Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 8983–8987, December 1988
Biochemistry

Characterization of a cDNA clone encoding the calmodulin-binding domain of mouse brain calcineurin

(protein phosphatase/enzyme structure)

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Communicated by Martha Vaughan, September 6, 1988

ABSTRACT **A cDNA clone corresponding to a portion of the catalytic subunit of calmodulin (CaM)-dependent phosphoprotein phosphatase (calcineurin) was isolated from a murine brain library by expression vector immunoscreening. A β-galactosidase fusion protein that reacted on Western blots with anti-calcineurin antibodies and biotinylated CaM was purified in preparative amounts using CaM-Sepharose affinity chromatography. Partial digestion of the hybrid protein with *Staphylococcus aureus* V-8 protease produced several immunoreactive peptides that appeared identical to fragments generated from authentic brain calcineurin. The 1111-base-pair (bp) *Eco*RI insert contained an open reading frame encoding a protein of 35 kDa followed by a 190-bp 3′ noncoding region; seven peptides obtained by partial amino acid sequencing of the bovine brain enzyme were found in the deduced sequence. A domain ≈12 kDa from the carboxyl terminus was deduced to be the CaM-binding site based on consensus structural features and a sequence of seven amino acids highly related to smooth muscle myosin light-chain kinase. Two regions with identity to protein phosphatases 1 and 2A were found in the amino half of the cloned sequence; however, the intervening sequence contained apparent insertions, suggesting splicing of subdomains. Thus, the structure of calcineurin is chimeric, consisting of conserved catalytic elements and a regulatory CaM-binding domain.**

Regulation of phosphoprotein metabolism by specific classes of protein kinases and phosphatases is important for a wide spectrum of biological activities (1–3). The classical studies on the modulation of kinases involved in control of glycolytic enzymes point to a central role for protein phosphorylation in maintenance of intermediary metabolism (for a review, see ref. 4). More recent observations regarding the role of phosphorylation in hormone receptor function (5) and in the expression of oncogenes (6, 7) suggest that specific biochemical transduction pathways utilize phosphorylation events to alter signaling responses. A role for Ca^{2+}-regulated phosphoproteins in neurotransmission has been implicated by the high concentrations of the calmodulin (CaM)-dependent protein kinase in synaptic densities (8, 9) and the phosphorylation of specific neuronotypic substrates under conditions of increased synaptic activity (10).

In general, much less information is available regarding the role of phosphoprotein phosphatases in regulation of cellular responses. The major subgroups of phosphatases, distinguished by their substrate specificity and regulation by peptide inhibitors (11), are present in various proportions in different tissues. One of these is a CaM-dependent form (12) also called calcineurin (13) or phosphatase 2B (14) that is present in highest concentration in nervous tissue. Calcineurin is composed of both catalytic and regulatory subunits (60 and 18 kDa, respectively), the latter itself a calcium-binding peptide with structural similarities to CaM (13, 15). The activity of this phosphatase is highly stimulated by divalent cations, such as Ni^{2+}, Mn^{2+}, and Co^{2+} (16–20), in a time-dependent fashion (18), suggesting metal-induced high activity conformations of the catalytic subunit. However, studies of the enzyme reconstituted from the dissociated subunits indicate that the isolated catalytic subunit has very low intrinsic activity, whether or not CaM is present, unless it is complexed to its regulatory subunit (21, 22). This suggests that the smaller subunit does not simply inhibit activity in the absence of added CaM but confers catalytic competence to the holoenzyme.

To investigate the structural features of the catalytic subunit that regulate activity and to compare the CaM-binding domain with those of other CaM-binding proteins, we have isolated a cDNA clone encoding the CaM-binding portion of the protein.¶ The present study describes the characterization of this clone and discusses its deduced structural features in relation to those of other CaM-regulated enzymes and protein phosphatases.

