Calcineurin Subunit Interactions: Mapping the Calcineurin B Binding Domain on Calcineurin A[†]

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Received February 27, 1995; Revised Manuscript Received April 25, 1995[⊗]

ABSTRACT: Recombinant forms of the A and B subunits of the protein phosphatase calcineurin were produced in *Escherichia coli*, reconstituted into a heterodimer and purified to homogeneity. The reconstituted heterodimer exhibited properties like that of bovine brain calcineurin. This included calmodulin-stimulated activity and a subunit stoichiometry and Stokes radius consistent with native-like structure. In order to map the region on the A subunit where calcineurin B binds, a series of overlapping 20-residue peptides corresponding to this putative domain were synthesized. Using isolated calcineurin A and B subunits, an assay that relied upon peptide inhibition of calcineurin B stimulation of calcineurin A activity was developed. All five peptides, but not a control peptide, inhibited calcineurin B-dependent stimulation of calcineurin A although with different potencies. The three most effective inhibitory peptides spanned calcineurin A residues 338−377. These three peptides also altered the electrophoretic mobility of the isolated calcineurin B subunit during native polyacrylamide gel electrophoresis indicating a direct interaction between these peptides and calcineurin B. The peptide corresponding to residues 348−367 was also able to block binding of calcineurin B to the catalytic subunit.

Calcineurin, also known as protein phosphatase 2B, consists of two tightly associated subunits. The 58 kDa A subunit contains the active site and shares extensive homologies with the family of serine/threonine protein phosphatases that also includes protein phosphatases 1 and 2A (Guerini & Klee, 1991). The 19 kDa B subunit, on the other hand, is a member of the family of Ca^{2+} binding proteins that includes calmodulin (CaM),[1] troponin C, and parvalbumin. By itself, the calcineurin heterodimer is relatively inactive but can be stimulated several fold in a reversible and Ca^{2+} dependent fashion by CaM. Although related, CaM and calcineurin B interact with the A subunit at distinct sites (Guerini & Klee, 1991).

The active site was first localized to calcineurin A in experiments which separated the holoenzyme into individual subunits using gel filtration chromatography in the presence of denaturants such as urea or SDS (Winkler et al., 1984; Gupta et al., 1985; Merat et al., 1985; Merat & Cheung, 1987). In these studies, the phosphatase activity was found to be associated with the 58 kDa A subunit, which, although slight, could be greatly stimulated upon reconstitution with calcineurin B in the presence of CaM. Several groups have confirmed this using isolated recombinant calcineurin A catalytic subunit (Perrino et al., 1992; Ueki & Kincaid, 1993; Haddy & Rusnak, 1994). The marked stimulation of phosphatase activity by calcineurin B (Winkler et al., 1984; Merat et al., 1985; Merat & Cheung, 1987), as well as the slight increase in activity upon Ca^{2+} binding to this subunit (Stewart & Cohen, 1988), has lead to its designation as the regulatory subunit of calcineurin. Unlike CaM, however, this subunit remains tightly associated with the catalytic subunit in the absence of Ca^{2+}.

The physiological role of calcineurin B has not yet been completely defined. Perrino et al. (1992) have demonstrated that the calcineurin heterodimer has increased catalytic efficiency (in terms of k_{cat}/K_m) relative to isolated calcineurin A. More recently, using a proteolyzed, CaM-independent form of calcineurin, it was shown that calcineurin B has a role in regulating activity by apparently influencing only the K_m of the enzyme for its substrate (Stemmer & Klee, 1994). Also of interest was the finding that the B subunit is required in order for calcineurin to interact with the immunosuppressant drug·immunophilin complexes, cyclosporin A·cyclophilin, and FK506·FKBP (Haddy et al., 1992; Haddy & Rusnak, 1994), recently supported by cross-linking and binding experiments (Li & Handschumacher, 1993; Husi et al., 1994; Clipstone et al., 1994).

A putative domain on the A subunit which interacts with calcineurin B was first identified by Guerini et al. (1992) by comparing rat and *Drosophila* calcineurin A sequences. In that report, it was hypothesized that a highly conserved region situated between the active site and CaM-binding domains represented the calcineurin B binding site. More

[†] This work was supported by grants from the Mayo Foundation, the American Heart Association (MHA90), and the National Institutes of Health (GM46865).

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[⊗] Abstract published in *Advance ACS Abstracts,* June 15, 1995.

[1] Abbreviations: BSA, bovine serum albumin; CaM, calmodulin; CNA, calcineurin A subunit; CNB, calcineurin B subunit; CsA, cyclosporin A; DEAE, diethylaminoethyl; DNase, bovine pancreatic deoxyribonuclease I, type II; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′N′*-tetraacetic acid; FKBP, FK506-binding protein; FMOC, 9-fluorenylmethoxycarbonyl; IPTG, isopropyl β-thio-D-galactoside; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; pNPP, *p*-nitrophenylphosphate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

0006-2960/95/0434-8348$09.00/0

recent experiments have also found this region to be required for binding calcineurin B but did not rule out the possibility that other residues may be involved (Husi et al., 1994; Clipstone et al., 1994).

In this report, using recombinant forms of both calcineurin subunits, we have reconstituted and purified a native-like calcineurin heterodimer and used the individual subunits to investigate the calcineurin B binding domain. The reconstituted enzyme exhibited phosphatase activity and native molecular mass comparable to those of native bovine brain calcineurin. Using synthetic peptides based on the putative calcineurin B binding domain, we have demonstrated that peptides representing calcineurin A residues 338−377 are most effective in inhibiting calcineurin B-dependent stimulation of calcineurin A phosphatase activity. We have also shown that these peptides interact directly with the B subunit and are able to inhibit heterodimer formation. These results confirm that the calcineurin A residues represented by these peptides contribute to the calcineurin B binding domain.

EXPERIMENTAL PROCEDURES

Materials. Blue Dextran, bovine serum albumin (BSA), DEAE Sepharose CL-6B, bovine pancreatic deoxyribonuclease I (DNase), *p*-nitrophenylphosphate (pNPP), bovine cardiac cyclic-AMP dependent protein kinase catalytic subunit, Sephacryl S300, and Sepharose 4B were purchased from Sigma (St. Louis, MO). CaM-Sepharose was prepared from purified bovine brain CaM (Dedman & Kaetzel, 1983; Gopalakrishna & Anderson, 1982) and cyanogen bromide activated Sepharose 4B (March et al., 1974) as described previously (Sharma et al., 1983). Adenosine 5′-[γ-^{32}P]-trisphosphate, triethylammonium salt (3000 Ci/mmol), was purchased from Amersham (Arlington Heights, IL). Sephadex G75 resin (10−40 μm) and the mono-Q HR5/5 column were obtained from Pharmacia (Piscataway, NJ). PM30 and YM3 ultrafiltration membranes were purchased from Amicon (Beverly, MA). The RAMPS multiple peptide synthesis system and Rapid Amide resin were obtained from E. I. DuPont (Willmington, DE).

