Overexpression and Purification of Human Calcineurin α from *Escherichia coli* and Assessment of Catalytic Functions of Residues Surrounding the Binuclear Metal Center†

Angelo Mondragon,‡,§ Eric C. Griffith,‡,§ Luo Sun,‡ Fei Xiong,‡ Christopher Armstrong,‡,§ and Jun O. Liu*,‡,§,||

Center for Cancer Research and Departments of Biology and Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received December 30, 1996; Revised Manuscript Received February 19, 1997⊗

ABSTRACT: Calcineurin is an important signal-transducing enzyme in many cell types including T lymphocytes and is a common target for the immunosuppressants cyclosporin A and FK506. The crystal structures of both calcineurin [Griffith et al. (1995) *Cell 82*, 507−522; Kissinger et al. (1995) *Nature 378*, 641−644] and a related enzyme, protein phosphatase-1 [Goldberg et al. (1995) *Nature 376*, 745−753], revealed that this class of serine/threonine phosphatases contain in their putative active sites a binuclear metal center formed by an Asn, two Asp, and three His residues. In addition, one His and two Arg residues lie in close vicinity of the binuclear metal centers. The importance of the binuclear metal center and its surrounding residues in catalysis by calcineurin has not been investigated experimentally. Herein, we report an efficient bacterial expression and purification system for human calcineurin α. Using this system, a systematic alanine-scan mutagenesis on the residues surrounding the putative active site was performed. It was found that an intact binuclear metal center is essential for the catalytic activity of the enzyme. In addition, His151, Arg122, and Arg254 also exhibited either a loss or a dramatic decrease in catalytic activity upon mutation into alanines. Interestingly, the Arg254Ala mutant retained a small but significant amount of catalytic activity toward the small substrate *p*-nitrophenyl phosphate, but is completely inactive toward a phosphopeptide substrate, suggesting that this arginine may be involved in the binding of phosphoprotein substrates as well as in catalysis. As all the residues in the putative active site are conserved between different eukaryotic serine/threonine phosphatases, these results should apply to all members of this family of protein phosphatases.

Calcineurin, or protein phosphatase-2B (PP-2B),[1] plays a key role in several intracellular signal transduction pathways, the most notable of which is the T cell receptor-mediated signal transduction in T lymphocytes (Schreiber & Crabtree, 1992; Crabtree & Clipstone, 1994). It is also the common molecular target for two clinically important immunosuppressive drugs, cyclosporin A (CsA) and FK506 (Liu et al., 1991; Friedman & Weissman, 1991). Extensive pharmacological and cell biological evidence has accumulated supporting the notions that calcineurin is a rate-limiting intracellular signaling enzyme involved in T cell receptor-mediated signal transduction (Clipstone & Crabtree, 1992; O'Keefe et al., 1992) and that CsA and FK506 block T cell activation by inhibiting the phosphatase activity of calcineurin (Liu et al., 1992; Nelson et al., 1993). In addition to its function in TCR-mediated signal transduction, calcineurin has also been implicated in various signal transduction pathways in neuronal (Stemmer & Klee, 1991) and kidney cells (Dumont et al., 1992; Aperia et al., 1992; Lea et al., 1994), responsible for the neurotoxic and nephrotoxic side effects of CsA and FK506. Calcineurin is highly conserved among eukaryotes from human to yeast; studies of yeast calcineurin and immunophilins have contributed substantially to our current understanding of the structure and function of calcineurin (Cyert et al., 1991; Foor et al., 1992; Cunningham & Fink, 1994; Cardenas et al., 1994). It has been shown that inhibition of calcineurin by immunophilin−immunosuppressant complexes leads to the cytotoxicity in CsA- and FK506-sensitive strains of yeast *Saccharomyces cerevisiae* (Breuder et al., 1994; Parent et al., 1993). Since both CsA and FK506 have severe side effects, new immunosuppressive agents of lower toxicity are needed. An understanding of the molecular mechanism of catalysis by calcineurin and its interactions with substrates is therefore essential for uncovering new signaling substrates of calcineurin that may serve as new molecular targets for designing more specific and less toxic immunosuppressants.

Calcineurin belongs to a family of serine/threonine phosphatases including PP-1 and PP2A (Klee et al., 1988; Cohen, P., & Cohen, 1989; Shenolikar, 1994). Another member thought to belong to this family is the bacteriophage λ phosphatase (Cohen, P. T. W., & Cohen, 1989). Calcineurin

† This work was supported by a Start-up Fund from the Center for Cancer Research, Massachusetts Institute of Technology, the Chicago Community Trust and the Rita Allen Foundation (to J.O.L.). A predoctoral fellowship from the Ford Foundation (to A.M.) is gratefully acknowledged. J.O.L. is a Pfizer−Laubach Career Development Assistant Professor of Biology and Chemistry.

* Correspondence should be addressed to this author at the Center for Cancer Research, MIT, 77 Massachusetts Ave., E17-128D, Cambridge, MA 02139. Telephone: 617-253-5750. Fax: 617-258-6172. Email: junliu@mit.edu.

‡ Center for Cancer Research, MIT.

§ Department of Biology, MIT.

|| Department of Chemistry, MIT.

⊗ Abstract published in *Advance ACS Abstracts,* April 1, 1997.

[1] Abbreviations: CN, calcineurin; CNAα, α isoform of calcineurin A subunit; CNα, holoenzyme of calcineurin Aα and calcineurin B; CaM, calmodulin; PNPP, *p*-nitrophenyl phosphate; DTT, dithiothreitol; PP-1, protein phosphatase-1; PP-2A, protein phosphatase-2A; PP-2B, protein phosphatase-2B.

S0006-2960(96)03193-5 CCC: $14.00

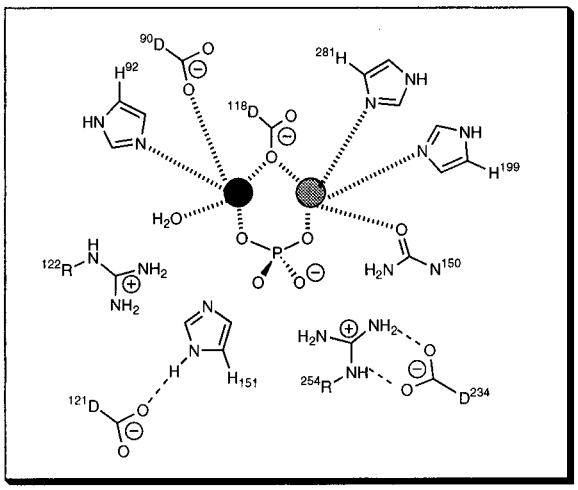

FIGURE 1: Diagram of the binuclear metal center of the active site of calcineurin α. The distances of the different residues to the metal ions are not drawn to scale. The two metal ions are represented by a black (putative Fe^{3+}) and a shaded (putative Zn^{2+}) ball, respectively. This is adapted from Griffith et al. (1995).

is distinct from other members of this phosphatase family in that its activity is modulated by calcium and calmodulin (Klee et al., 1988). This regulation is made possible by the interaction between the catalytic A subunit and the regulatory B subunit of the enzyme and the presence in the A subunit of calcineurin of several domains in addition to the catalytic domain, i.e., the calcineurin B binding domain (Guerini & Klee, 1989; Sikkink et al., 1995), the calmodulin (CaM) binding domain, and an autoinhibitory domain (Kincaid et al., 1988; Hubbard & Klee, 1989; Hashimoto et al., 1990). The CaM binding domain and the autoinhibitory domain together serve as a calcium-sensing "on-and-off" switch for the phosphatase activity of calcineurin. The B subunit of calcineurin appears to be involved in both substrate binding and catalysis (Stemmer & Klee, 1994; Perrino et al., 1995).