METHODS

Materials. A murine brain λgt11 library having 1.5×10^5 independent recombinants was obtained from the American Type Culture Collection; this library was donated by C. Puckett, J. Kamholz, and R. A. Lazzarini (National Institutes of Health). Cultures of *Escherichia coli* 1090r$^-$ and BNN-103 were the generous gifts of Edward Ginns (National Institute of Mental Health). Plasmid pUC19 and competent DH-5α cells were purchased from Bethesda Research Laboratories. CaM-Sepharose (3 mg of protein per ml of gel) was prepared as described (23) using CaM purified by melittin-Sepharose chromatography (24). Biotinylated CaM was prepared and used essentially as described (25).

Expression Vector Immunoscreening. Nutrient agar plates (diameter, 150 mm), seeded with ≈20,000 plaque-forming units of phage were induced and then overlaid with isopropyl β-D-thiogalactoside (IPTG)-saturated nitrocellulose filters as described (26). Duplicate lifts of 3 hr and 5 hr were washed in 50 mM Tris·HCl (pH 8) containing 150 mM NaCl (Tris-buffered saline; TBS) and then incubated for 20 min with blocking solution (5% nonfat dry milk in TBS plus 10 mM sodium azide and 0.01% thimerosal). Affinity-purified anti-CN antibody (27, 28) that had been adsorbed with immobi-

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Abbreviations: CaM, calmodulin; MLCK, myosin light-chain kinase; IPTG, isopropyl β-D-thiogalactoside.

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¶The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04134).

lized *E. coli* lysate (Promega Biotec, Madison, WI) was diluted into blocking solution and incubated with the plaque-containing filters (8 ml per filter) for 2.5 hr. After washing with TBS plus 0.05% Tween 20, filters were incubated for 1 hr with alkaline phosphatase-conjugated rabbit anti-goat IgG (Kirkegaard and Perry, Gaithersburg, MD) and washed as described above. Immunopositive signals were detected with the chromogens nitroblue tetrazolium and bromochloroindolyl phosphate, and the corresponding plaques were excised with sterile pipette tips. After overnight elution of phage in 1 ml of 10 mM Tris·HCl (pH 8) plus 10 mM $MgCl_2$ (SM), a portion was diluted 1:2000 and 20 μl was used to seed nutrient plates for testing of positives.

Amino Acid Sequence Analysis. Homogeneous bovine brain calcineurin was purified as described (29) and the 60-kDa catalytic subunit was separated from the 18-kDa subunit by reverse-phase chromatography on a Vydac-C4 high-performance column (Hesperia, CA) using a gradient of 30–60% acetonitrile in aqueous 0.1% trifluoroacetic acid. Lyophilized fractions were digested with trypsin [trypsin/subunit, 1:100 (wt/wt)] after solubilization in buffer containing 0.1% sodium dodecyl sulfate. Tryptic peaks were separated by using a C-18 reverse-phase column and the peptides were sequenced with an Applied Biosystems 470 gas-phase sequenator (Foster City, CA) with a model 120 phenylthiohydantoin amino acid analyzer. A total of 10 peptides were sequenced, 7 of which were found in the deduced sequence of the clone.

Expression of Recombinant Fusion Proteins. An overnight culture of the lysogen host BNN-103, grown in NZCYM medium (30) lacking ampicillin, was diluted 1:200 (10^7 cells per ml) and 0.2 ml of this was infected with 0.1 ml of amplified phage stock (30 min, 30°C). After diluting 1:5000, 100 μl was spread onto fresh plates and grown at 30°C. Individual colonies were transferred to duplicate plates at 30°C and 43°C and samples that did not grow at 43°C (lysogens) were selected. For expression of fusion protein, 500 ml of NZCYM medium was inoculated with 5 ml of an overnight culture and grown at 30°C until the OD_{660} was 0.5 (2–2.5 hr). After adding 2 ml of sterile 1 M IPTG, cultures were "heat-shocked" for 20 min at 43°C and incubated at 37°C for an additional 1–2 hr. Cells were centrifuged, frozen in a dry ice/ethanol mixture, and sonicated on ice in 6 vol of 10 mM Tris·HCl (pH 8) plus 1 mM EDTA (TE) containing 10 μg of soybean trypsin inhibitor per ml plus 20 μg each of leupeptin and pepstatin A per ml. Bacterial sonicates were centrifuged (20,000 × *g* for 30 min) and the supernatant was used for further analysis.

