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Functional Domain Structure of Calcineurin A: Mapping by Limited Proteolysis

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ABSTRACT: Limited proteolysis of calcineurin, the Ca^{2+}/calmodulin-stimulated protein phosphatase, with clostripain is sequential and defines four functional domains in calcineurin A (61 kDa). In the presence of calmodulin, an inhibitory domain located at the carboxyl terminus is rapidly degraded, yielding an M_r 57 000 fragment which retains the ability to bind calmodulin but whose *p*-nitrophenylphosphatase is fully active in the absence of Ca^{2+} and no longer stimulated by calmodulin. Subsequent cleavage(s), near the amino terminus, yield(s) an M_r 55 000 fragment which has lost more than 80% of the enzymatic activity. A third, slower, proteolytic cleavage in the carboxyl-terminal half of the protein converts the M_r 55 000 fragment to an M_r 42 000 polypeptide which contains the calcineurin B binding domain and an M_r 14 000 fragment which binds calmodulin in a Ca^{2+}-dependent manner with high affinity. In the absence of calmodulin, clostripain rapidly severs both the calmodulin-binding and the inhibitory domains. The catalytic domain is preserved, and the activity of the proteolyzed 43-kDa enzyme is increased 10-fold in the absence of Ca^{2+} and 40-fold in its presence. The calcineurin B binding domain and calcineurin B appear unaffected by proteolysis both in the presence and in the absence of calmodulin. Thus, calcineurin A is organized into functionally distinct domains connected by proteolytically sensitive hinge regions. The catalytic, inhibitory, and calmodulin-binding domains are readily removed from the protease-resistant core, which contains the calcineurin B binding domain. Calmodulin stimulation of calcineurin is dependent on intact inhibitory and calmodulin-binding domains, but the degraded enzyme lacking these domains is still regulated by Ca^{2+}.

Calcineurin, the major soluble calmodulin-binding protein in brain (Klee et al., 1979), is a Ca^{2+}/calmodulin-stimulated protein phosphatase (Stewart et al., 1982). It appears to be the neural specific isozyme of a widely distributed class of protein phosphatases [as reviewed by Klee et al. (1988) and Klee and Cohen (1988)]. The enzyme is a heterodimer of calcineurin A (61 kDa) and calcineurin B (19 kDa) which remains tightly associated in the presence or absence of Ca^{2+}. Calcineurin A contains the catalytic center (Merat et al., 1985; Gupta et al., 1985) and in the presence of micromolar Ca^{2+} binds calmodulin with high affinity, $K_d = 10^{-10}$ M (Hubbard & Klee, 1987), resulting in a reversible 10–20-fold increase of the phosphatase activity. Calcineurin B binds Ca^{2+} with high affinity and is believed to mediate a small (2–3-fold) Ca^{2+} stimulation of enzyme activity and the Ca^{2+}-dependent association of the two subunits of the enzyme (Merat et al., 1985; Klee et al., 1985). The amino acid sequence of calcineurin B, determined by Aitken et al. (1984), revealed the presence of four "EF-hand" Ca^{2+}-binding domains as defined by Kretsinger (1980). Despite its structural similarity with calmodulin, calcineurin B does not substitute for calmodulin in the stimulation of the phosphatase; conversely, calmodulin cannot replace calcineurin B in the Ca^{2+}-dependent reconstitution of calcineurin (Klee et al., 1983; Merat et al., 1985). Previous studies showed that limited degradation of calcineurin A by trypsin abolishes calmodulin binding and results in the irreversible activation of the phosphatase (Manalan & Klee, 1983; Li & Chan, 1984; Tallant & Cheung, 1984). Activation of calcineurin by limited proteolysis or by calmodulin is not due to a dissociation of calcineurin subunits (Manalan & Klee,

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1983). These observations led to the suggestion that the binding of calmodulin may relieve an intrinsic inhibition apparently dependent on an intact, but unoccupied calmodulin-binding domain.

We have now used limited proteolysis with clostripain to further probe the structure of calcineurin A. An inhibitory domain, distinct from the calmodulin-binding domain, has been identified. Using protein sequence analysis of isolated proteolytic fragments, we have mapped independent calcineurin A domains involved in catalysis, calcineurin B and calmodulin binding, and self-inhibition.

