luan, S., Li, W., Rusnak, F., Assmann, S. M., and Schreiber, S. L. (1993) Immunosuppressants implicate protein phosphatase regulation of K^+ channels in guard cells. *Proc. Natl. Acad. Sci. USA* **90,** 2202–2206.
108. Antoni, F. A., Shipston, M. J., and Smith, S. M. (1993) Inhibitory role for calcineurin in stimulus-secretion coupling revealed by FK506 and cyclosporin A in pituitary corticotrope tumor cells. *Biochem. Biophys. Res. Comm.* **194,** 226–233.
109. Hultsch, T., Rodriguez, J. L., Kaliner, M. A., and Hohman, R. J. (1990) Cyclosporin A inhibits degranulation of rat basophilic leukemia cells and human basophils. *J. Immunology.* **144,** 2659–2664.
110. Momayezi, M., Lumpert, C. J., Kersken, H., Gras, U., Plattner, H., Krinks, M. H., and Klee, C. B. (1987) Exocytosis induction in *Paramecium tetraurelia* cells by exogenous phosphoprotein phosphatase in vivo and in vitro: possible involvement of calcineurin in exocytotic membrane fusion. *J. Cell Biol.* **105,** 181–189.

111. Lawson, M. A. and Maxfield, F. R. (1995) Ca^{2+}-and calcineurin-dependent recycling of an integrin to the front of migrating neutrophils. *Nature* **377,** 75–79.
112. Fruman, D. A., Klee, C. B., Bierer, B. E., and Burakoff, S. J. (1992) Calcineurin phosphatase activity in T lymphocytes is inhibited by FK506 and cyclosporin A. *Proc. Natl. Acad. Sci. USA* **89,** 3686–3690.
113. Shibasaki, F., Kondo, E., Akagi, T., and McKeon, F. (1997) Suppression of signalling through transcription factor NF-AT by interactions between calcineurin and Bcl-2. *Nature* **386,** 728–731.
114. Shibasaki, F. and McKeon, F. (1995) Calcineurin functions in Ca^{2+} -activated cell death in mammalian cells. *J. Cell Biol.* **131,** 735–743.
115. Wolvetang, E. J., Larm, J. A., Moutsoulas, P., and Lawen, A. (1996) Apoptosis induced by inhibitors of the plasma membrane NADH-oxidase involves Bcl-2 and calcineurin. *Cell Growth Different.* **7,** 1315–1325.
116. Crabtree, G. R. (1989) Contingent genetic regulatory events in T lymphocyte activation. *Science* **243,** 355–361.
117. Shaw, J.-P., Utz, P. J., Durand, D. B., Toole, J. J., Emmel, E. A., and Crabtree, G. R. (1988) Identification of a putative regulator of early T cell activation genes. *Science* **241,** 202–205.
118. Flanagan, W. M., Corthésy, B., Bram, R. J., and Crabtree, G. R. (1991) Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. *Nature* **352,** 803–807.
119. Jain, J., McCaffrey, P. G., Valge-Archer, V. E., and Rao, A. (1992) Nuclear factor of activated T cells contains Fos and Jun. *Nature* **356,** 801–804.
120. McCaffrey, P. G., Perrino, B. A., Soderling, T. R., and Rao, A. (1993) NF-ATp, a T lymphocyte DNA-binding protein that is a target for calcineurin and immunosuppressive drugs. *J. Biol. Chem.* **268,** 3747–3752.
121. Jain, J., McCaffrey, P. G., Miner, Z., Kerppola, T. K., Lambert, J. N., Verdine, G. L., Curran, T., and Rao, A. (1993) The T-cell transcription factor $NFAT_p$ is a substrate for calcineurin and interacts with Fos and Jun. *Nature* **365,** 352–355.
122. Loh, C., Shaw, K. T.-Y., Carew, J.,Viola, J. P. V., Luo, C., Perrino, B., and Rao, A. (1996) Calcineurin binds the transcription factor NFAT1 and reversibly regulates its activity. *J. Biol. Chem.* **271,** 10,884–10,891.
123. Ruff, V. A. and Leach, K. L. (1995) Direct demonstration of $NFAT_p$ dephosphorylation and nuclear localization in activated HT-2 cells using a specific $NFAT_p$ polyclonal antibody. *J. Biol. Chem.* **270,** 22,602–22,607.
124. Shaw, K. T.-Y., Ho, A. M., Raghavan, A., J. Kim, J., Jain, J., Park, J., Sharma, S., Rao, A., and Hogan, P. G. (1995) Immunosuppressive

drugs prevent a rapid dephosphorylation of transcription factor NFAT1 in stimulated immune cells. *Proc. Natl. Acad. Sci. USA* **92,** 11,205–11,209.