The peptide DLDVPIPGRFDRRVSVAAE corresponding to the phosphorylation site of bovine cardiac cyclic-AMP dependent protein kinase regulatory subunit type II [R_{II} peptide (Blumenthal et al., 1986)] was synthesized and purified by HPLC reverse-phase chromatography by the Protein Sequence and Peptide Synthesis Facility at the Mayo Clinic. The 18-residue control peptide of Table 1 corresponding to residues 1675−1692 of mature human coagulation factor VIII (Toole et al., 1984) was obtained as a generous gift of Dr. David Fass, Mayo Clinic and Foundation.

Methods. Protein concentrations were measured using the Bio-Rad protein assay (Bradford, 1976) with BSA as a standard. Recombinant calcineurin A used in this study has a subunit molecular mass calculated from the cDNA sequence of 58 640 Da, including a nine amino acid fusion at the amino terminus (Haddy et al., 1992; Haddy & Rusnak, 1994). R_{II} peptide was phosphorylated with [^{32}P]ATP to a specific activity of 760 μCi/μmol using the catalytic subunit of bovine cardiac cyclic-AMP dependent protein kinase and purified as described (Hubbard & Klee, 1991). Positive ion electrospray-mass spectrometry of calcineurin B was performed at the Mass Spectrometry Core Facility at the Mayo Clinic & Foundation.

Purification of Recombinant Calcineurin A. Crude extract containing recombinant calcineurin A was prepared from *Escherichia coli* pCNAT77/BL21(DE3) cells grown at 23 °C in LB containing ampicillin (100 μg/mL) for 18 h after induction with isopropyl β-thio-D-galactoside (IPTG) essentially as described (Haddy & Rusnak, 1994) but without the addition of $FeCl_3$ or $ZnCl_2$ to culture media. After harvesting and washing, the cells from 9 L of culture were suspended (0.4 g wet cell weight/mL) in buffer containing 0.10 M Tris-HCl, 10.0 mM EDTA, 1.0 mM dithiothreitol, 75 mg/L phenylmethanesulfonyl fluoride, 10 mg/L trypsin inhibitor, 1.0 mg/L leupeptin, and 10 mg/L *N*-tosyl-L-phenylalanine chloromethyl ketone, pH 7.5. The cell suspension was then lysed by passage through a French pressure cell at 17 000 psi and DNase added to a final concentration of 5 μg/mL. The lysed cells were then centrifuged at 39000*g* at 4 °C for 30 min, and the supernatant was collected to give crude extract. Subsequent purification was carried out at 4 °C.

After adding $CaCl_2$ to a final concentration of 10 mM (equimolar with [EDTA]), crude extract containing recombinant calcineurin A was loaded onto a column (16 × 500 mm) containing CaM-Sepharose equilibrated with pH 7.5 buffer containing 20 mM Tris-HCl, 0.22 M NaCl, 1.0 mM Mg acetate, 1.0 mM dithiothreitol, and 10 μM $CaCl_2$. The CaM-Sepharose column was then washed with 200 mL of the same buffer and calcineurin A eluted from the column with buffer containing 0.10 mM EGTA in place of $CaCl_2$. Fractions containing calcineurin A were combined and concentrated to about 0.6 mg/mL using an Amicon pressurized filtration cell equipped with a PM30 membrane and frozen at −70 °C until further use.

Subcloning and Overexpression of Rat Calcineurin B. The cDNA for rat calcineurin B (Genbank accession number L03554), kindly provided by Drs. Brian Perrino and Thomas Soderling, Vollum Institute, was subcloned into the vector pT7-7 (Tabor, 1990) after amplification using the polymerase chain reaction and the following oligonucleotide primers: 5′-CCATGGATCCTCTAGAAGGAGATATA-CATATGGGAAATGAGGCAAGT-3′ and 5′-ACTAGTC-GACTCACACATCTACCACCAT-3′. These primers are homologous to opposite ends of the human calcineurin B gene sequence at their 3′ termini and contain additional restriction endonuclease sites for subcloning into pT7-7 at the 5′ termini. Following amplification, the PCR product was purified from a low melting point agarose gel followed by chromatography over an elutip column (Schleicher and Schuell, Keene, NH) according to the manufacturer's instructions. The purified fragment was subsequently digested with *Nde*I and *Sal*I and subcloned into *Nde*I−*Sal*I-digested pT7-7 to give the recombinant vector pRCNBT775-3. Recombinant vectors carrying the calcineurin B gene were identified by restriction digest analysis, and the sequence of the complete calcineurin B gene was verified by DNA sequencing. The recombinant plasmid was then transformed into competent *E. coli* BL21(DE3) cells (Studier et al., 1990).

Purification of Recombinant Calcineurin B. Recombinant calcineurin B was purified to homogeneity (Figure 1) from crude extracts of *E. coli* strain BL21(DE3) containing pCNBT775-3. Eighteen liters of BL21(DE3) cells containing pRCNBT775-3 was grown at 37 °C with shaking in Luria broth containing ampicillin (100 μg/mL) until the absorbance at 595 nm reached ≈0.7 at which point IPTG was added to

a final concentration of 1.0 mM. The cell cultures were grown for an additional 6 h and harvested by centrifugation at 3400*g* for 15 min. The cells were washed once in 0.10 M Tris-HCl, pH 7.5, and suspended in buffer containing 0.10 M histidine, 10.0 mM EDTA, and 1.0 mM dithiothreitol, pH 6.1, at about 0.4 g wet cell weight/mL. The cells were lysed by passage through a French pressure cell at 17 000 psi, and DNase was added to a concentration of 5 μg/mL. The cell extract was then centrifuged at 39000*g* at 4 °C for 30 min to obtain crude extract. Subsequent purification was carried out at 4 °C. The crude extract was loaded onto a column (26 × 300 mm) containing DEAE-Sepharose CL6B equilibrated with 25.0 mM histidine, 1.0 mM EDTA, and 1.0 mM dithiothreitol, pH 6.1, buffer. After loading of crude extract, the column was washed with 300 mL of equilibration buffer and the bound protein eluted with a 1000 mL gradient from 0.0 to 0.4 M NaCl in equilibration buffer. Column fractions were assayed for the presence of calcineurin B using 13% SDS−PAGE. Fractions containing calcineurin B eluting from about 0.25 to 0.35 M NaCl were pooled and concentrated using an Amicon pressurization cell equipped with a YM3 membrane. The concentrated calcineurin B fraction was then loaded onto a column (50 × 800 mm) containing Sephadex G75 resin (10−40 μm) equilibrated in buffer (25 mM histidine, 1.0 mM EDTA, 1.0 mM dithiothreitol, 0.15 M KCl, pH 6.1), and the protein was eluted with a flow rate of 1 mL/min with the same buffer. Fractions containing calcineurin B were assayed and selectively pooled. Subsequent purification to homogeneity was achieved using a mono-Q anion exchange column. The combined fractions from gel filtration chromatography were dialyzed against buffer (25 mM histidine, 1.0 mM EDTA, 1.0 mM dithiothreitol, pH 6.1) and loaded onto a mono-Q HR5/5 column equilibrated in the same buffer. After washing with 2.5 mL of equilibration buffer, the bound protein was eluted with a linear 0.0−0.4 M NaCl gradient.