Subcloning and cDNA Sequencing. Recombinant phage from clone CNα-1 were amplified by lysis on a 150-mm nutrient plate and collected in 10 ml of sterile SM. Phage were purified from the clarified extract with 200 μl of immobilized anti-phage antibody (LambdaSorb, Promega Biotec) and their DNA was prepared as described by the manufacturer. A portion (5–6 μg) of the phage DNA was digested with 50 units of *Eco*RI (Bethesda Research Laboratories), phenol/chloroform-extracted, and precipitated with 1 vol of 15% polyethylene glycol in 1.5 M NaCl. Restriction fragments were ligated, without additional purification, into dephosphorylated pUC19 (350 ng of phage DNA per 50 ng plasmid DNA) using 2 units of T-4 ligase (Bethesda Research Laboratories) per reaction. DNA was transfected into DH-5α cells, and transformants producing colorless colonies on 5-bromo-4-chloro-3-indolyl β-D-galactoside plates were selected.

DNA sequencing was done with plasmid DNA isolated by the alkaline lysis method (30) that was treated with RNase prior to denaturing with 0.2 M NaOH (31). Primer-directed dideoxy sequencing (32) with Klenow reagent kits from either New England BioLabs or Promega Biotec was carried out on 6% buffer gradient gels using the SequiGen system (Bio-Rad). Independent analysis was carried out by automated DNA sequencing with the DuPont Genesis 2000 System using Sequenase (United States Biochemical, Cleveland). Specific oligonucleotide primers were synthesized with a Beckman System-1 Plus DNA synthesizer.

RESULTS

Selection of cDNA Clones by Expression Vector Immunoscreening and Characterization of Fusion Protein. A murine brain library constructed in λgt11 was screened with affinity-purified antibody to bovine brain calcineurin. One immunopositive clone (CNα-1) was strongly inducible, the reaction on filters containing 10 mM IPTG being 3- to 4-fold greater than those lacking pyranoside (data not shown). When crude lysates of lysogen containing the recombinant phage were tested by immunoblotting procedures, the major calcineurin-immunoreactive band coincided with anti-β-galactosidase immunoreactivity. In addition, when electroblotted extracts were probed with biotin-labeled CaM (25), a Ca^{2+}-dependent interaction was observed, suggesting that the protein contained a functional CaM-binding domain.

Bacterial extracts from IPTG-induced cultures of BNN-CNα-1 were prepared in the presence of several protease inhibitors. Attempts to purify the fusion protein by using anti-β-galactosidase coupled to Sepharose 4B (Protosorb, Promega Biotec) were unsuccessful.[‖] However, since the fusion protein specifically interacted with CaM on electroblots, CaM-Sepharose chromatography was carried out. The supernatant from a 500-ml culture was adjusted to 2 mM Ca^{2+} and applied to 5 ml of the CaM affinity gel; >98% of the protein was not retained. After washing extensively with Ca^{2+}-containing buffers (23), the column was eluted with 5 mM EGTA, yielding 4 mg of a protein of ≈155 kDa (Fig. 1, lane 1). As suggested by earlier analytical experiments, this protein displayed immunoreactivity toward both the β-galactosidase and calcineurin antibodies and bound biotinylated CaM in a Ca^{2+}-specific fashion (lanes 2–7).