Materials and Methods

Bovine brain calcineurin and bovine testis calmodulin were purified as previously described (Klee et al., 1983; Newton et al., 1988). Clostripain, a product of Boehringer Mannheim Biochemicals, was activated by incubating a 6.4 mg/mL solution in 0.05 M Na_2HPO_4, pH 7.9, with 10 mM dithiothreitol, overnight at 0–4 °C (Porter et al., 1971). The activated protease was stored at −70 °C for up to 2 months without significant loss of activity. ^{125}I-Calmodulin containing 1.4–1.6 mol of iodine/mol (specific activity 1.3×10^5 dpm/pmol of calmodulin) was prepared as described by Klee et al. (1983). Poly(vinylidene difluoride) (PVDF)[1] membranes (Immobilon 0.45-μm pore size) were obtained from Millipore. Sequencing-grade reagents, glass fiber disks, and Polybrene were purchased from Applied Biosystems. Electrophoresis reagents were products of Polysciences Inc.

Enzyme Assays. Clostripain activity was assayed at 25 °C, using as substrate *N*-benzoyl-L-arginine methyl ester (0.25 mM) in 3 mL of 10 mM Tris-HCl buffer, pH 7.5, containing 0.3 mM $CaCl_2$ and 1.7 mM dithiothreitol, as described by Schwert and Takenata (1955). One unit is that amount of enzyme which gives a change in absorbance at 253 nm of 3×10^{-3} cm^{-1} min^{-1}. The *p*-nitrophenylphosphatase activity of calcineurin (Pallen & Wang, 1983) was monitored at 400 nm with a Cary 118 spectrophotometer equipped with a microcell attachment. The incubation mixtures (0.2 mL), containing 40 nM calcineurin in 40 mM Tris-HCl buffer, pH 8, 0.1 M NaCl, 0.5 mM dithiothreitol, and 0.1 mg/mL bovine serum albumin, were brought to 23 °C by a 5-min incubation. When present, [$MgCl_2$] was 6 mM, [$CaCl_2$] was 1 mM, [EGTA] was 1 mM, and [calmodulin] was 180 nM. The reaction was started by addition of *p*-nitrophenyl phosphate (final concentration 5 mM). One unit of enzyme catalyzes the formation of 1 μmol of *p*-nitrophenol/min at 23 °C, calculated with an extinction coefficient for *p*-nitrophenol, at 400 nm and at pH 8, of 15.4×10^3 (Bessey & Love, 1952).

Digestion of Calcineurin with Clostripain. Calcineurin (4.3–6.6 μM) in 40 mM Tris-HCl buffer, pH 8, containing 0.1 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 8 mM dithiothreitol, with and without 12.8–15.7 μM calmodulin, was brought to 30 °C by a 1-min incubation. Unless indicated otherwise, proteolysis was started by addition of clostripain to final concentrations of 10^3 and 0.7×10^3 units/mL in the presence and absence of calmodulin, respectively. Aliquots (0.1 mL), taken at various time intervals, were mixed with 20 μL of a 20 μg/mL solution of *N*-*p*-tosyl-L-lysine chloromethyl ketone (Sigma) to stop the digestion. The samples were either used directly or stored at −70 °C.

SDS–Polyacrylamide Gel Electrophoresis and ^{125}I-Calmodulin Gel Overlay. Protein samples were denatured by boiling for 1 min in 1% SDS and 0.5 M dithiothreitol and subjected to electrophoresis on gradients on polyacrylamide (7.5–15% T, 2.7% C) in the presence of 0.1% SDS, using the discontinuous buffer system of Laemmli (1970). Calmodulin binding was measured by the gel overlay method (Glenney & Weber, 1980; Carlin et al., 1981) using 10 nM ^{125}I-calmodulin, as described by Klee et al. (1983). Autoradiography was of the dried gels to increase sensitivity. Coomassie staining increased ^{125}I-calmodulin binding to the large calcineurin A polypeptides (61, 57, and 55 kDa) and decreased binding to the 8-kDa fragment and so was done on separate gels run in parallel or after measurement of ^{125}I-calmodulin binding. Protein concentration and calmodulin binding were quantitated by densitometry within the linear response range of the stained gels and autoradiograms, respectively. Separating gels used for the preparation of samples for protein sequencing were stored at 4 °C for 48 h prior to use. Stacking gels were polymerized 1 h before electrophoresis and contained 0.065% (w/v) ammonium persulfate. Electrotransfer, washing and staining of the protein bound to PVDF membranes was performed as described by Matsudaira (1987). Proteins were electrotransferred with 0.3A for 45 min at 4 °C.

Interaction with Calcineurin B. The interaction of the proteolytic derivatives of calcineurin A (42- and 46-kDa fragments obtained after 6- and 540-min incubation with clostripain in the absence and presence of calmodulin, respectively) with calcineurin B was assessed by gel filtration on a Sephadex G-100 column (0.6 × 20 cm) equilibrated in 40 mM Tris-HCl buffer, pH 8, containing 0.1 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM dithiothreitol. The eluate was monitored for absorbance at 230 and 280 nm. Calcineurin A, its derivatives, and calcineurin B were identified by SDS–polyacrylamide gel electrophoresis.