Reconstitution and Purification of Recombinant Calcineurin. Crude extract of recombinant calcineurin A in 0.10 M Tris-HCl, pH 7.5, 10.0 mM EDTA, 1.0 mM dithiothreitol, 75 mg/L phenylmethanesulfonyl fluoride, 10 mg/L trypsin inhibitor, 1.0 mg/L leupeptin, and 10 mg/L *N*-tosyl-L-phenylalanine chloromethyl ketone, and calcineurin B purified through the first DEAE-Sepharose CL-6B chromatography step as described above were combined and stirred on ice for 29 h. Reconstituted calcineurin (calcineurin A + calcineurin B) was purified using a CaM-Sepharose affinity step as described above for calcineurin A with the exception that $CaCl_2$ was added to the protein fraction to a final concentration of 6.0 mM just prior to affinity purification. Following chromatography, column fractions containing reconstituted calcineurin were combined and concentrated to 7.5 mL using an Amicon pressurized filtration cell equipped with a PM30 membrane. The concentrated protein fractions were then chromatographed on a column (26 × 905 mm) containing Sephacryl S-300 resin equilibrated with 20.0 mM Tris-HCl, 0.10 M KCl, 1.0 mM Mg acetate, 1.0 mM dithiothreitol, and 0.10 mM EGTA, pH 7.5, at a flow rate of 20 mL/h. Column fractions were assayed for phosphatase activity using *p*-nitrophenylphosphate (pNPP) as substrate as described below.

Calibration of Sephacryl S-300 Gel Filtration Column. The Sephacryl S-300 gel filtration column (26 × 905 mm) was calibrated at a flow rate of 20 mL/h in 33 mM potassium phosphate, 0.15 M KCl, pH 7.0, using Bio-Rad gel filtration molecular weight standards (Bio-Rad, Hercules, CA) and blue dextran. The standards (2.4 mg of blue dextran, 1.5 mg of thyroglobulin, 1.5 mg of bovine γ-globulin, 1.5 mg of chicken albumin, 0.75 mg of myoglobin, and 0.15 mg of cyanocobalamin) were brought up to 1.0 mL with gel filtration buffer and chromatographed, and the absorbance at 280 nm was monitored. The elution volume for blue dextran was used for the void volume and K_{av} values were calculated using the expression

$$K_{av} = (V_e - V_o)/(V_t - V_o)$$

where V_e is the elution volume, V_o is the void volume, and V_t is the total volume of the column.

Table 1: Amino Acid Sequences of Putative Calcineurin B Binding Peptides and Control Peptide

peptide name	peptide sequence	residue number[a]
CNAPEP1	VMNIRQFNCSPHPYWLPNFM	328−347
CNAPEP2	PHPYWLPNFMDVFTWSLPFV	338−357
CNAPEP3	DVFTWSLPFVGEKVTEMLVN	348−367
CNAPEP4	GEKVTEMLVNVLNICSDDEL	358−377
CNAPEP5	VLNICSDDELGSEEDGFDGA	368−387
control peptide	EDFDIYDEDENQSPKSFQ	1675−1692[b]

[a] Residue numbering of CNAPEP sequences based on rat calcineurin Aα primary sequence (Saitoh et al., 1991). [b] Control peptide numbering based on mature human factor VIII primary sequence (Toole et al., 1984).

Synthesis of CNA Peptides. Five overlapping, 20-residue peptides corresponding to a putative calcineurin B binding site (Guerini et al., 1992) on rat calcineurin A (Saitoh et al., 1991), as well as a control peptide (Toole et al., 1984), were synthesized by 9-fluorenylmethoxycarbonyl (FMOC) chemistry using DuPont RAMPS multiple peptide synthesis system and RapidAmide resin. The synthesis protocols of the manufacturer were followed. Protection of amino acid side chains during synthesis was as follows: *tert*-butyl for D, E, S, T, and Y; *tert*-butyloxycarbonyl for K; 2,2,5,7,8-pentamethylchroman-6-sulfonyl for R; and triphenymethyl for C and H. Cleavage of peptides was achieved using a mixture of trifluoroacetic acid (TFA), phenol, H_2O, thioanisol, and 1,2-ethanedithiol (82.5/ 5.0/ 5.0/ 5.0/ 2.5; v/v) (King et al., 1990). The sequence of the control peptide, as well as the five calcineurin A peptides and their position in the primary sequence of rat calcineurin A, are given in Table 1.

All peptides were purified using a Vydac Protein and Peptide C18 column (1.0 × 25.0 cm) with 214 nm detection using a H_2O/0.05% TFA−acetonitrile/0.05% TFA gradient. The major eluting peak was collected and lyophilized. Following purification, the purity and composition of each peptide was checked by Edman degradation, amino acid analysis, and mass spectrometry by the Mass Spectrometry Core Facility at the Mayo Clinic.

For enzyme assays, stock solutions of about 1 mM of each peptide were prepared as follows and stored at −20 °C until needed: Control peptide was dissolved in 0.1 M Tris-HCl, pH 7.5; CNAPEP1 was dissolved in water, CNAPEP2 was dissolved in 10% acetic acid, CNAPEP3 was dissolved in 5% acetic acid, CNAPEP4 was dissolved in 50 mM MOPS, pH 7.0, and CNAPEP5 dissolved in 33 mM Tris-HCl, pH 8.0. The concentration of each peptide was determined using the following extinction coefficients (calculated based on the

chromophores present): control peptide, $\epsilon_{257.4} = 396\ M^{-1}\ cm^{-1}$; CNAPEP1, $\epsilon_{280} = 7030\ M^{-1}\ cm^{-1}$; CNAPEP2, $\epsilon_{280} = 12\,660\ M^{-1}\ cm^{-1}$; CNAPEP3, $\epsilon_{280} = 5690\ M^{-1}\ cm^{-1}$; CNAPEP5, $\epsilon_{257.4} = 198\ M^{-1}\ cm^{-1}$ (Edelhoch, 1967; Gratzer, 1976). Due to a lack of chromophore, the concentration of CNAPEP4 solutions was determined by amino acid analysis.

Phosphatase Activity Using pNPP as Substrate. CaM-dependent phosphatase activity using pNPP as substrate was measured at 30 °C in 25 mM MOPS, 1.0 mM $MnCl_2$, 0.10 mM $CaCl_2$, 1.0 μM CaM, 10 mM pNPP, pH 7.0, and 85 nM reconstituted calcineurin. After incubation for 2 min at 30 °C, the reaction was initiated by the addition of pNPP. Specific activity was measured by following the increase in absorbance at 410 nm with time using a Cary1 UV/visible spectrophotometer. The temperature of both sample and reference cuvette was maintained using thermostatable cell holders attached to a Lauda RM6 circulating water bath water. The change in extinction at 410 nm was taken to be 7180 $M^{-1}\ cm^{-1}$ at pH 7.0 based on a pK_a of 7.17 and a measured extinction coefficient of 17 800 $M^{-1}\ cm^{-1}$ at 410 nm for the *p*-nitrophenolate anion.