The mechanism of catalysis by the protein serine/threonine phosphatases in general, and by calcineurin in particular, remained obscure for many years. Early efforts to detect covalent phosphoenzyme intermediates were unsuccessful, suggesting that calcineurin catalysis does not involve tandem phosphoryl transfer from the substrate to the enzyme and subsequent transfer from the enzyme to water (Martin & Graves, 1986). Several pieces of evidence suggested that metal ions other than calcium may play important roles in catalysis. Metal analysis of calcineurin purified from bovine brain revealed that it contains 1 molar equiv each of Zn^{2+} and Fe^{3+} (King & Huang, 1984). This result was recently confirmed by EPR spectroscopy (Qin et al., 1995; Yu et al., 1995), but how these metal ions participated in catalysis was unclear. Recently, the crystal structures of truncated bovine brain and full-length human calcineurin (α isoform) revealed that the catalytic domain of calcineurin A subunit contains a binuclear metal center located at the bottom of a putative polypeptide substrate binding groove (Griffith et al., 1995; Kissinger et al., 1995). The side chains of an asparagine, two aspartic acids, and three histidines were shown to be involved in metal ion binding (Figure 1). In addition, one Asp-His diad and two Arg residues were located next to the binuclear metal center. Strikingly, an identical binuclear metal center, along with the Asp-His diad and the two arginines, was found in PP-1 (Goldberg et al., 1995). Based on these structures, slightly different versions of a catalytic mechanism were proposed (Griffith et al., 1995; Goldberg et al., 1995). It was suggested that the binuclear metal center serves to bind the negatively charged phosphate, neutralizing its charge and facilitating the attack on the phosphate by a metal ion-bound water molecule. The Asp-His diad was proposed to serve as either a general acid, protonating the leaving alcoholic side chains of serine or threonine (Griffith et al., 1995; Goldberg et al., 1995), or a general base, deprotonating an iron-bound water molecule for nucleophilic attack on the phosphate (Kissinger et al., 1995). A third possible role for the Asp-His pair, though less likely, is to serve as a nucleophile to attack the phosphate directly, forming a phosphoenzyme intermediate (Goldberg et al., 1995). The two arginines were proposed to neutralize the developing negative charge on the oxygen of the phosphate during formation of a pentavalent transition state (Griffith et al., 1995; Goldberg et al., 1995). None of these hypotheses, however, have been tested experimentally.

Despite their evolutionary distance, the bacteriophage λ phosphatase possesses significant homology to the eukaryotic enzymes (Cohen, P. T. W., & Cohen, 1989) and was used as a model to perform structure/function studies on this family of serine/threonine phosphatases prior to the availability of structural information (Zhou et al., 1993, 1994). Like its eukaryotic counterparts, the λ phosphatase is also dependent on metal ions for its activity, suggesting a conservation of the catalytic mechanism. Indeed, several amino acid residues that are conserved among all members of this family were found by site-directed mutagenesis to be essential for both metal ion binding and catalysis in the λ phosphatase (Zhou et al., 1994). These residues correspond to Asp90 (Asp20 in λ phosphatase), His92 (λHis22), Asp118 (λAsp49) involved in metal ion coordination, and His151 (λHis76) and Arg122 (λArg53) in the vicinity of the binuclear metal center of human calcineurin Aα. However, the residues forming the putative Zn^{2+} binding site (Asn151, His199, and His281) in calcineurin were not mutated in the λ phosphatase due in part to the lack of apparent conservation of the two histidines between the λ phosphatase and other members of this family (Cohen, P. T. W., & Cohen, 1989; Zhou et al., 1993), leaving unanswered the question of how important these residues are for the catalytic activity of these enzymes. Although realignment of sequences of the λ phosphatase and other members of this family did reveal the presence of two histidines in the λ phosphatase that may correspond to those in calcineurin and PP-1 (Lohse et al., 1995), the distances between each of the two histidines in the λ phosphatase and the rest of the conserved metal-coordinating residues are shorter than those in either calcineurin or PP-1. Furthermore, Arg254, located close to the binuclear metal center, is not found in the λ phosphatase, raising the question of whether this arginine plays any role in catalysis.

To assess the importance of the binuclear metal center and the surrounding residues in catalysis by calcineurin, we established an efficient bacterial expression system for the α isoform of human calcineurin and performed an Ala-scanning mutagenesis. We found that the binuclear metal center, His151, and Arg122 are indispensable for the catalytic activity of calcineurin. In contrast, most residues are not critical for maintaining the structural integrity of the enzyme as measured by interactions between the alanine mutants and the FKBP−FK506 complex.

EXPERIMENTAL PROCEDURES

Materials. All reagents used in molecular biology protocols, including restriction enzymes, were purchased from New England Biolabs (Beverley, MA) or Boehringer Mannheim (Indianapolis, IN). The PCR kit was from Perkin-Elmer Roche (Branchberg, NJ). The pET15b vector was from Novagene (Madison, WI). Oligonucleotides were purchased from Bio·Synthesis, Inc. (Lewisville, TX). The NuSieve low-melting agarose gel was from FMC Bioproducts (Rockland, ME). Talon metal affinity resin was purchased from Clontech (Palo Alto, CA). The calmodulin−Sepharose was from Pharmacia Biotech (Piscataway, NJ). All protease inhibitors were from Sigma (St. Louis, MO). The RII peptide was made by the Biopolymer Lab at MIT. *p*-Nitrophenyl phosphate was from Gibco BRL (Grand Island, NY). The MESG-coupled EnzCheck Phosphate kit was from Molecular Probes (Seattle, WA). The anti-calcineurin monoclonal antibody used in Western blotting was from Pharmingen (San Diego, CA).