To further confirm that the fusion protein contained a portion of the catalytic subunit of calcineurin, it was subjected to partial digestion with staphylococcal V-8 protease and its fragments were compared to those of authentic bovine brain enzyme. Several proteolytic fragments from the cloned fusion protein reacted with anti-calcineurin antibody and appeared to have the same mobility as peptides of 30, 25, and 22 kDa produced by cleavage of the bovine brain enzyme (Fig. 2). This correspondence of immunoreactive peptides strongly supported the identity of the cloned fragment as being part of the 60-kDa subunit of calcineurin.

cDNA Sequence of CNα-1: Identification of the Putative CaM-Binding Domain and Conserved Phosphatase Regions. The *Eco*RI insert of CNα-1 (1111 bp) was sequenced after subcloning into pUC19, and specific oligonucleotides were used to prime sequencing reactions (Fig. 3). An open reading frame of 921 bp, encoding a protein of ≈35 kDa, was followed by a 3′ noncoding region of 190 bp that contained tandem polyadenylylation sites at nucleotides 1069 and 1075. However, the expected polyadenylylated "tail" was not observed, suggesting that this cDNA may have been primed from an internal adenosine-rich sequence, as seen by others (33). Within the coding region, sequences corresponding to 7 of the 10 peptides obtained by gas-phase sequencing were observed (see underlined regions), providing direct confirmation of the authenticity of the clone.

A search for regions similar to the CaM-binding domain described for other proteins revealed an area 110 residues

[‖] It is paradoxical that the same monoclonal antibody used for immunoblotting was ineffective as an immunoaffinity adsorbent. Presumably, the immobilized antibody was unable to interact with the free solution conformation of the fusion protein.

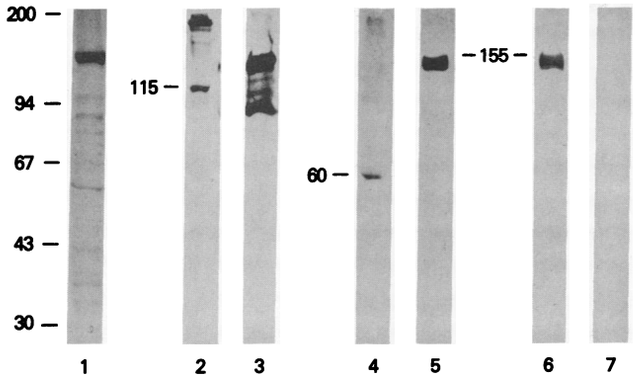

FIG. 1. Characterization of fusion protein from BNN-CNα-1 by immunoreactivity and specific binding to biotinylated CaM. Portions of fusion protein (300 ng) isolated by using CaM-Sepharose were electrophoresed on a sodium dodecyl sulfate gel (7% acrylamide) and electroblotted onto nitrocellulose. These were analyzed for specific immunoreactivity toward anti-β-galactosidase (Promega Biotec) (lane 3) and anti-calcineurin (lane 5) antibodies and binding to biotinylated CaM in the presence of 1 mM Ca^{2+} (lane 6) or 5 mM EGTA (lane 7). Detecting reagents were alkaline phosphatase conjugates of goat anti-mouse IgG (Promega Biotec), rabbit anti-goat (Kirkegaard and Perry), and streptavidin (Bethesda Research Laboratories), respectively. Lane 1 shows Coomassie blue staining of 1 μg of the CaM-Sepharose eluate; the positions of molecular mass markers (kDa) are given on the ordinate. Lanes 2 and 4 contain prestained standards (Bethesda Research Laboratories) containing 50 ng of β-galactosidase (115 kDa) and 50 ng of calcineurin (60 kDa), respectively, blotted in the same experiment. The additional bands in lane 3 represent degradation products of the fusion protein.

from the carboxyl terminus having the consensus amphipathic properties—i.e., groups of positively charged and hydrophobic amino acid side chains (Fig. 4). In fact, one 7-residue stretch, Ile-Arg-Ala-Ile-Gly-Lys-Met, contained a remarkable correspondence to the sequence from the smooth muscle myosin light-chain kinase (MLCK) domain, Val-Arg-Ala-Ile-Gly-Arg-Leu (33), having a 4-amino acid identity flanked by 3 conservative substitutions. The nucleotide sequence encoding this region showed only four differences, giving 81% identity; the significance of this strong relatedness is unclear. The predicted secondary structure of the larger