Protein Concentration. Calcineurin and calmodulin were quantitated spectrophotometrically using the extinction coefficients $\epsilon^{1\%}_{277nm} = 9.3$ for calcineurin (Klee et al., 1983) and $\epsilon^{1\%}_{277nm} = 1.8$ for calmodulin (Klee, 1977). The concentration of protein fragments was determined by amino acid analysis (Pico-Tag system of Waters Associates) following the protocol of Heinrikson and Meredith (1984). Transfer of polypeptides to the PVDF membranes was quantitated by densitometry using calcineurin A as a standard and correcting for molecular weight. Small amounts (<20%) of protein were still detectable in the gel after transfer, and about 20% of calcineurin or its proteolytic fragments passed through the membrane and were clearly seen on a nitrocellulose filter placed as a trap behind the PVDF membrane. Calmodulin was not retained on the PVDF membrane under these conditions.

Protein Sequence Analyses. A Model 477 Applied Biosystems protein sequenator was used. PVDF membranes with bound protein were placed in the sequencing cartridge and covered with a precycled Polybrene-coated, trifluoroacetic acid activated, glass fiber filter according to the manufacturer's instructions.[2] Initial yields were calculated on the basis of the protein quantitation described above and the amount of PTH derivatives, corrected for 100% injection, recovered at the first cycle.

Results

Limited Digestion of Calcineurin with Clostripain in the Absence of Calmodulin. Clostripain, an arginine-specific protease, was selected with the aim of obtaining fragments of calcineurin containing an intact calmodulin-binding domain(s). Such domains, when severed from the rest of the protein, are

[1] Abbreviations: PVDF, poly(vinylidene difluoride); EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate; PTH, phenylthiohydantoin.

[2] Applied Biosystems User Bulletin issue 32, 1987.

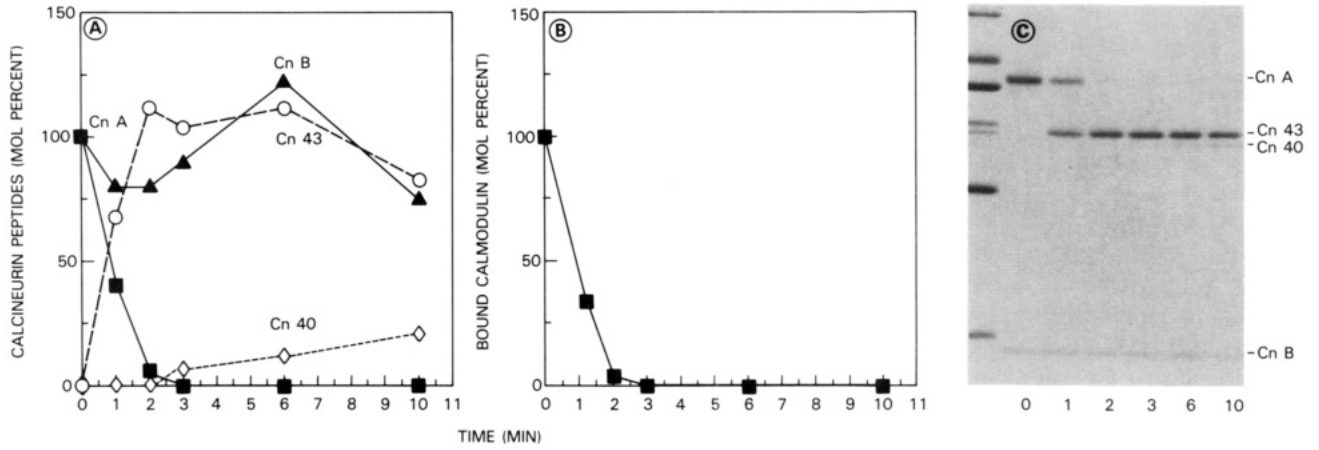

FIGURE 1: Time course of proteolysis of calcineurin in the absence of calmodulin. Calcineurin was digested with clostripain (0.7×10^3 units/mL) and proteolysis stopped at the times indicated as described under Materials and Methods. Samples, corresponding to 5 μg of calcineurin, were subjected to SDS–polyacrylamide gel electrophoresis, tested for ^{125}I-calmodulin binding, and then stained with Coomassie blue. (A) Densitometric analysis of the Coomassie-stained gel. (B) Densitometry of the ^{125}I-calmodulin overlay autoradiogram. Calcineurin A (■) was the sole calmodulin-binding species detected. The results are expressed as mole percent of the value obtained with the original amount of intact calcineurin. (C) Coomassie-stained gel. The positions of calcineurin A (Cn A), calcineurin B (Cn B), and calcineurin A fragments [Cn 43 (○), Cn 40 (◇) are as indicated. The molecular weight marker proteins in the left lane are as follows: phosphorylase *b*, 97K; bovine serum albumin, 67K; catalase, 58K; fumarase, 48K; actin, 42K; lactate dehydrogenase, 36K; and β-lactoglobulin, 17.5K.