*Construction of the Expression Vector pETCN*α *and the Expression E. coli Strain*. The expression cassette for CNAα was PCR-amplified from the vector using a 5′ primer (5′-ATCGCGGATCCCATATGTCCGAGCCCAAGGCA-3′) tagged with an *Nde*I (underlined) site and a 3′ primer (5′-GCATGCCTCGAGTCACTGAATATTGCTGCTA-3′) tagged with an *Xho*I (underlined) site. The expression cassette for CNB was PCR-amplified from reverse-transcribed mRNAs of Jurkat T cells using a 5′ primer (5′-ATCGCTCTCG*AG-GAG*ATATACATATGGGAAATGAGGCAAGTT-3′) tagged with an optimized Shine/Dalgarno sequence (in italics) and an *Xho*I (underlined) site, and a 3′ primer (5′-GCATGCCT-GCAGTCACACATCTACCACCATCTT-3′) tagged with a *Pst*I (underlined) site. To ensure the fidelity of the PCR reaction, a 1:8 mixture (in units) of Vent polymerase and Taq polymerase was used for all PCR amplifications. The CNB PCR fragment was first subcloned into pBluscript SK+ vector at the *Xho*I and *Pst*I sites. The resultant vector was digested with *Xho*I and *Bam*HI (derived from the polylinker region of pBluescript SK+) to yield a CNB expression cassette. The expression vector pET15b, the PCR fragments of CNAα digested with appropriate restriction enzymes, and the *Xho*I−*Bam*HI CNB expression cassette were purified from a NuSieve low-melting agarose gel. A three-fragment ligation followed by transformation and plasmid screening yielded a CNAα−CNB operon missing the N-terminal *Nde*I−*Nde*I fragment from CNAα, which was subsequently subcloned to give the desired expression vector pETCNα. After the sequences of CNAα and CNB were confirmed by sequencing, pETCNα was transformed into *E. coli* strain BL21(DE3/pLysS), and the transformants was subsequently transformed with pBB131 encoding the yeast myristoyl-CoA: protein *N*-myristoyltransferase (Duronio et al., 1990) to afford the strain BL21(pLysS)/pETCNα/pBB131.

Purification of Wild-Type Recombinant Human Calcineurin α. A single colony of the *E. coli* strain BL21-(pLysS)/pETCNα/pBB131 from a fresh plate (less than 1 week old) was inoculated into 10 mL of LB containing 100 μg/mL ampicillin and 50 μg/mL kanamycin (amp/kan) and incubated with shaking at 37 °C overnight and used to inoculate 1.5 L of LB (amp/kan). When A_{600} reached 1.0, IPTG and myristic acid were added to final concentrations of 0.6 mM and 0.2 mM, respectively. The culture was shaken at 37 °C for an additional 3 h. The cells were harvested by centrifugation at 4000*g* for 6 min and washed once with 30 mL of prechilled buffer A (25 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 2 mM EGTA, 2 mM EDTA, and 20 mM β-mercaptoethanol). All subsequent purification steps were carried out at 4 °C. The cell pellet was weighed, resuspended (at 5 mL/g of wet pellet) in lysis buffer B (buffer A + 1 mM PMSF, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 1 μg/mL pepstatin, and 2 μg/mL soybean trypsin inhibitor), and lysed by two passages through French press (15 000 psi at the orifice). The lysate was centrifuged at 20000*g* for 20 min. DNA was removed by mixing with protamine sulfate (final concentration of 0.2%, w/v) followed by centrifugation at 20000*g* for 20 min to yield a crude cell lysate which was subjected to the following purification steps.

(a) Ammonium Sulfate Precipitation. Ammonium sulfate powder was added in small batches to a stirred cell lysate to 45% saturation. The solution was stirred for another 20 min followed by centrifugation at 20000*g* for 20 min. The protein pellet was dissolved in 10 mL of buffer B and dialyzed against 3 L of buffer C (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 3 mM $MgSO_4$) for 16 h. The dialyzed protein solution was centrifuged at 20000*g* for 20 min to remove precipitates that formed during dialysis.

(b) Talon Affinity Chromatography. Two milliliters of Talon resin was washed in 12 mL of buffer C 3 times, each consisting of 5 min mixing followed by centrifugation in a tabletop centrifuge for 3 min at 1200*g*. To the washed Talon resin was added the dialyzed solution of proteins from (a) and incubated on a rotator for 30 min. The Talon resin was pelleted by centrifugation at 1200*g* for 3 min and washed sequentially with 2 × 12 mL of buffer C, 10 mL of 20 mM PIPES (pH 7.0)/100 mM NaCl, and 10 mL of 20 mM PIPES/100 mM NaCl (pH 5.6). During the last wash, the resin was transferred into a Bio-Rad Econo column. The 6xHis-tagged calcineurin was eluted by 10 mL of 50 mM imidazole in buffer C, and 1 mL fractions were collected. The amount and purity of recombinant calcineurin were analyzed by the absorbance at 280 nm and 12% SDS−PAGE. The fractions containing the highest amount of calcineurin were pooled and dialyzed against 3 L of buffer D (25 mM Tris-HCl, pH 7.4, 3 mM $MgSO_4$, 1 mM EGTA, and 0.5 mM DTT) overnight.

(c) CaM−Sepharose Chromatography (Perrino et al., 1995). Two milliliters of CaM−Sepharose was washed 3 times, each time with 12 mL of buffer E (25 mM Tris-HCl, pH 7.4, 3 mM $CaCl_2$, 0.1 mM EDTA, and 0.2 mM DTT) for 5 min. To the washed CaM−Sepharose was added dialyzed Talon affinity chromatography fractions and $CaCl_2$ to a final concentration of 3 mM, and the mixture was incubated on a rotator for 30 min. After centrifugation (1200*g* for 4 min) and removal of the supernatant, the CaM−Sepharose resin was washed sequentially with 12 mL of buffer E and 12 mL of buffer F (buffer E + 1 M NaCl) and 12 mL of buffer E. The CaM−Sepharose resin was loaded onto a column and eluted with 10 mL of buffer D, and 1-mL fractions were collected. After analysis of the purity of proteins on 12% SDS−PAGE, the fractions containing the purest recombinant calcineurin were pooled and dialyzed in buffer D containing 20% glycerol. The enzyme was stored at −20 °C before used for further characterization.

(d) Determination of the Amino-Terminal Sequence of Recombinant Human Calcineurin α. The purified cal-

cineurin α was dialyzed extensively against H_2O in a P-10 Centricon. The amino-terminal sequence of the calcineurin holoenzyme (with both A and B subunits) was determined by Edman degradation at the Biopolymer Lab at MIT.