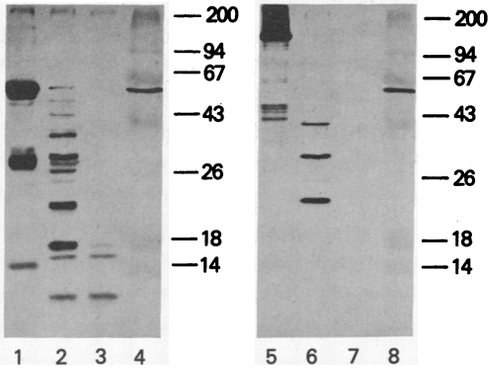

FIG. 2. Comparison of anti-calcineurin immunoreactivity of proteolytic fragments of calcineurin and isolated fusion protein. Samples (2 μg) of purified bovine brain calcineurin (lanes 1–3) and isolated fusion protein (lanes 5–7) were incubated in a vol of 50 μl (30 min, 37°C) with increasing concentrations of staphylococcal V-8 protease in 0.1% sodium dodecyl sulfate prior to electrophoresis (12% gels) and transfer to nitrocellulose. After blocking and incubation with goat anti-calcineurin antibody (28), blots were developed with alkaline phosphatase-conjugated antibodies. Lanes: 1 and 5, no protease added; 2 and 6, protease at 3 μg/ml; 3 and 7, protease at 10 μg/ml; 4 and 8, prestained protein standards (kDa) containing 50 ng of bovine brain calcineurin.

domain (24 amino acids) showed a defined region of α-helix from residues 5–22. Construction of a helical wheel of 18 amino acids (corresponding to 3.6 residues per helical turn) suggested, as noted by others (35, 36), the presence of dominant apposed cationic and hydrophobic environments on the helix (Fig. 4). Also, as observed for several other CaM-dependent enzymes, a consensus sequence for phosphorylation, Arg-Xaa-Xaa-Ser, was present near the carboxyl end of the domain (Fig. 4).

A comparison between the deduced amino acid sequences of CNα-1 and protein phosphatases 1 (37) and 2A (38–40) showed two small regions of striking identity (Fig. 5) and a larger segment between them with much less similarity. Over the entire region, the sequence of CNα-1 showed 36% identity with phosphatase 1 and 40% with phosphatase 2A (phosphatases 1 and 2A were 48% identical over this region). The two highly conserved areas, each having 10–14 residues, were separated by 65 amino acids in the calcineurin clone, while the corresponding intervening region in the other enzymes was 51 or 52 residues; when aligned to maximize identity, the deduced sequence of CNα-1 showed two apparent insertions. These data suggest that, in calcineurin, several subdomains have been spliced to give a region of catalytic function; this, in turn, is fused to a carboxyl domain containing a regulatory (i.e., CaM-binding) function.

DISCUSSION

We have isolated a cDNA clone that encodes the CaM-binding domain of the CaM-dependent phosphoprotein phosphatase. As in the cloning of two other CaM-dependent enzymes, MLCK (33) and the brain multifunctional protein kinase (34), a fusion protein selected by immunoreactivity retained its ability to bind a regulatory macromolecule—i.e., CaM—providing separate lines of evidence for its identity. In the present study, immunological criteria were combined with proteolytic peptide mapping (41) to confirm the authenticity of the purified fusion protein. Such an "immunofingerprinting" approach may be especially useful when it is difficult to obtain adequate amounts of purified protein for direct sequence determination. Finally, the positions of seven peptides obtained by partial sequencing of the 60-kDa catalytic subunit spanned nearly the entire coding region, providing for unambiguous interpretation of the deduced sequence. Three additional peptides that were not found probably are in the region of the catalytic subunit absent from this clone.