immediately cleaved further by trypsin (Manalan & Klee, 1983) or chymotrypsin.[3] As shown in Figure 1C, clostripain rapidly converts calcineurin A to an M_r 43 000 polypeptide. Upon further incubation, the 43-kDa fragment is further degraded to M_r 40 000 and 38 000 polypeptides (data not shown). The molecular weight of calcineurin B was not significantly affected throughout the course of proteolysis. The absence of intermediates with molecular weights between 61 000 and 43 000 indicates that calcineurin A is first cleaved at a highly susceptible site(s) located near one end of the molecule, yielding 43- and 19-kDa fragments. The absence of a detectable M_r 19 000 peptide suggests that this fragment is immediately proteolyzed further once severed from the protein. A quantitative analysis of these data is illustrated in Figure 1A. During the first 10 min of the digestion, the concentration of large calcineurin A fragments (sum of 43- and 40-kDa species) is not significantly different from the initial concentration of calcineurin A. Figure 1B shows that the degradation of calcineurin A is accompanied by a loss of calmodulin binding and that none of the resulting fragments bind calmodulin, as assessed by the ^{125}I-calmodulin overlay method. Thus, conversion of calcineurin A to the 43-kDa protein results in a complete loss of the calmodulin-binding domain. The M_r 43 000 peptide and calcineurin B coelute during gel filtration, indicating preservation of the calcineurin B binding domain on the 43-kDa fragment (data not shown).

The *p*-nitrophenylphosphatase activity of calcineurin and its proteolytic derivatives is illustrated in Figure 2. Native calcineurin is devoid of activity in the absence of Mg^{2+}, measured in 5 mM EDTA (data not shown). In the presence of 6 mM Mg^{2+} (with 1 mM EGTA to chelate Ca^{2+}), the enzyme has the low specific activity of 0.006 unit/mg. Addition of Ca^{2+} (1 mM) results in a 2-fold increase in activity to 0.013 unit/mg. Addition of Ca^{2+} and calmodulin gives a 20-fold enhancement to 0.12 unit/mg. Following a 0.5-min incubation with clostripain, which quantitatively converted calcineurin A to the 43-kDa fragment (Figure 2, inset), the Mg^{2+}-dependent activity is increased to 0.1 unit/mg, a specific activity similar to that of the Ca^{2+}/calmodulin-stimulated holoenzyme. Addition of Ca^{2+} produces a further 3-fold enhancement of activity to 0.35 unit/mg. As expected, calmodulin, which does not bind to this fragment, does not activate the enzyme. Instead, a small inhibition of the Ca^{2+}-stimulated activity was consistently observed as reported by Kincaid et al. (1986). A subsequent loss of the Mg^{2+} activity was observed after longer incubations. Similar results are obtained when the phosphatase activity is monitored by dephosphorylation of the phosphopeptide described by Blumenthal et al. (1986). Thus, native calcineurin A must contain an inhibitory domain which is indistinguishable from the calmodulin-binding domain, adjacent to it or located at the other end of the molecule. Both domains are exquisitely sensitive to proteolytic attack whereas the catalytic site and the calcineurin B binding site are relatively resistant to proteolysis under these conditions.

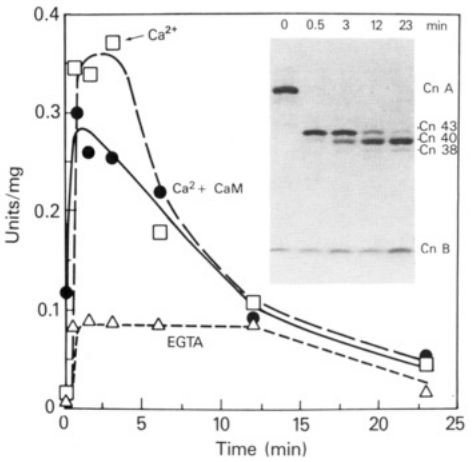

FIGURE 2: Effect of proteolysis of calcineurin, in the absence of calmodulin, on *p*-nitrophenylphosphatase activity. Calcineurin was digested with clostripain, in the absence of calmodulin, as described under Materials and Methods. Proteolysis was stopped at the times indicated, and aliquots were tested for phosphatase activity in the presence either of 1 mM EGTA and 6 mM $MgCl_2$ (△) or of 1 mM $CaCl_2$ and 6 mM $MgCl_2$ without (□) or with 0.17 μM calmodulin (●). The inset shows duplicate aliquots (3.5 μg of calcineurin) which were analyzed by SDS–polyacrylamide gel electrophoresis and stained with Coomassie blue. No significant change in activity was observed in the control sample incubated without clostripain.