Phosphatase Activity Using [^{32}P]R_{II} Peptide. Calcineurin phosphatase activity using [^{32}P]R_{II} peptide was measured in buffer containing 50 mM MOPS, pH 7.0, 1.0 mM $MnCl_2$, 0.1 mM $CaCl_2$, 1.0 mM dithiothreitol, 1.0 μM CaM, and 1.0 mg/mL BSA, in a final volume of 50 μL. After a 10 min incubation of enzyme in buffer at 30 °C, [^{32}P]R_{II} peptide was added to 1.1 μM and the reaction allowed to proceed for 10 min at 30 °C. The reaction was stopped by addition of 0.5 mL of 5% trichloroacetic acid containing 0.1 M KH_2PO_4, and the unreacted substrate was adsorbed onto Dowex AG50W-X4 cation exchange resin (H^+ form). After vortexing to mix, the slurry was incubated on ice for 5 min and then centrifuged in a microfuge for 1 min. An aliquot (0.35 mL) of the supernatant containing [^{32}P]orthophosphate was removed and added to 5 mL of scintillation fluid and counted on a Beckman LS 6000SC scintillation counter. For each set of assays, a control containing all reagents except enzyme was used to determine nonenzymatic phosphate release.

Peptide Inhibition Assays. The assay conditions described above for [^{32}P]R_{II} peptide were used to follow calcineurin inhibition by the peptides listed in Table 1 except that the MOPS concentration was 0.25 M, and BSA was excluded from the assay buffer. For each assay, calcineurin B (133 nM) was preincubated with peptide at 30 °C for 5 min after which calcineurin A was added and incubated for an additional 5 min at 30 °C. The enzyme assay was then initiated by addition of [^{32}P]R_{II} peptide and phosphate released measured as described above. The final assay concentrations of calcineurin A and calcineurin B were 50 and 100 nM, respectively. The peptide concentrations used to estimate the IC_{50} values listed in Table 2 represent the concentration of the peptide in the final assay volume.

Gel Shift CNAPEP-Calcineurin B Binding Assays. Gel shift assays investigating binding of peptides to calcineurin B subunit were carried out in a manner similar to that described for calmodulin-binding peptides (Head & Perry, 1974; Erickson-Viitanen & DeGrado, 1987). Slab gels (5.5 cm length, 0.75 mm thickness) of 7.5% polyacrylamide, 0.375 M Tris-HCl, pH 8.8, 40% glycerol, and 0.1 mM calcium lactate with a 4% acrylamide stacking gel in 0.125 M Tris-HCl, pH 6.8, containing 0.1 mM calcium lactate without glycerol were run at constant current (25 mA) at room temperature for 1 h in a Laemmli electrode buffer system (0.192 M glycine, 0.025 M Tris, pH 8.3) containing 0.1 mM calcium lactate. Calcineurin B (20 μM final concentration) was incubated for 30 min at 23 °C with various concentrations of peptide in a final volume of 50 μL in 0.5 M MOPS, 0.1 mM calcium lactate, and 1.0 mM dithiothreitol, pH 7.2. A solution (5 μL) of 0.2% bromophenol blue in 50% glycerol and 10% β-mercaptoethanol was added to the calcineurin B peptide sample, and 20 μL was loaded onto the gel. Gels were stained with 0.05% Coomassie blue in 50% methanol and 10% acetic acid and destained in 10% methanol and 10% acetic acid.

Table 2: IC_{50} Values for Inhibition of Calcineurin B-Mediated Stimulation of Calcineurin A Phosphatase Activity by CNAPEP Peptides

peptide name	IC_{50} value (M)[a]
CNAPEP1	80×10^{-6}
CNAPEP2	3.2×10^{-6}
CNAPEP3	16×10^{-6}
CNAPEP4	5.0×10^{-6}
CNAPEP5	$>100 \times 10^{-6}$

[a] The IC_{50} values reported represent the peptide concentration that leads to a 50% loss of phosphatase activity compared to the activity of calcineurin A/calcineurin B alone. Values were estimated from a graph of activity versus log [peptide] using a minimum of seven peptide concentrations, each run at least in duplicate. The following range of peptide concentrations were used: CNAPEP1, 1.0−200 μM; CNAPEP2, 0.1−50 μM; CNAPEP3, 1.0−100 μM; CNAPEP4, 0.1−50 μM; CNAPEP5, 20−100 μM. Calcineurin A and B concentrations were 50 and 100 nM, respectively.

Inhibition of Calcineurin A/B Association by CNAPEP3. To determine whether CNAPEP3 could block the physical association of calcineurin A and B subunits, calcineurin B (5 μM final concentration) was incubated with either CNAPEP3 (200 μM final concentration) or the control peptide (350 μM final concentration) in 0.2 M Tris-HCl, pH 7.5, 1.0 mM $CaCl_2$, and 1.0 mM dithiothreitol, for 30 min at 23 °C in a volume of 100 μL. Calcineurin A was then added to a final concentration of 5 μM and incubated for an additional 5 min before adding 200 μL of a slurry of CaM-Sepharose resin equilibrated in 25 mM Tris-HCl, 1 mM $MgCl_2$, 0.10 mM $CaCl_2$ and 1.0 mM dithiothreitol, pH 7.5. The slurry was mixed by gentle rocking for 10 minutes and centrifuged at 325*g* for 1 min to pellet the resin. The supernatant was removed and the resin washed three times with CaM-Sepharose equilibration buffer by centrifugation followed by removal of the supernatant each time. Calcineurin A and associated proteins was eluted from the resin with 50 μL of equilibration buffer with 10 mM EGTA substituted for $CaCl_2$. Aliquots of the supernatant were removed, and electrophoresed on 13% SDS−polyacrylamide gels, and silver stained, and the staining intensity was analyzed by densitometry of a scanned image. Staining intensity in parallel lanes was normalized to the intensity of the calcineurin A subunit in that lane.

RESULTS

Expression of Recombinant Calcineurin B Subunit in E. coli and Purification to Homogeneity. The gene for rat calcineurin B was subcloned into the vector pT7-7 and recombinant protein expressed in the *E. coli* strain BL21-(DE3). The recombinant protein was purified to homogeneity as judged by Coomassie blue staining of the protein

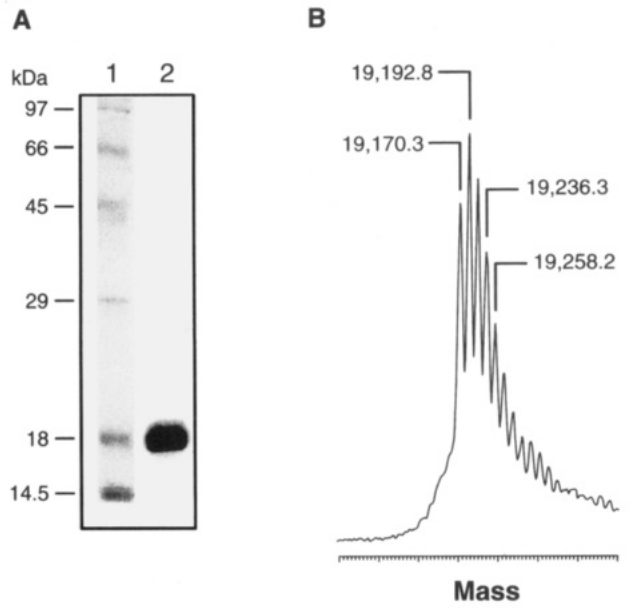

FIGURE 1: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and mass spectral analysis of purified recombinant rat calcineurin B. (A) 13% SDS−PAGE of recombinant calcineurin B. (Lane 1) Molecular mass standards: phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; β-lactoglobulin, 18 kDa; lysozyme, 14.5 kDa. (Lane 2) Calcineurin B (5 μg) purified as described under Experiment Procedures. (B) Positive ion electrospray-mass spectrum of calcineurin B with molecular masses of selected peaks indicated.

