Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 9183–9187, December 1989
Biochemistry

Cloning of human calcineurin A: Evidence for two isozymes and identification of a polyproline structural domain

(protein phosphatase/calmodulin-binding protein)

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Communicated by Maxine F. Singer, August 28, 1989 (received for review June 22, 1989)

ABSTRACT **Two types (I and II) of cDNAs encoding the large (A) subunit of calcineurin, a calmodulin-regulated protein phosphatase, were isolated from human basal ganglia and brainstem mRNA. The complete sequences of the two calcineurin clones are identical except for a 54-base-pair insert in the type I clone and different 3′ ends including part of the coding sequence for the C termini of the two proteins. These findings suggest that calcineurin A consists of at least two isozymes that may result from alternative splicing events. The two forms of the enzyme differ in the C terminus, which contains an inhibitory domain rapidly severed by limited proteolysis. With the exception of an 18-amino acid insert, the central parts of the molecules, which harbor the catalytic domains, are identical and show extended similarities with the entire catalytic subunits of protein phosphatases 1 and 2A, defining a distinct family of protein phosphatases. The 40-residue N-terminal fragment, specific for calcineurin, contains a sequence of 11 successive prolines that is also found in bovine brain calcineurin by peptide sequencing. A role in the calmodulin activation of calcineurin is proposed for this novel structural element.**

Calcineurin, the Ca^{2+}/calmodulin-regulated protein phosphatase, first detected in skeletal muscle and brain (1, 2), has been found in all cells from yeast (3) to mammals (4). It is a heterodimer of a 19-kDa Ca^{2+}-binding protein, calcineurin B, and a 61-kDa calmodulin-binding catalytic subunit, calcineurin A (4). Calcineurin B is a highly conserved protein. Antibodies to the bovine protein recognize calcineurin B from different sources, including yeast (ref. 4 and M. Cyert and J. Thorner, personal communication), and the sequence of the bovine protein is almost identical to the sequences of the human and mouse proteins deduced from their cDNA clones (5, 6). We report here that the sequences of two human calcineurins A deduced from their cDNA sequences* are similar to the partial sequence of bovine calcineurin A determined by protein sequencing and to the C-terminal sequence of the mouse protein reported by Kincaid *et al.* (7). Several forms of calcineurin A have been detected by electrophoresis and antigenic crossreactivity (8, 9). The molecular cloning of calcineurin A described in this paper suggests that different forms of calcineurin A may be the result of alternative splicing and reveals the strong homology between calcineurin and protein phosphatases 1 and 2A.

Functional domains of bovine brain calcineurin A identified by limited proteolysis (10) have been mapped along the calcineurin sequence. The C terminus of calcineurin contains an inhibitory domain whose effects are relieved upon calmodulin binding. The calmodulin-binding domain is immediately adjacent to the inhibitory domain. The catalytic site and the calcineurin B-binding site are located in a protease-resistant 40-kDa core that encompasses the rest of the molecule. A 20- to 30-residue N-terminal fragment is important for enzyme activity. Here we show that an unusual sequence of 11 successive prolines near the N terminus of the enzyme is found in the human and bovine proteins. We suggest a role in the calmodulin activation of calcineurin for this novel structural element.

MATERIALS AND METHODS

Chemicals. Rabbit brain and skeletal muscle poly(A)$^+$ RNA were purchased from Clontech. HeLa cell poly(A)$^+$ RNA was prepared by the method of Berger and Birkenmeier (11). The random primer kit was from Boehringer Mannheim. Oligodeoxynucleotides corresponding to the peptides Glu-Ala-Ile-Glu-Ala-Asp-Glu-Ala [5′-GAGGC(I or C)AT(A or T or C)GA(G or A)GC(I or C)GA(T or C)GA(G or A)GC-3′; probe 1] and Lys-Tyr-Glu-Asn-Asn-Val-Met-Asn-Ile [5′-AA(G or A)TA(T or C)GA(G or A)AA(T or C)AA(T or C)GT(T or C)ATGAACTA-3′; probe 2] of bovine brain calcineurin A were synthesized and purified by HPLC in the Laboratory of Molecular Pharmacology, Center for Drug and Biologics (Bethesda, MD). Oligonucleotides used for sequencing were synthesized by Michael Brownstein (Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, MD). Bovine brain calcineurin was purified as described (12). All other chemicals and cloning vectors were as previously reported (6).