125. Shibasaki, F., Price, E. R., Milan, D., and McKeon, F. (1996) Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4. *Nature* **382,** 370–373.
126. Beals, C. R., Sheridan, C. M., Turck, C. W., Gardner, P., and Crabtree, G. R. (1997) Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. *Science* **275,** 1930–1933.
127. Park, J., Yaseen, N. R., Hogan, P. G., Rao, A., and Sharma, S. (1995) Phosphorylation of the transcription factor NFATp inhibits its DNA binding activity in cyclosporin A-treated human B and T cells. *J. Biol. Chem.* **270,** 20,653–20,659.
128. Frantz, B., Nordby, E. C., Bren, G., Steffan, N., Paya, C. V., Kincaid, R. L., Tocci, M. J., O'Keefe, S. J., and O'Neill, E. A. (1994) Calcineurin acts in synergy with PMA to inactivate IkB/MAD3, an inhibitor of NF-kB. *EMBO J.* **13,** 861–870.
129. Kubo, M., Kincaid, R. L., and Ransom, J. T. (1994) Activation of the interleukin-4 gene is controlled by the unique calcineurin-dependent transcriptional factor NF(P)*. *J. Biol. Chem.* **269,** 19,441–19,446.
130. Tsuboi, A., Masuda, E. S., Naito, Y., Tokumitsu, H., Arai, K.-I., and Arai, N. (1994) Calcineurin potentiates activation of the granulocyte-macrophage colony-stimulating factor gene in T cells: involvement of the conserved lymphokine element 0. *Mol. Biol. Cell.* **5,** 119–128.
131. Yoshida, T., Toda, T., and Yanagida, M. (1994) A calcineurin-like gene *ppb1*$^+$ in fission yeast: mutant defects in cytokinesis, cell polarity, mating and spindle pole body positioning. *J. Cell Sci.* **107,** 1725–1735.
132. Odom, A., Poeta, M. D., Perfect, J., and Heitman, J. (1997) The immunosuppressant FK506 and its nonimmunosuppressive analog L-685,818 are toxic to *Cryptococcus neoformans* by inhibition of a common target protein. *Antimicrob. Agents Chemother.* **41,** 156–161.
133. Fracasanu, I. C., Hirata, D., Tsuchiya, B., Nishiyama, F., and Miyakawa, T. (1995) Protein phosphatase 2B of *Saccharomyces cerevisiae* is required for tolerance to manganese, in blocking the entry of ions into the cells. *Eur. J. Biochem.* **232,** 712–717.
134. Hirata, D., Harada, S.-I., Namba, H., and Miyakawa, T. (1995) Adaptation to high-salt stress in *Saccharomyces cerevisiae* is regulated by Ca^{2+}/calmodulin-dependent phosphoprotein phosphatase (calcineurin) and cAMP-dependent protein kinase. *Mol. Gen. Genet.* **249,** 257–264.

135. Marquez, J. A. and Serrano, R. (1996) Multiple transduction pathways regulate the sodium- extrusion gene *PMR2/ENA1* during salt stress in yeast. *FEBS Lett.* **382,** 89–92.
136. Douglas, C. M., Foor, F., Marrinan, J. A., Morin, N., Nielsen, J. B., Dahl, A. M., Mazur, P., Baginsky, W., Li, W., El-Sherbeini, M., Clemas, J. A., Mandala, S. M., Frommer, M. B., and Kurtz, M. B. (1994) The *Saccharomyces cerevisiae FKS1 (ETG1)* gene encodes an integral membrane protein which is a subunit of 1,3-β-D-glucan synthase. *Proc. Natl. Acad. Sci. USA* **91,** 12,907–12,911.
137. Eng, W.-K., Faucette, L., McLaughlin, M. M., Cafferkey, R., Koltin, Y., Morris, R. A., Young, P. R., Johnson, R. K., and Livi, G. P. (1994) The yeast *FKS1* gene encodes a novel membrane protein, mutations in which confer FK506 and cyclosporin A hypersensitivity and calcineurin- dependent growth. *Gene* **151,** 61–71.
138. Mazur, P., Morin, N., Baginsky, W., El-Sherbeini, M., Clemas, J. A., Nielsen, J. B., and Foor, F. (1995) Differential expression and function of two homologous subunits of yeast 1,3-β-D-glucan synthase. *Mol. Cell. Biol.* **15,** 5671–5681.
139. Moser, M. J., Geiser, J. R., and Davis, T. N. (1996) Ca^{2+}-calmodulin promotes survival of pheromone-induced growth arrest by activation of calcineurin and Ca^{2+}-calmodulin-dependent protein kinase. *Mol. Cell. Biol.* **16,** 4824–4831.
140. Garrett-Engele, P., Moilanen, B., and Cyert, M. S. (1995) Calcineurin, the Ca^{2+}/calmodulin dependent protein phosphatase, is essential in yeast mutants with cell integrity defects and in mutants that lack a functional vacuolar H^+-ATPase. *Mol. Cell Biol.* **15,** 4103–4114.
141. Tanida, I., Hasegawa, A., Iida, H., Ohya, K. Y., and Anraku, Y. (1995) Cooperation of calcineurin and vacuolar H^+-ATPase in intracellular Ca^{2+} homeostasis of yeast cells. *J. Biol. Chem* . **270,** 10,113–10,119.
142. Cunningham, K. W. and Fink, G. R. (1994) Calcineurin-dependent growth control in *Saccharomyces cerevisiae* mutants lacking PMC1, a homolog of plasma membrane Ca^{2+} ATPases. *J. Cell Biol.* **124,** 351–363.
143. Cunningham, K. W. and Fink, G. R. (1996) Calcineurin inhibits VCX1-dependent H^+/Ca^{2+} exchange and induces Ca^{2+} ATPases in *Saccharomyces cerevisiae. Mol. Cell. Biol.* **16,** 2226–2237.
144. Pozos, T. C., Sekler, I., and Cyert, M. S. (1996) The product of *HUM1*, a novel yeast gene, is required for vacuolar Ca^{2+}/H^+ exchange and is related to mammalian Na^+/Ca^{2+} exchangers. *Mol. Cell. Biol.* **16,** 3730–3741.
145. Tanida, I., Takita, Y., Hasegawa, A., Ohya, Y., and Anraku, H. (1996) Yeast Cls2p/Csg2p localized on the endoplasmic reticulum membrane regulates a non-exchangeable intracellular Ca^{2+} pool cooperatively with calcineurin. *FEBS Lett.* **379,** 38–42.