Generation and Purification of Active Site Mutants of Recombinant Human Calcineurin α. All calcineurin mutants were created by a tandem PCR procedure using a single mutagenic primer (Sarker & Sommer, 1990; Liu et al., 1991b). The mutagenic primers used were (mutated nucleotides are underlined) as follows: D90A, 5′-GTTTGTGGGGCCATTCATGG-3′; H92A, 5′-GGGGACATTGCTGGACAATTC-3′; D118A, 5′-TTCTTAGGGGCCTATGTTGAC-3′; R122A, 5′-CTATGTTGACGCAGGGTACTTC-3′; N150A, 5′-CTTCGTGGAGCTCATGAATG-3′; H151A, 5′-CGTGGAAATGCTGAATGTAG-3′; H199A, 5′-CTGTGTGTGGCTGGTGGTTTG-3′; H281A, 5′-CTCCGAGCCGCCGAAGCCC-3′; R254A, 5′-CAACACAGTCGCGGGGTGTTC-3′. To simplify the subsequent subcloning, pETCNα was used as a template, and the T7 primer hybridized to a region upstream of the T7 promoter was used together with the 3′ primer for the wild-type enzyme. The PCR fragments were digested with *Nco*I and *Kpn*I and subcloned into pETCNα digested with the same restriction enzymes to replace the *Nco*I−*Kpn*I fragment in the wild-type CNAα. The sequence of each mutant was verified by sequencing. The mutant expression vectors were transformed into BL21(DE3, pLysS) followed by transformation with pBB131, induced with 0.6 mM IPTG in the presence of myristic acid, and purified in a manner similar to the wild-type enzymes.

Determination of k_{cat} and K_m of Wild-Type and Mutant Enzymes. Kinetic constants (k_{cat} and K_m) were measured for both the small-molecule substrate *p*-nitrophenyl phosphate (PNPP) and the phosphorylated RII peptide. Phosphatase assays for both substrates were carried out in a buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1.5 mM $CaCl_2$, 6 mM $MgCl_2$, 0.5 mg/mL BSA, 1 mM dithiothreitol (DTT), and 67 μg/mL CaM in a final volume of 1 mL. For PNPP assays, varying concentrations of calcineurin were preincubated in the buffer for 5 min at 30 °C. The reaction was initiated by addition of PNPP stock solution (in H_2O) to final concentrations ranging from 20 to 80 mM. The progress of the reaction was followed at 410 nm using a Hewlett-Packard Diode Array UV spectrophotometer at 30 °C. The data were collected every 0.5 s for a total of 60−90 s and were iteratively fitted with a first-order rate constant by using the HP kinetic program. A molar extinction coefficient of 17 800 M^{-1} cm^{-1} was used to convert the absorbance values into molar concentrations. PhosphoRII peptide substrate was prepared and purified by HPLC as previously described (Ezkorn et al., 1995). A coupled assay was used with phosphate release followed by the method of Webb (1992) using the EnzCheck Phosphate Assay kit according to the manufacturer's instructions, except that a 5× reaction buffer was used with the buffer and water volumes were adjusted accordingly. The concentrations of phospho-RII peptide used ranged from 20 to 120 μM.

Determination of BD_{50} of Wild-Type and Mutant Calcineurin for the FKBP−FK506 Complex. To a 950 μL solution containing recombinant wild-type or mutant calcineurin (4 μg/mL) and GST−FKBP [1 μM; purified as previously described in Liu et al. (1991a)] in buffer G (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1.5 mM $CaCl_2$, 6 mM $MgCl_2$, and 0.1 mg/mL BSA) was added FK506 to final concentrations ranging from 0 to 100 μM. After incubation at 4 °C for 20 min, 50 μL of a glutathione−Sepharose bead suspension in buffer G (1:1) was added to the tube, and incubation at 4 °C was continued for an additional 20 min. The beads were pelleted at 10000*g* for 20 s and washed twice with buffer G. The beads were boiled in 20 μL of 1 × SDS protein loading buffer, and the same volume of binding solution from each tube was subjected to 10% SDS−PAGE followed by blotting onto a nitrocellulose membrane. Calcineurin was detected by a monoclonal anti-calcineurin antibody and anti-mouse IgG coupled to horseradish peroxidase using the ECL system per manufacturer's instructions. The film was digitized using DeskScan II software, and the calcineurin band was quantitated using NIH Image 1.60. The data were fitted to the Hill equation using DeltaGraph Pro 3.5. Apparent BD_{50} values were defined as the FK506 concentrations at which 50% of the immobilized FKBP−FK506 complex is bound by calcineurin or its mutants.

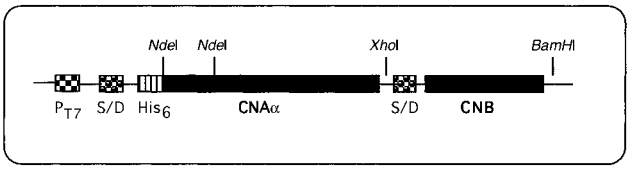

FIGURE 2: Configuration of the tandem expression construct pETCNα. Restriction sites used in vector construction are shown at the top, and the sequence features are indicated below. Abbreviations: P_{T7}, bacteriophate T7 promoter; S/D, Shine/Dalgarno sequence; His6, the 6xHis tag.

RESULTS

Overexpression and Purification of Human Calcineurin α from E. coli. Previous efforts to express mammalian calcineurin in a heterologous system, particularly in bacteria, have met with limited success. Calcineurin from rat has been expressed in baculovirus-infected Sf9 insect cells and purified to near-homogeneity (Perrino et al., 1992, 1995). This system suffered from the incomplete myristoylation of the recombinant B subunit, yielding a mixture of calcineurin B with both N-myristoylated and nonmyristoylated forms (Perrino et al., 1995). The rat calcineurin A subunit has also been expressed in *E. coli*, but was found to be extremely unstable with the majority trapped in inclusion bodies; the soluble recombinant rat calcineurin Aα had low phosphatase activity (Haddy & Rusnak, 1994). Upon addition of exogenous calcineurin B, the specific activity increased, but only to ca. 30% that of native calcineurin.

Our strategy was to coexpress both A and B subunits of calcineurin, since it is known that the A subunit of calcineurin is unstable in the absence of the B subunit (Klee, 1988). We designed an artificial operon consisting of both calcineurin Aα and calcineurin B cDNAs in tandem (Figure 2). To facilitate purification of the recombinant protein, we used the pET15b expression vector so that the recombinant calcineurin Aα contained at its N-terminus a poly-His tag which could be used to purify recombinant calcineurin by metal affinity chromatography. Both calcineurin Aα and calcineurin B subunits were amplified by PCR using primers tagged with unique restriction sites and expression signals

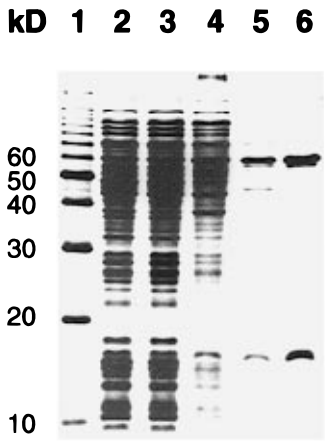

FIGURE 3: Recombinant human calcineurin α at different stages of purification. Lanes 1, molecular mass markers; 2, crude bacterial lysate; 3, supernatant after precipitation of nucleic acids with 0.4% protamine sulfate; 4, precipitate with 45% ammonium sulfate; 5, eluate from Talon metal affinity column; 6, eluate from calmodulin−Sepharose column. Samples were subjected to SDS−PAGE (12%), and the gel was stained with Coomassie blue before it was photographed.