cDNA Library Screening. The human brainstem and basal ganglia λgt11 libraries (13) were kindly provided by Robert Lazzarini (Mount Sinai Hospital, New York). Replica filters from 150-mm plates containing 5–6 × 10^4 phage were prepared as described (14). Baked filters, washed with 50 mM Tris·HCl, pH 8/1 mM EDTA/0.1% SDS/1 M NaCl at 37°C and prehybridized for 3–6 hr in 3× SSC (0.45 M NaCl/0.045 M sodium citrate, pH 7)/0.1% SDS/0.1% Ficoll/0.1% polyvinylpyrrolidone/0.1% bovine serum albumin containing denatured salmon sperm DNA at 30 μg/ml, were hybridized at 37–39°C overnight in the same solution containing salmon sperm DNA at 100 μg/ml and labeled oligonucleotides (1–3 × 10^7 cpm/ml). The filters were washed with 2× SSC/0.1% SDS twice for 20 min at room temperature, once for 15 min at 37°C, and twice for 10 min at 45°C. Positive clones detected with probe 2 were tested for the ability to hybridize with probe 1. Additional clones were obtained by screening the libraries under more stringent hybridization conditions (incubation at 65°C with 2–8 × 10^5 cpm/ml in the same solvent as above and three washes at 55°C with 0.1× SSC/0.1% SDS) with cDNA probes derived from the original clones labeled by nick-translation (15) or random primer extension (16).

Subcloning and Sequencing of cDNA Fragments. Phage DNA was prepared (17) and DNA fragments generated by *Eco*RI digestion were inserted into GeneScribe vectors (United States Biochemical). The chain-terminator method of

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*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M29550 and M29551).

Sanger *et al.* (18) as modified by Biggins *et al.* (19) was used to sequence denatured double-stranded plasmids prepared by the method of Hattori and Sakaki (20). Sequencing in both directions was done with synthetic oligonucleotide primers and nested deletions (21).

Northern Blots. Poly(A)$^+$ RNAs were separated in 1% agarose/2.2 M formaldehyde gels, transferred to nitrocellulose, and hybridized overnight at 65–68°C.

Protein Sequencing. Bovine brain calcineurin (20 nmol in 5.7 ml) was digested with trypsin (1 μg/ml) for 25 min at 30°C under the conditions used for clostripain digestion (10). The reaction was stopped by addition of 2 μl of diisopropyl fluorophosphate. Peptides were resolved from the trypsin-resistant 40-kDa core, associated with calcineurin B, by gel filtration on a 1.5 × 30 cm Sephadex G-100 column in 40 mM Tris·HCl, pH 8/0.2 M NaCl/1 mM $MgCl_2$/1 mM dithiothreitol. Column fractions were monitored by SDS/PAGE. Small peptides (M_r < 10,000) were separated by HPLC on a C_{18} column (Waters Associates) using a 0–50% linear gradient of CH_3CN in 0.1% trifluoroacetic acid over 50 min at a flow rate of 1.5 ml/min. Peptides not resolved in the first HPLC step were subjected to an additional HPLC step with a shallower gradient of CH_3CN. A model 477A Applied Biosystems protein sequenator was used to determine the sequence of the peptides. The N-terminal sequence of the clostripain-resistant 43-kDa core was determined as described (10). The partial sequence of the bovine brain protein is based on the alignment of the peptides along the sequence of the human protein deduced from the cDNA clone.