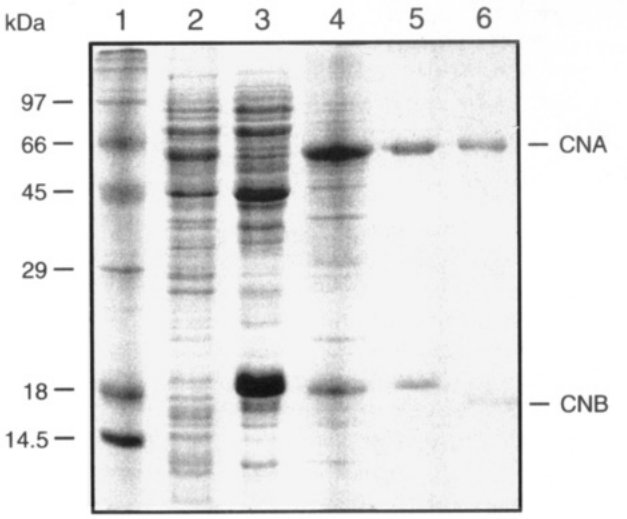

FIGURE 2: Purification of reconstituted calcineurin as followed by 14% SDS−polyacrylamide gel electrophoresis. Protein from various stages of purification was loaded into each lane. (Lane 1) Molecular mass standards (same as Figure 1). (Lane 2) Crude extract containing calcineurin A subunit from *E. coli* pCNAT77/BL21-(DE3) cells (1.7 μg). (Lane 3) Calcineurin B purified over DEAE-Sepharose CL-6B resin as described under Experimental Procedures (4 μg). (Lane 4) Reconstituted calcineurin after CaM-Sepharose chromatography (2 μg). (Lane 5) Reconstituted calcineurin after Sephacryl S-300 gel filtration chromatography (2 μg). (Lane 6) Bovine brain calcineurin (1 μg). The positions of calcineurin A and B subunits are indicated by arrows. Recombinant calcineurin B exhibits a slight decrease in mobility compared to native calcineurin B subunit (see Results and footnote 2). CNA, calcineurin A subunit; CNB, calcineurin B subunit.

following SDS−polyacrylamide gel electrophoresis (Figure 1a). Calcineurin B migrated with a molecular mass of 18−19 kDa when compared to molecular weight standards. Mass spectrometry of the purified protein using electrospray ionization yielded a molecular mass of 19 170.3 Da (Figure 1B) compared to an expected molecular mass of 19 169.9 Da calculated from the amino acid composition derived from the cDNA sequence for the protein missing the amino terminus methionine residue. The envelope of peaks obtained in Figure 1B are separated on average by 22 mass units and are diagnostic for sodium incorporation into protein. N-terminal sequencing of the purified protein gave the expected sequence, G-N-E-A-S-Y, indicating that the first methionine residue was removed during expression in *E. coli*.

Reconstitution and Purification of Recombinant Calcineurin Heterodimer. Reconstitution of recombinant calcineurin subunits was carried out by incubation of *E. coli* crude extract containing the A subunit with calcineurin B that had been partially purified (≈50% homogeneous) by anion exchange chromatography. Since both the calcineurin heterodimer as well as isolated calcineurin A bind CaM, while calcineurin B does not, an excess of the B subunit was used in the reconstitution to assure maximum yield and a stoichiometric association of subunits following CaM-Sepharose purification. Excess Ca^{2+} was added prior to CaM-Sepharose chromatography to assure binding of calcineurin B to calcineurin A (Milan et al., 1994) as well as binding of calcineurin to the resin. Purification of the reconstituted protein mixture using CaM-Sepharose affinity chromatography and Sephacryl S-300 gel filtration chromatography yielded a highly purified protein fraction that was ≥95% homogeneous and exhibited a 1.0 calcineurin A/calcineurin B subunit ratio based on densitometric analysis of Coomassie-stained 13% SDS−polyacrylamide gels (Figure 2). This two-step purification resulted in a 22-fold increase in the specific activity toward pNPP over that observed in crude extracts with an overall yield of ≈25% (data not shown). A significant level of purification was achieved initially by use of CaM-Sepharose affinity chromatography with stoichiometric quantities of both A and B subunits copurifying after reconstitution. Gel filtration chromatography was used as a final step to resolve calcineurin from several minor contaminants. During gel filtration chromatography, a small portion (≈20% of calcineurin A) dissociated into individual A and B subunits. For subsequent characterizations, the major eluting peak containing the intact heterodimer was collected. A slight increase in CaM stimulation in both CaM-Sepharose and gel filtration fractions was observed over that for crude extract, presumably due to the separation of calcineurin from contaminating *E. coli* phosphatases during purification. Qualitatively, the increase in specific activity at each stage of the purification paralleled the purity of calcineurin heterodimer as judged by SDS−PAGE shown in Figure 2, indicating that the reconstituted enzyme activity is stable during the course of the purification.

Characterization of Reconstituted Calcineurin. The calmodulin-dependent phosphatase activity of reconstituted calcineurin was comparable to that of native bovine calcineurin. Thus, using 1.1 μM [^{32}P]R_{II} peptide, reconstituted calcineurin had a specific activity of 10.4 nmol $^{32}P_i$ released·min^{-1}·mg^{-1}, compared to 17.1 nmol of $^{32}P_i$ released·min^{-1}·mg^{-1} for native calcineurin. With 10 mM *p*-nitrophenylphosphate (pNPP) as substrate, the activity of reconstituted calcineurin was slightly higher than that of native enzyme. With pNPP, reconstituted calcineurin had a

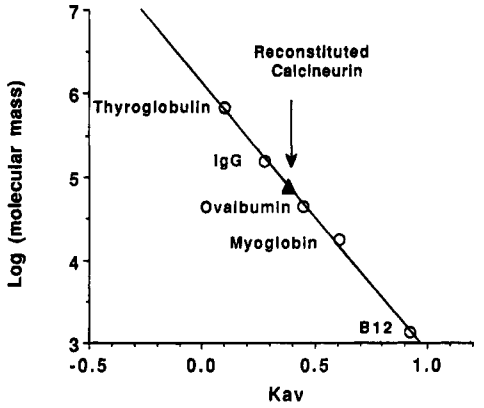

FIGURE 3: Plot of log(molecular mass) versus K_{av} for molecular mass standards and recontituted calcineurin heterodimer. K_{av} values were calculated from the elution times during gel filtration chromatography on Sephacryl S-300 as described under Experimental Procedures. The open circles represent K_{av} values for the molecular mass standards: thyroglobulin (6.7×10^5 Da); bovine γ-globulin (IgG), (1.58×10^5 Da); chicken ovalbumin (4.4×10^4 Da); myoglobin (1.7×10^4 Da); and cyanocobalamin (B12) (1.35×10^3 Da). The filled triangle represents the K_{av} value for reconstituted calcineurin (indicated by the arrow in the figure).

specific activity of 1.76 μmol·min^{-1}·mg^{-1} in the presence of CaM. Native bovine calcineurin, on the other hand, had a specific activity of 0.3−1.0 μmol·min^{-1}·mg^{-1}, depending upon the preparation.