(Figure 2 and Experimental Procedures for details). The presence of intact calcineurin Aα and calcineurin B in pETCNα was confirmed by sequencing. Subsequently, pETCNα was transformed into competent BL21(DE3/pLysS) that harbors an IPTG-inducible T7 RNA polymerase and T7 lysozyme that suppresses expression of calcineurin in the absence of IPTG (Sodeoka et al., 1993). Induction of BL21(DE3/pLysS)/pETCNα with IPTG yielded a clearly visible band at ca. 60 kDa and a less visible band at ca. 15 kDa on Coomassie blue-stained polyacrylamide gels. These bands were confirmed to be calcineurin Aα and calcineurin B, respectively, by Western blot analysis using antibodies specific for calcineurin Aα and calcineurin B (data not shown). A significant portion of the induced calcineurin α was in insoluble fractions upon bacterial cell lysis, suggesting that coexpression of the two subunits of calcineurin is not sufficient to overcome the insolubility problem.

It was known that CNB is myristoylated at its N-terminal glycine. We reasoned that reconstitution of the N-terminal myristoylation of calcineurin B may help to form more soluble calcineurin. The *Saccharomyces cerevisiae* myristoyl-CoA:protein *N*-myristoyltransferase (yNMT) has been cloned and extensively characterized (Duronio et al., 1990). A dual-plasmid expression system that coexpresses yNMT and mammalian *N*-myristoylproteins in *E. coli* has also been developed (Duronio et al., 1990). In this system, the yNMT in the plasmid pBB131 is under the control of the tac promoter. The vector pBB131 has a p15A origin of replication and confers kanamycin resistance to host cells. These features are compatible with the pETCNα expression vector which has a ColE1 origin of replication and confers ampicillin resistance to host cells. We transformed competent BL21(DE3/pLysS)/pETCNα with pBB131 (Duronio et al., 1990) and selected tranformants on an LB plate containing both ampicillin and kanamycin. When the culture of BL21(DE3/pLysS)/pETCNa-pBB131 was induced with IPTG, the amount of soluble calcineurin α was increased severalfold as judged by both SDS−PAGE (Figure 3, lane 2) and phosphatase activity in crude cell lysates using *p*-nitrophenyl phosphate as a substrate.

To purify the recombinant human calcineurin α, the bacterial lysate from IPTG-induced BL21(DE3/pLysS)/pETCNα-pBB131 was prepared by two passages through a French press. Nucleic acids were removed with 0.4% protamine sulfate. Addition of ammonium sulfate to 45% saturation precipitated most of the recombinant calcineurin, leaving many other proteins in the supernatant (Figure 3, lane 4). Substantial purification was achieved with non-nickel TALON metal affinity chromatography (Figure 3, lane 5). Most of the remaining high and low molecular weight contaminants from the TALON column eluate were removed through calmodulin−Sepharose chromatography (Figure 3, lane 6). Using this procedure, we can routinely purify 2−3 mg of recombinant human calcineurin α from 1 L of bacterial culture (Table 1). As shown in Table 2, the purified recombinant calcineurin α is fully active, with a K_m of 37 mM and a k_{cat} of 14.1 s^{-1} when *p*-nitrophenyl phosphate was used as a substrate, comparable to those reported for calcineurin purified from bovine brain (Klee et al., 1988).

The identity of the purified recombinant calcineurin α (composed of both calcineurin Aα and calcineurin B) was further confirmed by both gel mobility shift and N-terminal sequencing. It has been demonstrated that non-myristoylated CNB from baculovirus-infected Sf9 cells migrates more slowly on the SDS−polyacrylamide gel than the myristoylated form (Perrino et al., 1995). Calcineurin B is also known to exhibit a calcium-dependent gel mobility shift; it migrates more slowly on the SDS−polyacrylamide gel in the absence of calcium. As shown in Figure 4, the recombinant calcineurin B purified from bacteria coexpressing yeast *N*-myristoyltransferase migrates faster than that from bacteria which express calcineurins Aα and B only (Figure 4, lane 2 versus lane 4 and lane 3 versus lane 5). Both forms of calcineurin B exhibit a calcium-dependent mobility shift to a lower apparent molecular mass in the presence of calcium (Figure 4, lane 2 versus lane 3 and lane 4 versus lane 5). When yeast *N*-myristoyltransferase is coexpressed, we observed no unmyristoylated calcineurin B copurifying with calcineurin Aα. When the purified recombinant calcineurin α was subjected to Edman degradation, it gave only the N-terminal sequence of the 6xHis tag of calcineurin Aα, but not that of the calcineurin B subunit, suggesting that calcineurin B is completely myristoylated.

Assessment of Catalytic Functions of Residues Surrounding the Binuclear Metal Binding Site. The crystal structures of both calcineurin α (Griffith et al., 1995; Kissinger et al., 1995) and PP-1 (Goldberg et al., 1995) revealed a binuclear metal center with all surrounding residues conserved between calcineurin and PP-1. Among these residues, the importance of His199, His281, Asn150, and Arg254 has not been investigated by mutagenesis in the λ phosphatase, although an equivalent mutant of His281 of the β isoform of calcineurin has been created and shown to be inactive (Shibasaki et al., 1996). As a first step to assess the importance of the residues at the putative active site of calcineurin, we performed a systematic alanine-scan mutagenesis of all residues shown to be involved in the coordination of metal ions as well as His151, Arg122, and Arg254. As shown in Table 2, all alanine mutants were inactive toward PNPP, except for the Arg254Ala mutant, which retained about 0.6% of the wild-type phosphatase activity. Interestingly, whereas the Arg254Ala mutant retained some phosphatase activity toward PNPP, a small

Table 1: Purification of Recombinant Human Calcineurin α from *E. coli*

step	volume (mL)	concentration (mg/mL)	total protein (mg)	specific activity[a]	purification (*x*-fold)	yield[b] (%)
crude extract	16	9.1	145.6	18.3	1	100
protamine sulfate	17	9.8	166.6	12.5	0.7	78
0−45% ammonium sulfate	10	5.4	54.0	73.9	4	150
Talon affinity resin	6	0.98	5.9	768.0	42	170
CaM−Sepharose	5	0.54	2.7	835.0	46	85

[a] The relative specific activity [converted to nmol/(min·mg)] is measured using PNPP at a final concentration of 20 mM. [b] There is some inhibitory activity yet to be characterized for the phosphatase activity of calcineurin in crude extract that was removed upon further purification of calcineurin. The yield is therefore not an accurate measure of calcineurin recovery.