RESULTS

Isolation of Two Types of Calcineurin A Clones. Two clones coding for the C terminus of calcineurin A were obtained by using probes 1 and 2 to screen 6 × 10^5 phage derived from the brainstem library. They were used as sources of probes to isolate 31 calcineurin clones by two successive screenings of the two libraries (1.2 × 10^6 phage each time) with cDNA probes corresponding to amino acids 294–398 and 104–288. A fourth screening of 3.6 × 10^6 phage with a 114-base-pair (bp) probe (corresponding to residues 51–89), obtained by exonuclease III digestion of the longest clone, was needed to isolate four cDNAs containing the initiator codon. The 31 clones formed two groups on the basis of restriction mapping and sequence analysis (Fig. 1). The type I clones (3 isolates) have a relatively short 3′ noncoding sequence and contain an *Eco*RI and an additional *Pst* I site not present in the type II clones. The latter (28 isolates) have a long 3′ noncoding sequence with an additional *Sac* I site. A full-length type I clone (2457 bp) was sequenced in both directions. It contains a 107-bp leader sequence, the coding sequence for a 514-residue protein (calcineurin A-1) and an 808-bp 3′ untranslated region terminated by a polyadenylylation signal and a poly(A) tail (Fig. 2). Twenty-four type II clones, lacking the *Eco*RI site, encoded fragments of another form of calcineurin A (calcineurin A-2) whose C terminus was different from that of calcineurin A-1. Two clones that contained an initiator codon were tentatively identified as type II by sequence analysis. The sequence of the first 1215 nucleotides of these two clones is identical to that of the type I cDNA but they contain the 54-nucleotide deletion characteristic of the type II cDNA. Clones encoding the N or the C terminus of calcineurin A-2, with a perfect 913-nucleotide overlap, yielded the sequence of the type II cDNA coding for a 524-amino acid protein (Fig. 2). The 3′ ends of the type I and II clones are different; with the exception of a 54-bp insert at position 411, the sequences of type I and II cDNAs from nucleotide −107 to 1420 (type I clone) are identical. With the exception of two clones (one of each type) that contained an additional codon (GTA) at position 1239 (type I clone), the sequences of all the clones tested so far are identical to either one of the sequences shown in Fig. 2. A 3200-nucleotide mRNA, whose size corresponds to that of the type II clone, was detected in poly(A)$^+$ RNA preparations from rabbit brain, mouse skeletal muscle, and HeLa cells with a probe corresponding to a sequence common to the two classes of cDNA. These RNAs failed to hybridize to a probe specific for the type I cDNA, probably because of the low abundance of type I mRNA.

Primary Structure of Calcineurins A-1 and A-2. The protein sequences deduced from the cDNA sequences illustrated in Fig. 2 are shown in Fig. 3. Calcineurin A-2, encoded by the more abundant, type II cDNA, has a molecular mass of 59 kDa, similar to the reported (4) apparent size of bovine brain calcineurin A. The less abundant, type I cDNA codes for a 58-kDa protein, calcineurin A-1. The two proteins differ by an 18-amino acid insert, between Glu-137 and Cys-138 in calcineurin A-1, and have different C-terminal sequences starting at Ala-456. The amino acid sequences of peptides derived from bovine brain calcineurin A, also shown in Fig. 3, are almost completely identical with the deduced sequence of human calcineurin A-2. Only 15 amino acid substitutions are found over 216 residues, spanning half of the sequence of the protein. The C terminus of the bovine protein resembles calcineurin A-2 more than calcineurin A-1, but substitutions in the last 20 amino acids suggest that the bovine protein may represent yet a third calcineurin isozyme. The sequence reported for the C-terminal half of the mouse protein (residues 224–560) (7) is also of type 2. With the exception of a short sequence (residues 387–396) and 5 additional amino acids at the C terminus, the mouse protein is very similar to the human protein and even more so to the bovine protein.

Identification of Functional Domains. The functional domains identified by limited proteolysis with clostripain can now be precisely located along the amino acid sequence.

Regulatory domains. The rapid loss of the inhibitory domain in the presence of calmodulin is most likely the result of proteolysis at the cluster of basic residues between amino acids 474 and 496. A 14-kDa calmodulin-binding peptide, which is protected against proteolysis by calmodulin, encompasses residues 342–474/496 (M. Hubbard, D.G., D. Campbell, M. Krinks, P. Cohen, and C.K., unpublished data). The