The behavior of reconstituted calcineurin during gel filtration chromatography also was indistinguishable from that of native calcineurin. Thus, reconstituted calcineurin eluted with a molecular mass of 77 kDa during gel filtration chromatography on Sephacryl S-300 (Figure 3), consistent with the formation of a native-like heterodimer.

Inhibition of Calcineurin Phosphatase Activity with Calcineurin B Binding Peptides. Five overlapping, 20-residue peptides (Table 1) comprising the putative calcineurin B-binding domain of calcineurin A were synthesized and tested for their ability to bind to calcineurin B and effect heterodimer formation. An 18-residue peptide representing the sequence from mature human factor VIII was used as a negative control. Incubation of purified recombinant calcineurin A catalytic subunit with purified recombinant calcineurin B markedly enhanced the phosphatase activity, with a >20-fold stimulation observed over the phosphatase activity of calcineurin A alone (Figure 4). In the presence of each of the five calcineurin A peptides, but not the control peptide, calcineurin B-mediated stimulation of calcineurin phosphatase activity toward [^{32}P]R_{II} peptide was notably inhibited, although the concentration of peptide required for maximum inhibition varied for each peptide.

Dose−response curves for each peptide representing inhibition of calcineurin B-dependent stimulation of calcineurin A phosphatase activity were obtained. The IC_{50} values reported in Table 2 represent the concentration of peptide that resulted in 50% inhibition of the phosphatase activity of calcineurin A in the presence of calcineurin B. The most effective peptides were CNAPEP4 and CNAPEP2, with IC_{50} values of 3.2 and 5.0 μM, respectively (Table 2). CNAPEP3 also inhibited calcineurin B stimulation of phosphatase activity with a slightly higher IC_{50} value of 16 μM. The peptides with the weakest affinity for calcineurin B were CNAPEP1 and CNAPEP5. CNAPEP1 had an IC_{50} value of 80 μM while a value for CNAPEP5 was not determined

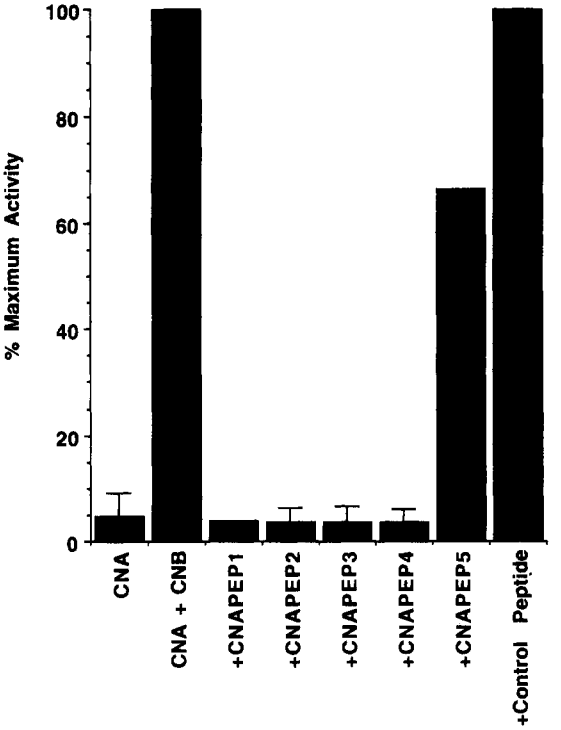

FIGURE 4: Inhibition of calcineurin B stimulation of calcineurin A phosphatase activity by the peptides listed in Table 1. Phosphatase assays used [^{32}P]R_{II} peptide of calcineurin A alone, calcineurin A combined with calcineurin B, and calcineurin A combined with calcineurin B in the presence of various concentrations of CNAPEP1−5 or the control peptide. The concentrations of each peptide used are as follows: CNAPEP1, 200 μM; CNAPEP2, 20 μM; CNAPEP3, 100 μM; CNAPEP4, 20 μM; CNAPEP5, 100 μM; control peptide, 700 μM.

due to the high concentration of peptide required. From Figure 4, the activity in the presence of 100 μM CNAPEP5 was 66% of the activity in the absence of peptide indicating an IC_{50} > 100 μM.

Peptide Binding to Calcineurin B. A gel shift assay using nondenaturing polyacrylamide gels was employed in order to demonstrate peptide binding to calcineurin B. Calcineurin B incubated with either CNAPEP3 or CNAPEP4 exhibited an increase in electrophoretic mobility versus calcineurin B alone (Figure 5), indicating a direct physical association between calcineurin B and these peptides. The electrophoretic mobility of calcineurin B increased with increasing peptide concentration for both peptides. For CNAPEP3 diffuse yet distinct bands corresponding to free calcineurin B and calcineurin B·CNAPEP3 complex were observed in some gels (data not shown). CNAPEP2 also interacted with calcineurin B to form a complex which, however, did not enter the gel during electrophoresis.

Calcineurin B did not undergo any change in electrophoretic mobility in the presence of CNAPEP1 up to 200 μM. Gel shifts with CNAPEP5 were not attempted since, from the IC_{50} value listed in Table 2, it was estimated that peptide concentrations >200 μM range would be required before any significant shift in electrophoretic mobility would be observed. Lastly, gel shift experiments with all five peptides failed to show any complex formation with CaM (data not shown).