Table 2: Phosphatase Activity and Affinity for FKBP−FK506 of Calcineurin Aα Mutants[a]

	PNPP		phospho-RII		
CN mutants	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	BD_{50}[b] (nM)
WT	37 ± 20	14.1 ± 3.7	0.372 ± 0.017	2.30 ± 0.42	17
D90A	ND	$<2.60 \times 10^{-3}$	ND	ND	11
H92A	ND	$<2.60 \times 10^{-3}$	ND	ND	35
D118A	ND	$<2.60 \times 10^{-3}$	ND	ND	127
R122A	ND	$<2.60 \times 10^{-3}$	ND	ND	34
N150A	ND	$<2.60 \times 10^{-3}$	ND	ND	392
H151A	ND	$<2.60 \times 10^{-3}$	ND	ND	31
H199A	ND	$<2.60 \times 10^{-3}$	ND	ND	87
R254A	90.0 ± 39.0	0.077 ± 0.037	ND	ND	49
H281A	ND	$<2.60 \times 10^{-3}$	ND	ND	82

[a] ND, not determinable. [b] Concentration of FK506 at which 50% of the immobilized FKBP−FK506 is bound by calcineurin or mutants.

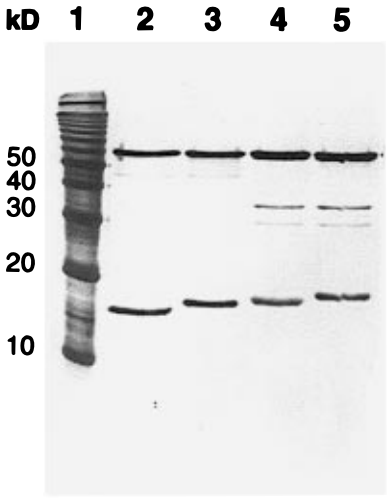

FIGURE 4: Calcium-dependent gel mobility shift of recombinant calcineurin B with and without N-terminal myristoylation. Lane 1, molecular mass markers; 2, myristoylated recombinant human calcineurin α + Ca^{2+}; 3, myristoylated recombinant human calcineurin α + EGTA; 4, nonmyristoylated recombinant human calcineurin α + Ca^{2+}; 5, nonmyristoylated recombinant human calcineurin α + EGTA.

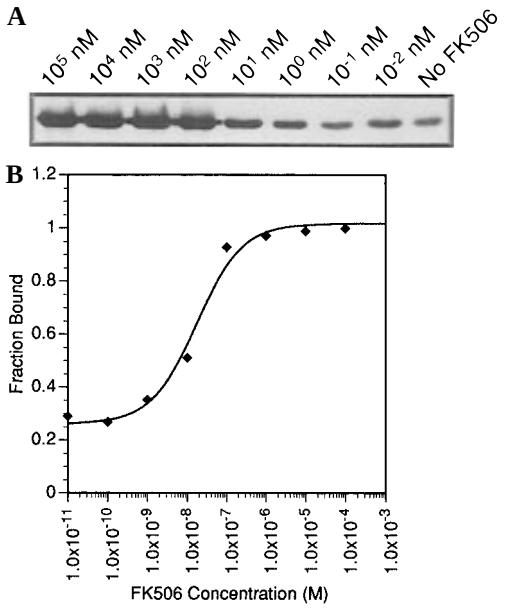

FIGURE 5: Determination of the binding affinity of wild-type calcineurin for FKBP−FK506 complex. (A) Western blot analysis of recombinant human calcineurin α bound by GSTFKBP12−FK506 affinity matrices at various concentrations of FK506. (B) Plot of relative amount of calcineurin α bound by varying concentrations of GSTFKBP12−FK506 fitted to Hill's equation.

substrate, it is completely inactive toward the phospho-RII peptide substrate, suggesting that Arg254 may be involved in peptide substrate recognition as well as in catalysis.

To assess the effect of alanine mutation on the structural integrity of calcineurin, we employed an FKBP−FK506 binding assay (Liu et al., 1991a) in which FKBP fused to glutathione *S*-transferase was used as an affinity reagent to bind to wild-type and mutant calcineurin in the presence of varying concentrations of FK506 (Figure 5). As FKBP was shown to interact not only with the interface between calcineurin B and the calcineurin B binding domain in calcineurin A in the FKBP−FK506−calcineurin complex but also with residues in the catalytic domain of calcineurin A close to the active site (Kissinger et al., 1995), this assay should provide a measure of the structural integrity of the calcineurin mutants. The apparent BD_{50} values of the different CNα mutants and wild-type enzyme for the FKBP−FK506 complex (Table 2) indicate that there was limited structural disturbance to the structure of calcineurin upon mutation of most of the residues in the active site of the enzyme. Two exceptions are N150A and D118A, which have apparent affinity values that are 23-fold and 7-fold lower than wild-type calcineurin, respectively, suggesting that these mutants may possess altered structures, which is partly

responsible for their lack of catalytic activity. As the FKBP−FK506 complex binds to the catalytic domain of calcineurin A as well as to the composite surface formed by calcineurins A and B (Griffith et al., 1995; Kissinger et al., 1995), it is possible that alteration of the binuclear metal center in N150A and D118A mutants leads to the dislocation of the residues from the catalytic domain that interact with FKBP in the FKBP−FK506−CN complex.

DISCUSSION

In this paper, we report an efficient bacterial expression system for the α isoform of human calcineurin, which allows for the purification to near-homogeneity of sufficient quantities of recombinant calcineurin for further biochemical characterization. We were able to optimize the soluble portions of recombinant calcineurin by coexpression of both A and B subunits of calcineurin and by reconstitution of the N-terminal myristoylation of calcineurin B. The recombinant calcineurin α thus purified is fully active. Compared to the expression system in which calcineurin A and calcineurin B were expressed separately (Haddy & Rusnak, 1994), this system yields higher amounts of fully active enzyme. Unlike the baculovirus-infected Sf9 insect cell system (Perrino et al., 1995), the N-terminal myristoylation of calcineurin B was almost quantitative by coexpressing yeast *N*-myristoyltransferase and adding free myristic acid to the IPTG-induced bacterial culture. Although no physiological functions have been ascribed to the N-terminal myristoyl group on calcineurin B (Zhu et al., 1995), its presence increased the concentration of soluble recombinant calcineurin in *E. coli* lysate. The increased solubility of myristoylated calcineurin may be attributed to its increased stability (Kennedy et al., 1996). We have recently employed the same strategy to express and purify the β isoform of calcineurin from *E. coli* (L. Sun, Eddy Chen, and J. O. Liu, unpublished data), thus allowing a systematic comparison of the enzymatic properties of the different isoforms of calcineurin. We note that a bacterial expression system coexpressing the two subunits of calcineurin was recently reported, though the myristoylation of calcineurin B subunit was not reconstituted in that system, likely yielding lower amounts of purified enzymes based on our aforementioned observations (Lewis et al., 1995).