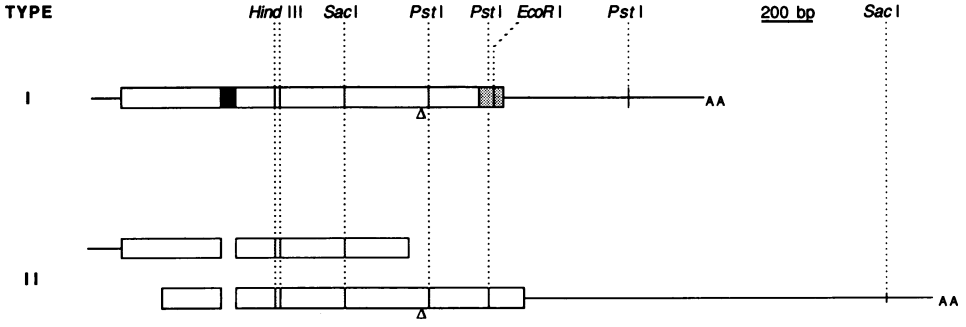

FIG. 1. Restriction map of calcineurin A clones. The coding sequences are shown as bars, with the 54-bp insert, and the different 3′ ends of the coding sequences are denoted by heavy and light stippling, respectively. Triangles indicate the position of the GTA insert.