Limited Proteolysis of Calcineurin with Clostripain in the Presence of Calmodulin. Clostripain digestion of calcineurin in the presence of a 3-fold molar excess of calmodulin proceeds

[3] M. J. Hubbard and C. B. Klee, unpublished observation.

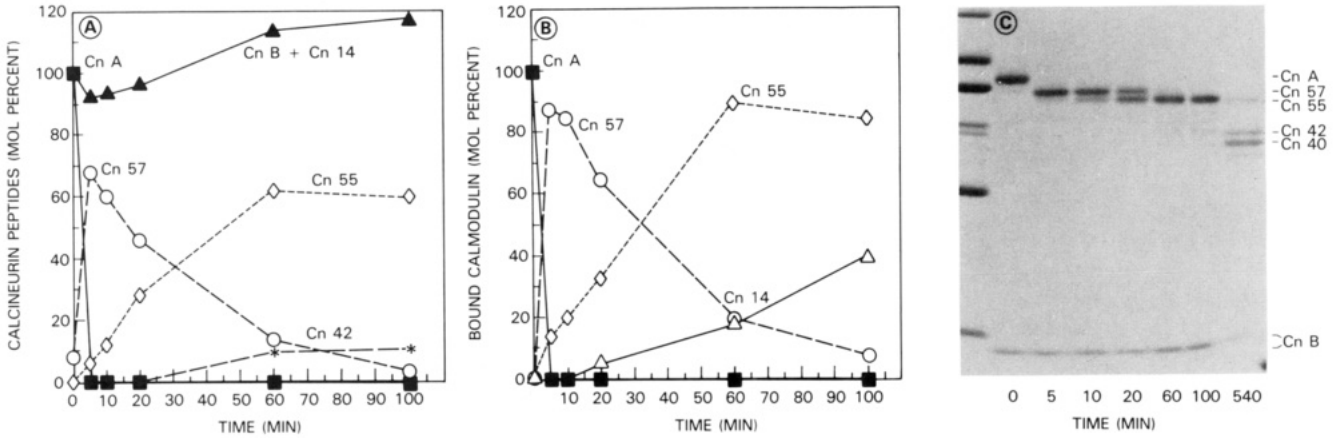

FIGURE 3: Time course of proteolysis of calcineurin in the presence of calmodulin. Proteolysis was carried out as described under Materials and Methods, in the presence of 13.6 μM calmodulin and 1.3×10^3 units of clostripain/mL. The data were analyzed and illustrated as in Figure 1. The identity of calcineurin A (■) and its fragments [Cn 57 (○), Cn 55 (◇), Cn 42 (*), Cn 40 and Cn 14 (▲)] is indicated on the figure. Calmodulin, calmodulin fragments 1–74 and 75–148, and other small peptides diffuse out of the gel during the ^{125}I-calmodulin overlay procedure and so are not visible on the gel shown here.

more slowly and generates different fragments than does digestion in the absence of calmodulin (Figure 3C). Proteolysis affects only calcineurin A and occurs in a stepwise fashion. Calcineurin A is first rapidly (<5 min) and quantitatively converted to a 57-kDa polypeptide which, over the next 55 min, is fully converted to an M_r 55 000 species. After prolonged incubation (>5 h), the latter is progressively degraded to 42- and 40-kDa peptides (Figure 3C). Quantitation of these fragments (Figure 3A) shows that the total of the molar concentrations of calcineurin A and its large proteolytic fragments does not change significantly during the course of the reaction, indicating that a portion of calcineurin A, corresponding to two-thirds of the molecule, is resistant to proteolysis under these conditions. The protease-resistant core remains associated with intact calcineurin B upon gel filtration (data not shown) and must, therefore, contain the calcineurin B binding site. Quantitative analyses of calmodulin binding (Figure 3B) show that the 57- and 55-kDa polypeptides bind calmodulin tightly whereas the 42- and 40-kDa fragments bind calmodulin very weakly.