With the bacterial expression system established, we assessed the importance of amino acid residues surrounding the putative binuclear metal center of calcineurin by mutating each residue to an alanine. We found that an intact binuclear metal center is necessary for the phosphatase activity of calcineurin, as mutation of any single residue involved in coordination of the metal ions leads to complete abrogation of the enzymatic activity. These observations are consistent with the recent report that calcineurin is sensitive to inactivation by oxidative damage which can be prevented by superoxide dismutase (Wang et al., 1996). Among the residues that are not involved in metal ion coordination, His151 in calcineurin is juxtaposed to serve as a general acid to facilitate the departure of the alcoholic side chain of serine or threonine from the peptide substrate, or as a general base to deprotonate a metal ion-bound water molecule to attack the phosphate, or as a nucleophile for direct attack on the phosphate undergoing hydrolysis. That the His151Ala mutant lacks phosphatase activity suggests that His151 plays a critical role in catalysis, but does not distinguish between these mechanistic possibilities. The inability to detect a phosphoenzyme intermediate (Martin & Graves, 1986) and the lack of stereochemical retention of labeled phosphate during the hydrolysis by the structurally related purple acid phosphatase (Mueller et al., 1993) argue against the possibility that His151 serves as a nucleophile in catalysis. But this possibility cannot yet be ruled out as the phosphoenzyme intermediate could be too unstable to be detected and the purple acid phosphatase may not employ the same mechanistic strategy as calcineurin and related serine/threonine phosphatases. Had the His151Ala mutant retained some phosphatase activity, the determination of the pH dependence of the mutant in comparison to the wild-type enzyme may have offered clues to the true catalytic function of His151. Unfortunately, this mutant is completely inactive toward either PNPP or phospho-RII peptide substrate, making it impossible to use the pH dependence of kinetic parameters to probe its role in catalysis. We note that a His151Gln equivalent mutant of the β isoform of calcineurin has been previously made and shown to be completely inactive (Shibasaki et al., 1996), in agreement with our observation. The other two residues close to the binuclear metal center with potentially active side chains are Arg122 and Arg254. A likely role of these positively charged arginines is to neutralize the developing negative charge on the phosphate undergoing hydrolysis. But why two arginines located at the two opposite sides of the binding groove are needed remains unknown. The partial retention of phosphatase activity of Arg254Ala mutants using PNPP as a substrate suggests that it does play an important but not an essential role in catalysis. The complete abrogation of the phosphatase activity of this mutant toward the phospho-RII peptide substrate suggests that it may be involved in phosphoprotein substrate binding as well as catalysis. Whether Arg254 is involved in substrate recognition remains to be verified using different phosphoprotein substrates.

Based on significant homology with calcineurin and other mammalian serine/threonine phosphatases, the bacteriophage λ phosphatase has been used as a model to study the mechanism of catalysis of this superfamily of phosphatases. Mutagenesis in the λ phosphatase of residues that are conserved among all members of this family revealed that residues corresponding to Asp90, Asp118, His92, His151, and Arg122 of calcineurin Aα are important for catalysis (Zhou et al., 1994), leaving undetermined the importance of Asn150, His199, His281, and Arg254 in catalysis by calcineurin. In addition to confirming the mutagenesis results from the λ phosphatase, we further demonstrated that three amino acids involved in the coordination of the putative Zn^{2+} ion in calcineurin are equally critical for the catalytic activity of calcineurin. Early sequence comparison between the λ phosphatase and calcineurin did not identify the two histidines in the λ phosphatase corresponding to His199 and His281 in calcineurin (Cohen, P. T. W., & Cohen, 1989; Zhou et al., 1993, 1994). Realignment of sequences in light of the active site structures of calcineurin and PP-1 revealed the presence of the two histidines in the λ phosphatase that may participate in the coordination of a second metal ion (Lohse et al., 1995). The positions of the two histidines in the λ phosphatase, however, are much closer to the rest of the conserved metal ion coordinating residues than the corresponding histidines are in calcineurin in their primary

sequences. It remains to be determined whether His140 and His187 in λ phosphatase indeed form part of a second metal ion binding site and whether the λ phosphatase utilizes a binuclear metal center for catalysis.

Calcineurin is a cytosolic signaling enzyme that induces the activation of transcription factors in the nucleus of T cells including NFAT, NF-κB, AP-1, and Oct1/Jun (Schreiber & Crabtree, 1992; Liu, 1993; Crabtree & Clipstone, 1994). Although recent evidence suggests that calcineurin modulates the activity of NFAT by physically associating with NFAT (Wesselborg et al., 1996; Loh et al., 1996) and translocating with NFAT4 (one of the four known isoforms of NFAT) into the nucleus (Shibasaki et al., 1996), how calcineurin modulates the activity of the other transcription factors remains unknown. The identification of additional substrates for calcineurin will be critical for a full understanding of those signaling pathways. Since wild-type calcineurin, like other phosphatases, presumably associates with substrates transiently, with NFAT being a possible exception, a catalytically inactive mutant of calcineurin that retains substrate binding may be extremely useful for identification of new substrates for calcineurin as has been shown for the active site mutants of protein tyrosine phosphatases (Sun et al., 1993; Furukawa et al., 1994; Herbst et al., 1996). The active site mutants reported in this paper will thus be useful as biochemical probes for discovering new calcineurin substrates involved in different signal transduction pathways, including those in T lymphocytes and kidney and neuronal cells.

ACKNOWLEDGMENT

We are grateful to Dr. Randy Kincaid for a generous gift of the cDNA clone of calcineurin Aα. We thank Ms. Suming Kong for excellent technical assistance. We thank John Newman and Peter Kim at the Whitehead Institute for help in curve fitting.

REFERENCES

Aperia, A., Ibarra, F., Svensson, L.-B., Klee, C., & Greengard, P. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89,* 7394−7397.

Breuder, T., Hemenway, C. S., Movva, N. R., Cardenas, M. E., & Heitman, J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91,* 5372−5376.

Cardenas, M., Lorenz, M., Hemenway, C. S., & Heitman, J. (1994) *Perspect. Drug Discovery Des. 2,* 103−126.

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

Cohen, P., & Cohen, P. T. W. (1989) *J. Biol. Chem. 264,* 21436−21438.

Cohen, P. T. W., & Cohen, P. (1989) *Biochem. J. 260,* 931−934.

Crabtree, G. R., & Clipstone, N. A. (1994) *Annu. Rev. Biochem. 63,* 1045−1083.

Cunningham, K. W., & Fink, G. R. (1994) *J. Cell Biol. 124,* 351−363.

Cyert, M. S., Kunisaaw, R., Kaim, D., & Thorner, J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88,* 7376−7380.