Inhibition of Calcineurin A/B Association by CNAPEP3. To determine whether one of the inhibitory peptides of Table

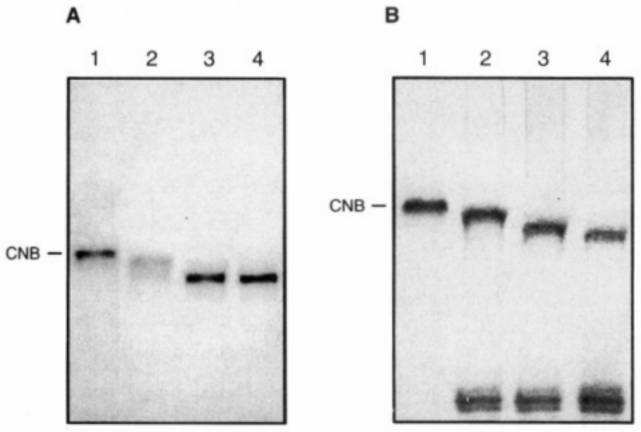

FIGURE 5: Mobility shift of calcineurin B during native polyacrylamide gel electrophoresis. (A) Calcineurin B in the presence of CNAPEP3: lane 1, 20 μM calcineurin B alone, lanes 2−4, 20 μM calcineurin B in the presence of 20, 100, and 200 μM CNAPEP3, respectively. (B) Calcineurin B in the presence of CNAPEP4: lane 1, 20 μM calcineurin B alone; lanes 2−4, 20 μM calcineurin B in the presence of 93, 175, and 248 μM CNAPEP4, respectively. The bands appearing at the bottom of the gel in B are from CNAPEP4.

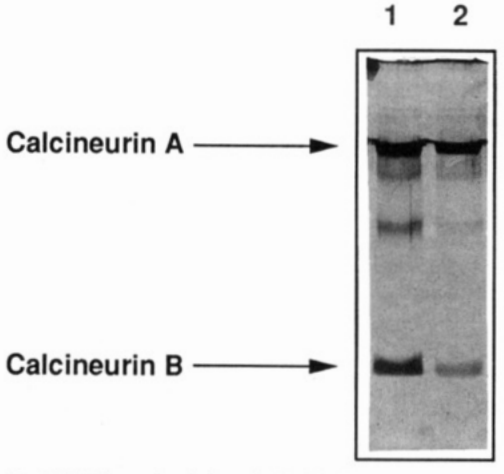

FIGURE 6: Inhibition of calcineurin B binding to calcineurin A by CNAPEP3. Calcineurin B was preincubated with either 350 μM control peptide (lane 1) or 200 μM CNAPEP3 (lane 2) prior to adding calcineurin A and the mixture subsequently purified via calmodulin-Sepharose affinity chromotography. Calcineurin A and associated proteins were eluted with EGTA-containing buffer, electrophoresed on 13% SDS−polyacrylamide gels, and silver stained. The positions of calcineurin A and B subunits are indicated with arrows.

1 could block physical association of the calcineurin heterodimer, CNAPEP3 was preincubated with calcineurin B in Ca^{2+}-containing buffer prior to adding calcineurin A. After a 5 min incubation, the mixture was adsorbed onto CaM-Sepharose resin and washed with buffer containing Ca^{2+}, and calcineurin A and associated proteins (e.g., calcineurin B) were eluted with buffer containing EGTA. CNAPEP3, but not the control peptide, significantly blocked heterodimer formation as evidenced by the decrease in calcineurin B coeluting with calcineurin A (Figure 6). Densitometric analysis of the polyacrylamide gel in Figure 6 indicated that 68% less calcineurin B copurified with calcineurin A in the presence of CNAPEP3 versus the control peptide.

DISCUSSION

Although calcineurin can be obtained from bovine brain, the use of a fully recombinant form of calcineurin has obvious advantages for future mutagenesis work. Furthermore, since native bovine calcineurin appears to be a mixture of several isoforms (Billingsley et al., 1985), experiments which require homogeneous preparations such as protein crystallography and peptide mapping are best undertaken using recombinant enzyme. In this report, recombinant forms of both calcineurin A and B subunits have been produced in *E. coli* and purified as isolated subunits as well as a reconstituted heterodimer.

Recombinant rat calcineurin A produced in *E. coli* exhibits CaM-dependent phosphatase activity, and its properties have already been described (Haddy et al., 1992; Haddy & Rusnak, 1994). Recombinant calcineurin B produced here also was functional, since, upon its addition to isolated recombinant calcineurin A, a substantial increase in phosphatase activity occurred as expected. Interestingly, the recombinant calcineurin B subunit produced in this study exhibited a notable difference in electrophoretic mobility during SDS−PAGE compared to bovine brain calcineurin B subunit. Thus, recombinant rat calcineurin B migrated with an expected molecular mass of 18−19 kDa as shown in Figures 1A and 2. Calcineurin B from bovine brain, whose N-terminus is myristoylated (Aitken et al., 1984), migrated with an aberrant molecular mass of 16 kDa. Edman degradation of recombinant calcineurin B gave the correct N-terminus while mass spectrometry yielded the expected mass for a full length polypeptide missing the first methionine residue. The difference in electrophoretic mobility between recombinant calcineurin B versus native bovine brain calcineurin is due solely to the lack of cotranslational N-terminus myristoylation of the recombinant subunit.[2]

Using bacculovirus expressed calcineurin A reconstituted with calcineurin B isolated from native bovine brain, Perrino et al. (1992) found activities of 0.38−0.68 μmol·min^{-1}·g^{-1} under V_{max} conditions for both native and reconstituted calcineurin using either [^{32}P]R_{II} peptide or pNPP as substrate. In this study, the phosphatase activity of recombinant calcineurin was also comparable to bovine brain calcineurin using either of these substrates. The 100-fold difference in activity of either bovine brain or recombinant calcineurin toward [^{32}P]R_{II} peptide versus pNPP is due to the fact that the concentration of R_{II} peptide used in this study ($\approx$1 μM) was less than the K_m of that substrate for calcineurin [26 μM (Blumenthal et al., 1986)]. In fact, the activities of both native and recombinant calcineurin increased correspondingly at higher R_{II} peptide concentration (data not shown).

In addition to its native-like phosphatase activity, the reconstituted enzyme exhibited a molecular mass during gel filtration chromatography of $\approx$77 kDa[3] indicating a native-like $\alpha\beta$ heterodimer; bovine brain calcineurin chromatographs similarly (Wallace et al., 1978a,b; Sharma et al., 1979).

[2] Recombinant myristoylated rat calcineurin B exhibits an electrophoretic mobility on SDS−PAGE identical to that of myristoylated native bovine calcineurin B (Aitken et al., 1984). Furthermore, mass spectrometry of recombinant myristoylated rat calcineurin B yields a molecular mass of 19 380.7 (calculated: 19 380.0) (M. Kennedy and F. Rusnak, manuscript in preparation).

[3] It is more appropriate to correlate the elution volume of macromolecules during gel filtration chromatography with the Stokes radius rather than the molecular mass. For proteins that possess similar frictional ratios, partial specific volumes, and sedimentation coefficients, the elution volume can be used to estimate molecular mass (Siegel & Monty, 1966).

The marked stimulation of phosphatase activity upon addition of calcineurin B provides a convenient assay to probe calcineurin A−calcineurin B interactions. We have used this assay and a series of overlapping peptides to investigate the binding site of calcineurin B on the A subunit. The choice of the five peptides listed in Table 1 was based on sequence alignments of calcineurin A from mouse (Kincaid et al., 1988; Ito et al., 1989; Kuno et al., 1989; Muramatsu et al., 1992), human (Guerini & Klee, 1989; Kincaid et al., 1990; Muramatsu & Kincaid, 1993), rat (Saitoh et al., 1991), *Neurospora crassa* (Higuchi et al., 1991), *Saccharomyces cerevisiae* (Liu et al., 1991; Cyert et al., 1991), and *Drosophila melanogaster* (Guerini et al., 1992) that revealed a highly conserved region of circa 50 residues between the active site and CaM-binding domains. Guerini et al. (1992) proposed a minimal 40-residue domain on the basis of a similar analysis and indicated in a footnote that calcineurin B could interact with a 27-residue peptide within this domain.