A small calmodulin-binding peptide (M_r 14 000) becomes apparent after 20-min digestion and appears to be the product of the degradation of the 55-kDa fragment to the 42-kDa species (Figure 3B). The small difference in expected molecular weight (14 000 as opposed to 13 000) is well within the experimental errors in the molecular weight determinations by SDS-gel electrophoresis. As shown in Figure 4, the 14-kDa calmodulin-binding fragment does not accumulate but is further degraded to smaller peptides (M_r 12 000 and 8000). These peptides also bind calmodulin but more weakly than does the 14-kDa fragment. None of these small calmodulin-binding polypeptides were observed when calcineurin was omitted from the incubation mixture, thus ruling out the possibility that they were derived from the autolysis of clostripain in the presence of calmodulin (data not shown).

The calmodulin-binding properties of calcineurin A and of its proteolytic derivatives are summarized in Table I. When calcineurin A has been fully converted to the 55-, 42-, 40-, and 8-kDa fragments, 57% of the original calmodulin-binding capacity is preserved. The 55-kDa fragment binds calmodulin at least as tightly as calcineurin A whereas the 42- and 40-kDa polypeptides bind calmodulin with a greatly reduced efficiency (<10% of calcineurin A). The 14-kDa calmodulin-binding peptide binds calmodulin with high affinity (Figure 4), but the specific binding activity could not be determined because

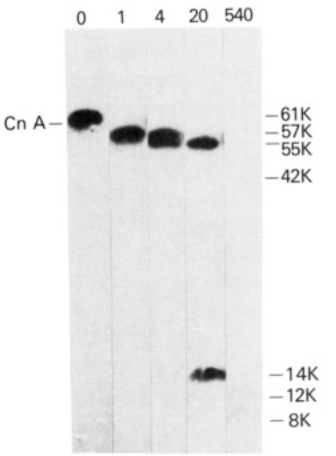

FIGURE 4: ^{125}I-Calmodulin binding to calcineurin A fragments generated in the presence of calmodulin. Calcineurin was digested with clostripain in the presence of 12.8 μM calmodulin, as described in the legend to Figure 3, for the times indicated (in minutes) and 3.5-μg aliquots were subjected to the ^{125}I-calmodulin gel overlay assay and autoradiography. The low level of radioactivity associated with 42-, 12-, and 8-kDa bands is not clearly seen on the photograph of the autoradiogram. The molecular weights of the calmodulin-binding species are indicated on the right.

Table I: ^{125}I-Calmodulin Binding to Clostripain Fragments of Calcineurin[a]

calcineurin A derivatives (M_r)	protein[b] (pmol)	^{125}I-CaM[c]	
		units	units/pmol
61 000	70	70	1.00
55 000	15	21	1.40
42 000	43	4	0.09
40 000	17	0.6	0.03
14 000	nd[d]	12.2	
8 000	nd	2.2	

[a] The Coomassie staining intensity and ^{125}I–CaM binding capacity of calcineurin A and its derivatives obtained after a 40-min clostripain digestion of calcineurin were quantitated after SDS–polyacrylamide gel electrophoresis, as described under Materials and Methods. [b] Calcineurin A and derivatives were quantitated on the basis of direct proportionality between size and staining intensity. [c] One unit is arbitrarily defined as that amount of ^{125}I-CaM bound to 1 pmol of calcineurin A. [d] nd, not determined.

of its comigration with calcineurin B. The protein content of the M_r 8000 and 12 000 peptides was too low to be quantitated, preventing the measurement of specific binding activity by this approach.

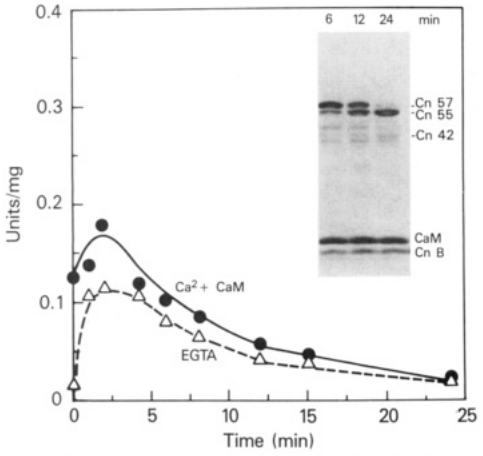

FIGURE 5: Effect of proteolysis of calcineurin, in the presence of calmodulin, on *p*-nitrophenylphosphatase activity. The time course of proteolysis was carried out as described under Materials and Methods, and the samples were analyzed as described in the legend to Figure 2. CaM = calmodulin. No significant change in activity was observed in the control sample incubated for 25 min in the absence of clostripain.