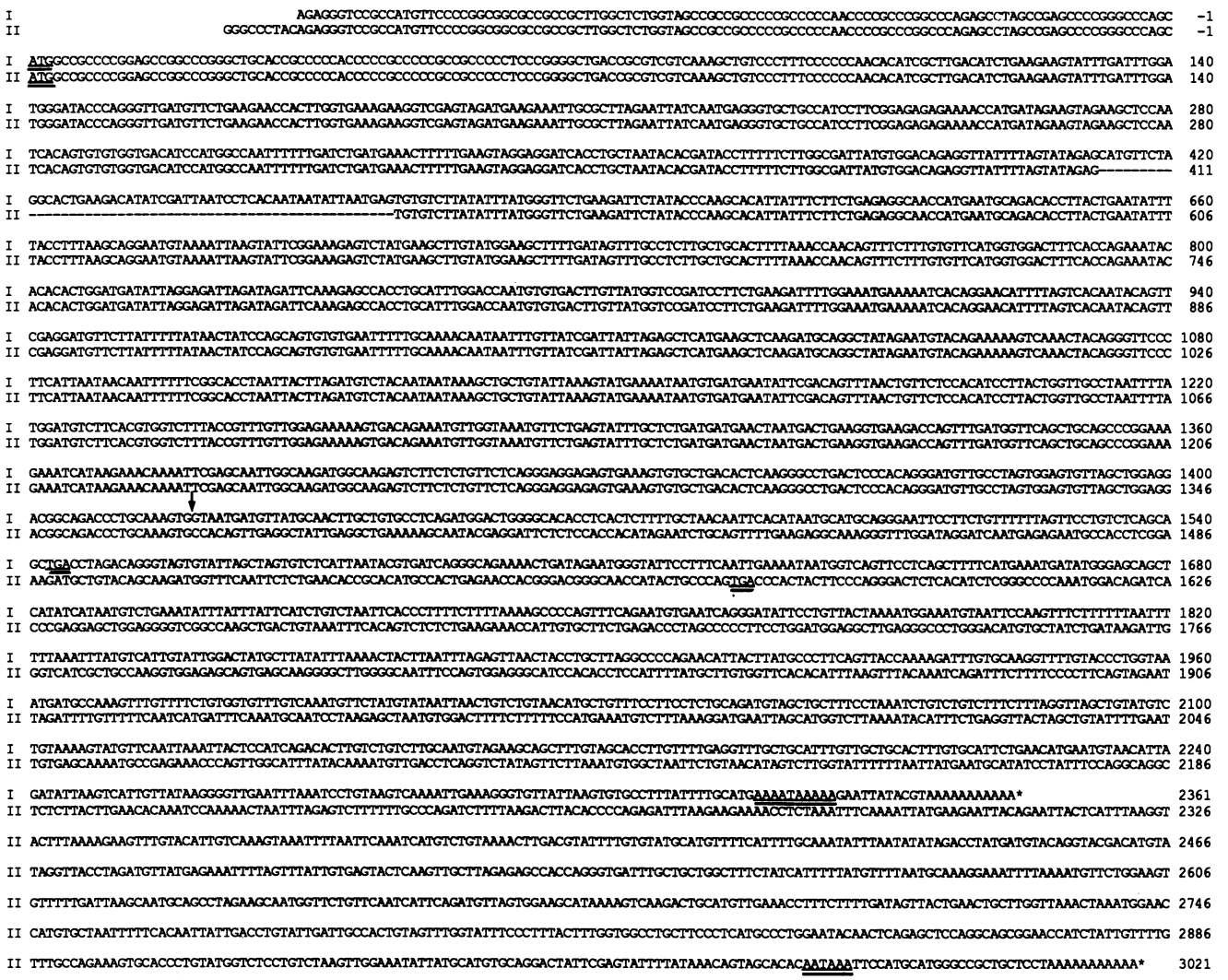

FIG. 2. Nucleotide sequences of the two human calcineurin A clones. The ATG initiator codons, the TGA terminator codons, and the polyadenylylation signals are underlined. Arrow indicates the end of the homologous regions.

calmodulin-protected peptide is one of the most highly conserved parts of the molecule and contains a putative calmodulin-binding domain (residues 401–423) whose high content of basic and hydrophobic residues is reminiscent of the calmodulin-binding peptides isolated from other calmodulin-binding proteins (27–32).

Catalytic domain. The catalytic site and the calcineurin B-binding site are contained in the protease-resistant 43-kDa core of calcineurin A (10) comprising residues 1–341. The striking similarities of residues 42–338 of calcineurin A to protein phosphatases 1 and 2A are illustrated in Fig. 3. The aligned sequences of the proteins show 83 identities and 54 conservative replacements. Two inserts (residues 256–262 and 305–310) unique to calcineurins A are potential binding sites for calcineurin B.

N-terminal domain. A unique feature of calcineurin A is the sequence of 11 prolines close to the N terminus. This unusual sequence revealed by the DNA sequencing exactly confirmed the structure deduced from peptide sequencing of the bovine protein.

DISCUSSION

The partial sequence of the mouse calcineurin A determined by Kincaid *et al.* (7) revealed some similarity between the catalytic subunit of calcineurin and protein phosphatases 1 and 2A. We show here, by elucidation of the complete sequence of two forms of human brain calcineurin A by cloning and by peptide sequencing of the bovine protein, that the similarities among the three proteins extend throughout almost the entire sequence of the catalytic subunits of protein phosphatases 1 and 2A and cover the region of the calcineurin molecule containing the catalytic site. The alignment scores of forms 1 and 2 of calcineurin determined from the National Biomedical Research Foundation ALIGN program are 53.03 and 46.91 with protein phosphatase 1 (33) and 52 and 46.15 with protein phosphatase 2Aα (34), respectively, as opposed to a score of 75.5 for alignment of protein phosphatases 1 and 2Aα with each other. The similarities may in fact be greater, since these numbers are affected by deletions in protein phosphatases 1 and 2Aα that may correspond to the calcineurin B-binding site of calcineurin A. These protein phosphatases, two of them under the direct or indirect control of second messengers (Ca^{2+} for calcineurin and cAMP for protein phosphatase 1), form a family distinct from the acid and alkaline phosphatases isolated from bacterial or mammalian sources. The latter enzymes exhibit less similarity among themselves (alignment scores <25) and no detectable similarity with the protein phosphatases mentioned above (scores <1).

As opposed to the high degree of conservation of the catalytic domains of the protein phosphatases, molecular cloning of calcineurin A uncovered the heterogeneity and potential diversity of the inhibitory domain of calcineurin A. Two classes of clones were isolated. Their DNA sequences