The assignment of a 24-amino acid sequence 12 kDa from the carboxyl terminus as the CaM-binding domain was based on consensus charge properties reported by others; indeed, no other region in the clone contained a comparable grouping of positively charged amino acids. As shown in Fig. 4, the CaM-binding domains all have a cluster of cationic residues at the beginning of the region, after which there are periodic positively charged residues in some, but not all, cases. Although the topology of the deduced CaM-binding site appears to fit the general pattern observed for several CaM-activated protein kinases (33, 34, 42–44) and the erythrocyte Ca^{2+} pump (35), the presence of a single glutamic acid residue in the domain appears to be a major difference. However, it appears that such a residue can be tolerated since studies indicate that the synthetic peptide can block the interaction of CaM with the catalytic subunit (R.L.K. and B.M.M., unpublished data). Since the properties of this site may relate to a specific enzymatic function (e.g., conformation-dependent binding of substrate or cofactor), it will be important to note whether other CaM-regulated enzymes possess acidic residues in this region. It is possible that the deduced CaM-binding domain on calcineurin in fact binds to the smaller (β) subunit of this enzyme, although we have no

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CTA GAC CGA TTC AAA GAA CCA CCG GCT TAT   GGG CCC ATG TGT GAC ATC CTA TGG TCA GAC   CCC CTG GAG GAC TTT GGA AAT GAG AAG ACT  90
 L  D  R  F  K  E  P  P  A  Y    G  P  M  C  D  I  L  W  S  D    P  L  E  D  F  G  N  E  K  T  30

CAG GAA CAT TTC ACT CAC AAC ACA GTC AGA   GGC TGT TCG TAC TTC TAC AGT TAC CCA GCT   GTG TGT GAC TTC CTG CAG CAT AAT AAT TTG 180
 Q  E  H  F  T  H  N  T  V  R    G  C  S  Y  F  Y  S  Y  P  A    V  C  D  F  L  Q  H  N  N  L  60
                      (P)

TTG TCC ATA CTC CGC GCC CAC GAA GCC CAG   GAT GCA GGG TAC CGC ATG TAC AGG AAA AGC   CAA ACA ACA GGC TTC CCG TCT CTA ATT ACA 270
 L  S  I  L  R  A  H  E  A  Q    D  A  G  Y  R  M  Y  R  K  S    Q  T  T  G  F  P  S  L  I  T  90

ATC TTC TCG GCA CCA AAT TAC TTA GAT GTG   TAC AAT AAC AAA GCT GCA GTG TTG AAG TAC   GAG AAC AAT GTG ATG AAC ATC AGG CAG TTC 360
 I  F  S  A  P  N  Y  L  D  V    Y  N  N  K  A  A  V  L  K  Y    E  N  N  V  M  N  I  R  Q  F  120

AAC TGC TCC CCG CAT CCG TAC TGG CTC CCA   AAT TTC ATG GAT GTT TTC ACC TGG TCG CTG   CCA TTT GTT GGG GAG AAA GTG ACT GAG ATG 450
 N  C  S  P  H  P  Y  W  L  P    N  F  M  D  V  F  T  W  S  L    P  F  V  G  E  K  V  T  E  M  150

CTG GTC AAT GTT CTC AAC ATC TGC TCC GAC   GAT GAA CTG GGG TCA GAA GAA GAT GGA TTT   GAC GGA GCC ACG GCC GCA GCC CGG AAG GAA 540
 L  V  N  V  L  N  I  C  S  D    D  E  L  G  S  E  E  D  G  F    D  G  A  T  A  A  A  R  K  E  180
                                                                                    ============

GTC ATC AGA AAC AAG ATC CGA GCA ATA GGC   AAA ATG GCC AGA GTG TTC TCA GTT CTC AGA   GAA GAG AGT GAG AGT GTC CTG ACA CTG AAG 630
 V  I  R  N  K  I  R  A  I  G    K  M  A  R  V  F  S  V  L  R    E  E  S  E  S  V  L  T  L  K  210
=========================================================================

GGC CTG ACC CCA ACT GGC ATG CTC CCC AGC   GGA GTG CTC TCT GGC GGG AAA CAG ACT CTG   CAA AGC GCT ACT GTT GAG GCT ATT GAG GCT 720
 G  L  T  P  T  G  M  L  P  S    G  V  L  S  G  G  K  Q  T  L    Q  S  A  T  V  E  A  I  E  A  240