As shown in Figure 5, conversion of calcineurin A to the 57-kDa fragment is accompanied by a loss of Ca^{2+} dependence and a 10-fold enhancement of the *p*-nitrophenylphosphatase activity. Addition of Ca^{2+} in the presence of calmodulin produces very little stimulation despite the presence of a functional calmodulin-binding domain. In contrast, dephosphorylation of the phosphopeptide of Blumenthal et al. (1986) by the 57-kDa fragment is stimulated only 2-fold in the absence of Ca^{2+}, and full activity requires addition of Ca^{2+}. Further proteolysis to the 55-kDa fragment results in the almost complete (80%) inactivation of the enzyme. The proteolytic conversion of calcineurin A to a 57-kDa fragment with constitutively activated *p*-nitrophenylphosphatase activity and a fully preserved calmodulin-binding domain demonstrates that the inhibitory and calmodulin-binding domains are distinct. The loss of phosphatase activity accompanying conversion to the 55-kDa fragment indicates that another part of calcineurin A, whose integrity is critical to preserve enzyme activity, is either adjacent to the inhibitory domain or at the opposite end of the molecule. This region of the molecule is apparently destabilized upon calmodulin binding and is removed by the protease prior to removal of the calmodulin-binding site, which requires several hours.

Mapping of the Functional Domains of Calcineurin A. The stepwise degradation of calcineurin A, yielding a limited number of clearly identifiable peptides, allowed mapping of the functional domains of calcineurin A. The proteolytic products of calcineurin, resolved from each other by SDS–polyacrylamide gel electrophoresis, were subjected to automated Edman degradation as described under Materials and Methods. The results of these experiments are summarized in Table II. The amino terminus of the native protein was blocked to Edman degradation as expected on the basis of earlier end group analyses (Klee et al., 1983). Likewise, analysis of 200 pmol of the 43-kDa fragment, derived from proteolysis in the absence of calmodulin, did not reveal any PTH derivative. Thus, the 19-kDa fragment removed by clostripain must come from the carboxyl-terminal region, and both the inhibitory and calmodulin-binding sites must be located in the carboxyl-terminal part of the molecule. The M_r 57 000 fragment which lacks the inhibitory domain was also blocked to the Edman reaction, confirming the localization of the inhibitory domain to within 40–50 residues of the carboxyl terminus. The amino-terminal sequence of the 55-kDa fragment is shown in Table II. The greatly reduced (>80%) enzymatic activity of this species suggests that a portion of the active site [catalytic center or the binding site of a metal cofactor (Pallen & Wang, 1985)] is located within 20–30 residues of the amino terminus of calcineurin A. Alternatively, this portion of the molecule may be essential to stabilize the active conformation of the enzyme. The amino-terminal sequence of the 42-kDa fragment, a degradation product of the 55-kDa fragment, overlaps with that of its parent molecule, placing the site of the cleavage yielding the 42-kDa fragment and the 14-kDa calmodulin-binding peptide within the carboxyl-terminal third of calcineurin A, adjacent to the inhibitory domain. The relative positions of the functional domains of calcineurin A, as elucidated in these studies, are illustrated schematically in Figure 6.

Table II: Amino-Terminal Sequences of Calcineurin A and Its Proteolytic Derivatives[a]

calcineurin A derivatives (M_r)	amount sequenced (pmol)	amino-terminal sequence
61 000	180	none detected
57 000	180	none detected
55 000	40	V V K A V P
42 000	40	A V P
43 000	200	none detected
14 000	nd[b]	Q F N – S P

[a] Calcineurin A and derivatives following digestion with clostripain (1- and 20-min digests with calmodulin and 0.5-min digest without calmodulin) were separated by SDS–polyacrylamide gel electrophoresis and subjected to sequence analysis as described under Materials and Methods. The amount of each peptide analyzed is indicated. The initial yields for the 55- and 42-kDa fragments were 36 and 28% of the protein applied to the gels, respectively. [b] The amount of 14-kDa fragment could not be determined since it comigrated with calcineurin B. Calcineurin B has a blocked amino terminus (Aitken et al., 1984) and so did not interfere with the sequencing of the 14-kDa fragment. The fourth residue of the 14-kDa fragment was not conclusively identified.

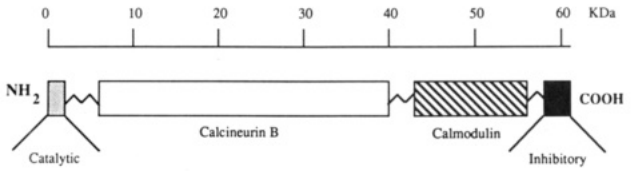

FIGURE 6: Schematic representation of the functional domains of calcineurin A. Mapping of calcineurin A is based on protein sequence analyses of the proteolytic derivatives described in the text. The functional domains, indicated by bars, are identified as follows: catalytic, a domain which contains residues important for or critical to preserve phosphatase activity; calcineurin B, the proteolytically resistant core which contains the calcineurin B binding domain; calmodulin, the calmodulin-binding domain associated with calmodulin-dependent stimulation of the phosphatase. The connecting lines represent proteolytically susceptible hinge regions between the functional domains. The scale bar indicates the approximate size of each domain as assessed by SDS-polyacrylamide gel electrophoresis. NH_2 and COOH denote the amino and carboxyl termini, respectively.