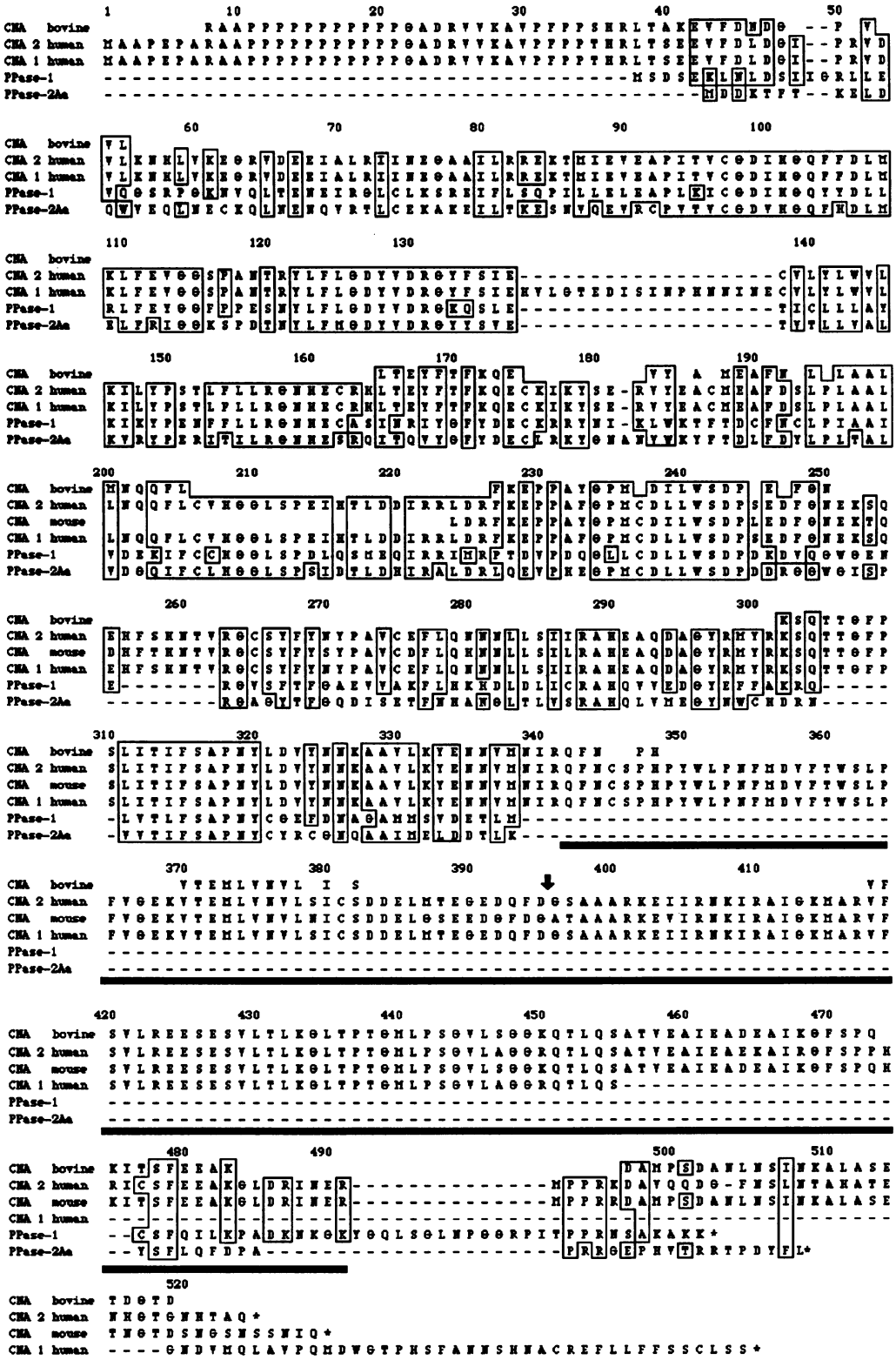

FIG. 3. Alignment of the peptide sequences of bovine brain calcineurin A (CNA) with the deduced protein sequences of human brain calcineurins A-2 and A-1, mouse calcineurin A (7), rabbit muscle protein phosphatase (PPase) 1, and rabbit muscle protein phosphatase 2Aα. For clarity the sequences of protein phosphatase 2Aα and 2Aβ (22–25) and the yeast protein phosphatase sit4 (26) are not included. Identities or conservative replacements shared by calcineurin with at least one of the other two phosphatases are boxed. The bar shows the calmodulin-binding peptide (10) and the arrow the single amino acid insert. Numbers correspond to the sequence of calcineurin A-2.

are identical with two exceptions: (*i*) the presence in cDNA for calcineurin A-1 of a 54-bp insert coding for 18 additional amino acids 137 residues away from the N terminus and (*ii*) two different C termini and two completely different 3′ noncoding regions. We suggest that forms 1 and 2 of calcineurin A are the result of two alternative splicing events. Consistent with this proposal a single gene was detected in preliminary gene mapping experiments (W. McBride, D.G., and C.K., unpublished data). Skipping of an exon could explain the deletion in the type II clone (35). Splicing from