GAT GAA GCC ATC AAA GGA TTT TCA CCA CAA   CAT AAG ATC ACT AGC TTC GAG GAG GCC AAG   GGC TTA GAC CGA ATT AAC GAG AGG ATG CCA 810
 D  E  A  I  K  G  F  S  P  Q    H  K  I  T  S  F  E  E  A  K    G  L  D  R  I  N  E  R  M  P  270

CCT CGC AGA GAC GCC ATG CCC TCT GAC GCC   AAC CTT AAC TCC ATC AAC AAG GCT CTC GCC   TCA GAG ACT AAC GGC ACG GAC AGC AAT GGC 900
 P  R  R  D  A  M  P  S  D  A    N  L  N  S  I  N  K  A  L  A    S  E  T  N  G  T  D  S  N  G  300
                                                  (R)

AGT AAT AGC AGC AAT ATC CAG TGA CCA CTT   CCT GTT CAC TTT TTT TTT TTT TTG AGC TGC   AGG GCA TGA TGG GAT TGC TGC ATC TCA GCA 990
 S  N  S  S  N  I  Q  *

GTT GGA TGT TCT TGC CTC TGA AGG TAG CTT   GTT TGC TCT GGG GGC CAG GAA TTG GAT TCA   GTT TAC ACT ATC ATG AAA AAT AAA AAT AAA 1080
                                                                                    --- --- --- ---

AAA AGA GGG AGA GAG ATA ATA AAC TAT ATT   G  1111
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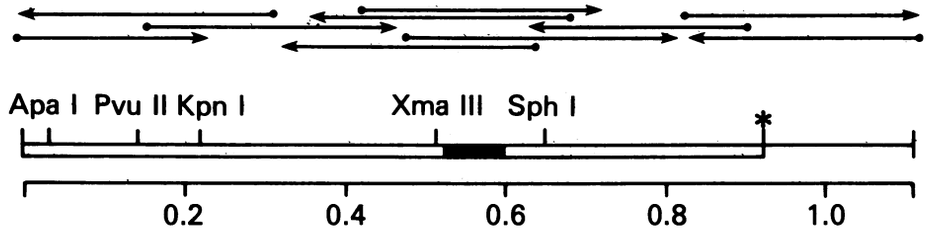

FIG. 3. Nucleotide and deduced amino acid sequence (single-letter code) of CNα-1, a cDNA encoding the CaM-binding region of calcineurin. Deduced sequences corresponding to peptides determined by gas-phase microsequencing of bovine brain α-subunit are underlined; amino acid differences are indicated in parentheses below the residue. Presumptive polyadenylylation sites are indicated by single dashed lines, and the stop codon is denoted by an asterisk. The putative CaM-binding domain is underlined with a double dashed line. A partial restriction map showing the positions of specific oligonucleotide primers is shown at the bottom of the figure; the distance from the 5′ end is given in kilobases. Shaded area between the restriction sites for *Xma* III and *Sph* I corresponds to the CaM-binding region.

evidence for interaction with the fusion protein or competition for CaM binding. Additional studies will be required to establish unequivocally the site of association with this subunit.

Although the mechanism underlying CaM regulation of phosphatase activity is not known, several deduced structural features may be relevant. In the case of MLCK, it appears that the CaM-binding domain, itself, may control activity by acting as a pseudosubstrate (45). In this regard, it is interesting that a potential phosphorylation site is present in the putative CaM-binding region of calcineurin, as in several other enzymes. With MLCK, phosphorylation of this

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MLCK (Sm.musc)   A R R K W Q K T G H A V R A I G R L S S M A M I
Calcineurin      A R K E - - V I R N K I R A I G K M A R V F S V L R
Ca2+- ATPse      L R R G Q I L W F R G K N R I Q T Q I K V V N A
CaM-kinase II    A R R K L K G A I L T T M L A T R N F S G G K G
Phos.b kinase    P R G K F K V I C L T V L A S V R I Y Y
MLCK (Sk.musc)   M K R R W K K N F I A V S A A N R F K K
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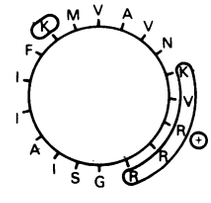