Studies investigating the interaction of calcineurin with either CsA·cyclophilin or FK506·FKBP immunosuppressant drug complexes (Husi et al., 1994; Clipstone et al., 1994) provided the first qualitative data that the highly conserved region corresponding to residues 333−391 of rat calcineurin A (Kincaid et al., 1988) comprises the calcineurin B binding site. Thus, using deletion mutants of calcineurin A, Clipstone et al. (1994) found that calcineurin B coimmunoprecipitated with calcineurin A only when residues 355−376 were present. The calcineurin B binding domain of calcineurin A might therefore be represented at least in part by these residues. Interestingly, cross-linking between calcineurin B and FKBP does not occur in the absence of a 59-residue peptide comprising this region or in the presence of two smaller fusion proteins representing the amino and carboxy termini of the full-length 59-residue putative calcineurin B binding polypeptide (Husi et al., 1994). Although these shorter versions still appeared to interact with the B subunit, it was not possible to precisely define the exact number or positions of calcineurin A residues in contact with calcineurin B.

In the present study, the five overlapping peptides listed in Table 1 span the entire 59 residues of human calcineurin A identified by Husi et al. (1994) and the domain noted by Guerini et al. (1992). Data generated using these shorter peptides provide unequivocal evidence that this region represents the calcineurin B binding domain. Thus, these peptides were able to block CNB-dependent stimulation of catalytic subunit phosphatase activity. From the inhibition data of Table 2, the two abutting peptides, CNAPEP2 and CNAPEP4, which together span 40 residues of rat calcineurin A and represent the "core" sequence of the five peptides, have the most potent affect on calcineurin B stimulation of catalytic subunit phosphatase activity. Not surprisingly, CNAPEP3 also inhibits the stimulation of phosphatase activity by calcineurin B with a comparable IC_{50} value. CNAPEP1 and CNAPEP5, which overlap CNAPEP2 and CNAPEP4, respectively, by 10 residues, have a much reduced potency for inhibiting calcineurin B mediated stimulation. It would thus appear that the flanking 10 residues unique to CNAPEP1 (328−337) and CNAPEP5 (378−387) do not contribute significantly to inhibition and may not be involved in binding to calcineurin B. For peptides 1−4, the residual phosphatase activity in the presence of saturating concentrations of these peptides (≈4%, Figure 4) corresponds to the activity of the calcineurin A subunit alone from that figure, suggesting that these peptides inhibit by binding to calcineurin B, preventing it from enhancing the phosphatase activity of the catalytic subunit.

Although these peptides block calcineurin B stimulation of calcineurin A activity, these data alone do not prove they represent to the calcineurin B binding domain. Alternatively, they could exert their inhibitory activity by interacting with another region on calcineurin A such as the active site. Therefore, it is important to show that these peptides bind directly to calcineurin B. An interaction between calcineurin B and CNAPEP2, CNAPEP3, and CNAPEP4 was demonstrated using native polyacrylamide gel electrophoresis, a technique that has been used to characterize CaM-binding peptides (Head & Perry, 1974; Erickson-Viitanen & DeGrado, 1987). With the peptides used in the CaM studies, the CaM·peptide complex was stable during the course of electrophoresis, and two bands corresponding to free CaM and the CaM·peptide complex were observed. With the peptides CNAPEP3 and CNAPEP4, however, typically only one band was observed and the migration of this band varied with increasing peptide concentrations (Figure 5). This behavior is expected if the half-life for dissociation of the calcineurin B·peptide complex was comparable to the time required for electrophoresis. Thus, dissociation during electrophoresis would preclude the observation of distinct free calcineurin B and calcineurin B·peptide complexes. Rather, an electrophoretic shift dependent upon the concentration of peptide would occur and also result in a "smearing" of the calcineurin B band as it migrated through the gel. Binding of CNAPEP2 to calcineurin B was also evident although, with that peptide, the complex did not enter the gel during electrophoresis. CNAPEP2 has limited solubility in aqueous solutions, a result of its hydrophobic nature. One possible explanation for the native gel electrophoresis results with CNAPEP2 could be that its interaction with calcineurin B leads to aggregation.

Native gel electrophoresis could not confirm calcineurin B binding to CNAPEP1 or CNAPEP5. With CNAPEP5, the concentration of peptide required for formation of a calcineurin B·CNAPEP5 complex could not be attained due to limitations in the amount of peptide available. Calcineurin B in the presence of CNAPEP1 did not undergo a shift in mobility during native PAGE, even at 200 μM peptide concentration. With an IC_{50} value of 80 μM from phosphatase inhibition assays, this concentration should have been sufficient to observe a calcineurin B·CNAPEP3 complex. It is possible that this peptide inhibits by interacting with a region on the A subunit rather than calcineurin B.

Further confirmation that these peptides represent a calcineurin B binding motif comes from experiments which show that one peptide was able to block calcineurin A·B heterodimer formation. Thus, incubation of calcineurin B with CNAPEP3 prior to reconstitution with calcineurin A dramatically reduced the amount of calcineurin B that copurified with the catalytic subunit following affinity chromatography on CaM-Sepharose. The ability of CNAPEP3 to block subunit association was significant but not complete. This probably results from the fact that CNAPEP3 does not represent the complete binding motif and has to compete with the intact catalytic subunit which has an affinity for

the B subunit that has been estimated to be nanomolar or less (Hao & Klee, 1993).

From these data it appears that CNAPEP2, CNAPEP3, or CNAPEP4 are equivalent in their abilities to bind calcineurin B and affect its interaction with calcineurin A. From that we conclude that the minimum calcineurin B binding domain is comprised of calcineurin A residues 338−377 but do not rule out the possibility that other calcineurin A residues could participate in binding calcineurin B. Since none of the individual peptides utilized in this study spanned this entire region, it is not surprising that they inhibit with micromolar inhibition constants. As a result, longer peptides representing the full length domain would be expected to bind tighter. In fact, while this paper was being revised, Watanabe et al. (1995) have found that a glutathione S-transferase fusion protein with calcineurin A residues 328−390 inhibited activity with an IC_{50} of 50−100 nM.

ACKNOWLEDGMENT

We are gratefully indebted to Dr. Brian Perrino and Dr. Thomas Soderling for providing cDNA encoding rat calcineurin A and calcineurin B subunits. We thank Drs. Stephen Naylor and Ken Johnson of the Mass Spectrometry Core Facility at the Mayo Clinic and Foundation for their assistance with mass spectrometry of calcineurin B. We also acknowledge Mr. Christopher Czaja for his assistance with reconstitution and purification of recombinant calcineurin.

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BI9504421