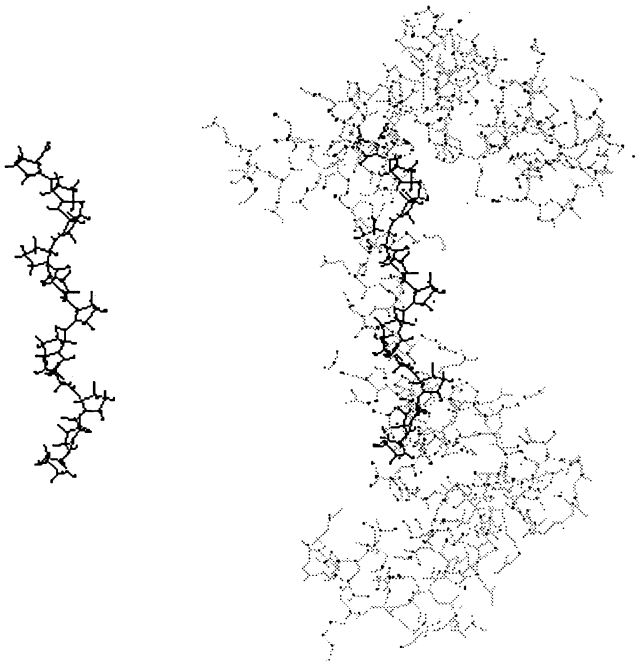

FIG. 4. Eleven proline residues in a polyproline II helix alone (*Left*) and docked along the central helix of calmodulin (*Right*). The molecular modeling was done with the program CHEM-X, developed and distributed by Chemical Design (Oxford, England). The coordinates of calmodulin (41) were made available to us by W. J. Cook (University of Alabama).

different transcripts of a gene with multiple polyadenylylation sites can be invoked to explain the different 3′ ends. Alternative splicing at the C terminus results in calcineurins selectively modified in the inhibitory domain. If this domain acts as a pseudosubstrate, as has been proposed for the calmodulin-regulated kinases (36–38), the multiple calcineurin isozymes could exhibit different substrate specificity. Such calcineurins may be differentially expressed in different cells or during development as are the protein phosphatase 2A isozymes (39). The origin of the additional variants with a single amino acid insert is not clear. These findings may explain the antigenic and electrophoretic microheterogeneity of calcineurin previously reported (8, 9). The differences among the bovine and mouse brain proteins and human proteins (derived from cDNA libraries corresponding to specific brain areas) may reflect not only species variations but also cell specificity and distribution.

In contrast to the variability of the inhibitory domain, the calmodulin-binding domain and a third functional domain located at the N terminus of the protein are highly conserved. The N terminus of calcineurin A, destabilized upon calmodulin binding, is rapidly cleaved by proteases resulting in a significant loss of activity. As shown above, this fragment (residues 1–24), which is perfectly conserved in the two human calcineurins A and the bovine calcineurin A, has an unusual sequence including a stretch of 11 prolines. Very few proteins contain polyproline sequences and the maximal length previously reported (protein E4 of papillomavirus) is 8 residues (ref. 42; sequence data base search done in June 1989). It is attractive to propose that this novel structural element plays a role in the mechanism of the activation of calcineurin by calmodulin. Two different conformations of polyproline in solution have been characterized, an extended type II helix with the α carbon in the trans configuration and a compact type I helix in the cis configuration (40). An 11-residue polyproline helix in the type II configuration will precisely span the length of the central helix of calmodulin (Fig. 4). The rigid structure could act as a spacer to maintain the central helix in an extended conformation and allow precise interaction of calcineurin bound to the C-terminal half of calmodulin with a second site in the N terminus of calmodulin.

We thank Dr. Robert Lazzarini for providing the cDNA libraries; Dr. Michael Brownstein and Judith Regan for the synthesis of the oligonucleotides; Drs. Bruce Paterson, Dean Hamer, and Werner Klee for helpful advice throughout the course of this work; and May Liu for expert editorial assistance. During part of this work, D.G. was a recipient of a fellowship from the National Swiss Foundation.

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