Dumont, F. J., Staruch, M. J., Koprak, S. L., Siekierka, J. J., Lin, C. S., Harrison, R., Sewell, T., Kindt, V. M., Beattie, T. R., Wyvratt, M., & Sigal, N. H. (1992) *J. Exp. Med. 176,* 751−760.

Duronio, R. J., Rudnick, D. A., Johnson, R. L., Linder, M. E., & Gordon, J. I. (1990) *Methods: Companion Methods Enzymol. 1,* 253−263.

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

Friedman, J., & Weissman, I. (1991) *Cell 66,* 799−806.

Furukawa, T., Itoh, M., Krueger, N. X., Streuli, M., & Saito, H. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91,* 10928−10932.

Goldberg, J., Huang, H.-B., Kwon, Y.-G., Greenbard, P., Nairn, A. C., & Kuriyan, J. (1995) *Nature 376,* 745−753.

Griffith, J. P., Kim, J. L., Kim, E. E., Sintchak, M. D., Thomson, J. A., Fitzgibbon, M. J., Fleming, M. A., Caron, P. R., Hsiao, K., & Navia, M. A. (1995) *Cell 82,* 507−522.

Guerini, D., & Klee, C. B. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86,* 9183−9187.

Haddy, A., & Rusnak, F. (1994) *Biochem. Biophys. Res. Commun. 200,* 1221−1229.

Hashimoto, Y., Perrino, B. A., & Soderling, T. R. (1990) *J. Biol. Chem. 265,* 1924−1927.

Herbst, R., Carroll, P. M., Allard, J. D., Schilling, J., Raabe, T., & Simon, M. A. (1996) *Cell 85,* 899−909.

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

Kennedy, M. T., Brockman, H., & Rusnak, F. (1996) *J. Biol. Chem. 271,* 26517−26521.

Kincaid, R. L., Nightingale, M. S., & Martin, B. M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85,* 8983−8987.

King, M. M., & Huang, C. Y. (1984) *J. Biol. Chem. 259,* 8847−8856.

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, E. W., Gastinel, L. N., Habuka, N., Chen, X., Maldonado, F., Barker, J. E., Bacquet, R., & Villafranca, J. E. (1995) *Nature 378,* 641−644.

Klee, C. B., & Cohen, P. (1988) *Adv. Second Messenger Phosphoprotein Res.* 225−247.

Lea, J. P., Sands, J. M., McMahon, S. J., & Tumlin, J. A. (1994) *Kidney Int. 46,* 647−652.

Lewis, C., Gastinel, L., Habuka, N., Tucker, K., Chen, X., Maldonado, F., & Villafranca, J. E. (1995) *FASEB J. 9,* A1346.

Liu, J. (1993) *Immunol. Today 14,* 290−296.

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

Liu, J., Chen, C.-M., & Walsh, C. T. (1991b) *Biochemistry 30,* 2306−2310.

Liu, J., Albers, M. W., Wandless, T. J., Luan, S., Alberg, D. G., Belshaw, P. J., Cohen, P., MacKintosh, C., Klee, C. B., & Schreiber, S. L. (1992) *Biochemistry 31,* 3896−3901.

Loh, C., Shaw, K. T.-Y., Carew, J., Viola, J. P. B., Luo, C., Perrino, B. A., & Rao, A. (1996) *J. Biol. Chem. 271,* 10884−10891.

Lohse, D. L., Denue, J. E., & Dixon, J. E. (1995) *Structure 3,* 987−990.

Martin, B. L., & Graves, D. J. (1986) *J. Biol. Chem. 261,* 14545−14550.

Mueller, E. G., Crowder, M. W., Averill, B. A., & Knowles, J. R. (1993) *J. Am. Chem. Soc. 115,* 2974−2975.

Nelson, P. A., Akselband, Y., Kawamura, A., Su, M., Tung, R. D., Rich, D. H., Kishore, V., Rosborough, S. L., DeCenzo, M. T., Livingston, D. J., & Harding, M. W. (1993) *J. Immunol. 150,* 2139−2147.

O'Keefe, S. J., Tamura, J., Kincaid, R. L., Tocci, M. J., & O'Neill, E. A. (1992) *Nature 357,* 692−694.

Parent, S. A., Nielsen, J. B., Morin, N., Chrebet, G., Ramadan, N., Dahl, A. M., Hsu, M.-J., Bostian, K. A., & Foor, F. (1993) *J. Gen. Microbiol. 139,* 2973−2984.

Perrino, B. A., Fong, Y.-L., Brickey, D. A., Saitoh, Y., Ushio, Y., Fukunaga, K., Miyamoto, E., & Soderling, T. R. (1992) *J. Biol. Chem. 267,* 15965−15969.

Perrino, B. A., Ng, L. Y., & Soderling, T. R. (1995) *J. Biol. Chem. 270,* 340−346.

Qin, K.-F., Khangulov, S., Liu, C., & Huang, C. Y. (1995) *FASEB J. 9,* A1347.

Sarker, G., & Sommer, S. S. (1990) *BioTechniques 8,* 404−407.

Schreiber, S. L., & Crabtree, G. R. (1992) *Immunol. Today 13,* 136−142.

Shenolikar, S. (1994) *Annu. Rev. Cell Biol. 10,* 55−86.

Shibasaki, F., Price, E. R., Milan, D., & McKeon, F. (1996) *Nature 382,* 370−373.

Sikkink, R., Haddy, A., MacKelvie, S., Mertz, P., Litwiller, R., & Rusnak, F. (1995) *Biochemistry 34,* 8348−8356.

Sodeoka, M., Larson, C. J., Chen, L., LeClair, K. P., & Verdine, G. L. (1993) *Bioorg. Med. Chem. Lett. 3,* 1089−1094.

Stemmer, P., & Klee, C. B. (1991) *Curr. Opin. Neurobiol. 1,* 53−64.
Stemmer, P. M., & Klee, C. B. (1994) *Biochemistry 33,* 6859−6866.
Sun, H., Charles, C. H., Lau, L. F., & Tonks, N. K. (1993) *Cell 75,* 487−493.
Wang, X., Culotta, V. C., & Klee, C. B. (1996) *Nature 383,* 434−437.
Webb, M. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89,* 4884−4887.
Wesselborg, S., Fruman, D. A., Sagoo, J. K., Bierer, B. E., & Burakoff, S. J. (1996) *J. Biol. Chem. 271,* 1274−1277.
Yu, L., Haddy, A., & Rusnak, F. (1995) *J. Am. Chem. Soc. 117,* 10147−10148.
Zhu, D., Cardenas, M. E., & Heitman, J. (1995) *J. Biol. Chem. 270,* 24831−24838.
Zhuo, S., Clemens, J. C., Hakes, D. J., Barford, D., & Dixon, J. E. (1993) *J. Biol. Chem. 268,* 17754−17761.
Zhuo, S., Clemens, J. C., Stone, R. L., & Dixon, J. E. (1994) *J. Biol. Chem. 269,* 26234−26238.

BI9631935