FIG. 4. Amino acid sequences (single-letter code) for the CaM-binding domains of several enzymes. Shaded areas represent positively charged amino acids, and underlined sequences are potential sites for phosphorylation by protein kinases. Spaces have been inserted in the sequence for calcineurin to align the region of similarity to MLCK. A helical wheel representing positions 5–22 of the calcineurin sequence is shown on the right. The sequences listed are from ref. 34, except those for the Ca^{2+}-ATPase (35) and calcineurin (this paper).

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1 -170  IMRPTDVPDQGLLCDLLWSDPDKDVQGWGEND-------
CN- 1   LDRFKEPPAYGPMCDILWSDPLEDFGNEKTQEHFTHNTV
2A-183  LDRLQEVPHEGPMCDLLWSDPD-DRGGWGISP-------

1 -202  RGVSFTFGAEVVAKFLHKHDLDLICRAHQVVEDGYEFFA
CN- 40  RGCSYFYSYPAVCDFLQHNNLLSILRAHEAQDAGYRMYR
2A-214  RGAGYTFGQDISETFNHANGLTLVSRAHQLVMEGYNWCH

1 -241  KRQ------LVTLFSAPNYCGEFDNAGAMMSVD....
CN- 79  KSQTTGFPSLITIFSAPNYLDVYNNKAAVLKYE....
2A-253  DRN------VVTIFSAPNYCYRCGNQAAIMELD....
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FIG. 5. Comparison of deduced primary structures of phosphoprotein phosphatases 1 and 2A to that of CNα-1 (calcineurin). Amino acid identities (single-letter code) between CNα-1 (residues 1–111) and phosphatases 1 (residues 170–267) and 2A (residues 183–280) are indicated by shading; gaps in the sequences of phosphatases 1 and 2A have been made to maximize similarities. Regions of identity between phosphatase 1 and 2A are indicated by solid lines above and below the residues. Sequence data for protein phosphatases 1 and 2A are from ref. 37.

site (42) is known to greatly decrease the binding affinity for CaM (46), while for CaM kinase II (34), it has been suggested that this site may be involved in the autophosphorylation known to render the enzyme insensitive to CaM. Whether phosphorylation of the site plays any comparable role in calcineurin is not known. The finding that the CaM-binding site is 12 kDa from the carboxyl terminus suggests that tryptic (47, 48) or chymotryptic cleavage (49) of the enzyme causes activation by removal of an inhibitory carboxyl-terminal domain of the enzyme. However, since some proteolytically activated species may be inhibited by CaM (49), catalytic regulation may be more complex; the use of recombinant proteins expressing different and/or altered forms of the catalytic subunit may help to elucidate some of these mechanisms.

It was shown recently that the similarly sized catalytic subunits of phosphatases 1 and 2A are closely related (37), having ≈50% sequence identity over an internal 280-residue region. From the present study, it is clear that calcineurin can be included in this protein phosphatase superfamily. The lower amount of identity with phosphatase 1 compared with phosphatase 2A may suggest that it more closely related to the latter; however, this must await comparison of the complete sequence. The strong relatedness that occurs in the two small subdomains suggests conservation of functions of crucial importance for enzyme activity. Since the amount of polypeptide separating these conserved regions differs, the inserted residues in calcineurin may impart a distinctive regulatory property to this phosphatase. Future studies to characterize full-length cDNA clones of calcineurin may provide insights into structural and evolutionary aspects of protein phosphatases.

We thank Dr. Martha Vaughan, in whose laboratory much of the initial work was conducted, for her generous support and encouragement. We thank Dr. Edward Ginns for valuable advice and discussions, William Eliason for his participation in determination of protein sequence, and Dr. Doug Amorese for sequence analysis with the automated DNA sequencer.

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