Discussion

Limited proteolysis with trypsin converts calcineurin into an activated phosphatase which is no longer dependent on calmodulin for optimum activity. The proteolyzed calcineurin A remains associated with the B subunit which itself is undegraded (Manalan & Klee, 1983).[4] Clostripain, an argi-

[4] Quantitative peptide mapping by HPLC revealed less than 10% proteolysis of calcineurin B after complete conversion of calcineurin A to the 40-kDa fragment.

nine-specific protease (Mitchell, 1977), cleaves calcineurin more selectively than does trypsin, allowing the isolation of calcineurin derivatives differentially affected in their activity or their regulation by Ca^{2+} and calmodulin.

These studies demonstrate the presence of a domain in calcineurin A which inhibits the phosphatase activity. This inhibitory domain is distinct from the calmodulin-binding domain and is located within the carboxyl-terminal 40–50 residues of calcineurin A. However, it should be borne in mind that neither this polypeptide nor any of the functional domains identified in this paper necessarily represent the entire functional domains of the native protein. Selective removal of the inhibitory domain renders calcineurin almost as active, in the absence of Ca^{2+}, as the native enzyme is in the presence of Ca^{2+} and calmodulin when *p*-nitrophenyl phosphate is used as a substrate. The modified enzyme is no longer stimulated by calmodulin even though it retains the calmodulin-binding domain. Thus, in the native protein, the inhibitory domain exerts a negative influence on the activity which is relieved by calmodulin binding to a distinct calmodulin-binding site. Limited proteolysis of the erythrocyte plasma membrane ATPase (Benaim et al., 1984) and myosin light chain kinases from skeletal (Edelman et al., 1985) and smooth (Ikebe et al., 1987) muscle has also revealed the existence of distinct but overlapping calmodulin-binding and inhibitory domains. A putative ATPase inhibitory domain shows some homologies with calmodulin, raising the possibility that the inhibitory domain of the enzyme interacts with its own calmodulin-binding domain (Brandt et al., 1988). In contrast, the primary structures of the calmodulin-binding domains of skeletal and smooth muscle myosin light chain kinases are similar to the phosphorylation sites of the substrates of these enzymes, suggesting that the calmodulin-binding domains themselves act as pseudosubstrate inhibitors (Kemp et al., 1987; Kennelly et al., 1987). A region adjacent to the postulated calmodulin-binding domain of Ca^{2+}/calmodulin-dependent protein kinase II has also been shown to inhibit kinase activity toward some peptide substrates although its primary structure does not suggest a pseudosubstrate mechanism of inhibition (Payne et al., 1988). The primary structure of the inhibitory domain of calcineurin remains to be determined. It is unlikely to be a phosphopeptide analogous to its substrate since, as isolated, calcineurin contains only 0.2–0.3 mol of phosphate/mol (King & Huang, 1984),[5] the binding of calmodulin has little effect on the K_m of calcineurin toward a variety of substrates (Klee et al., 1988), and only a 2-fold stimulation of the dephosphorylation of the phosphopeptide accompanies removal of the inhibitory domain. Prolonged exposure of calcineurin to clostripain, in the presence of calmodulin, severs the calmodulin-binding domain (14-kDa fragment) from the rest of the molecule (42-kDa fragment). The time course of proteolysis, the conservation of mass (55 kDa → 42 kDa + 14 kDa), the preservation of calmodulin-binding capacity, and the overlapping amino-terminal sequences of the 55- and 42-kDa fragments confirm that the 55-kDa species is the parent of the 42- and 14-kDa fragments. The first three residues (Val-Val-Lys) of the 55-kDa polypeptide are missing from the 42-kDa product, indicating a slow clostripain cleavage after lysine (Mitchell, 1977).

Three lines of evidence show that the 14-kDa fragment and its 8-kDa derivative contain at least a part of the calmodulin-binding domain of calcineurin: (1) these fragments, both in the protein and in isolation, are protected by calmodulin from proteolytic attack; (2) the appearance of free calmodulin-binding peptides is accompanied by a loss of calmodulin binding to the remaining core protein. The residual weak calmodulin-binding capacity of the 42-kDa species suggests that a fraction of the calmodulin-binding domain may remain associated with this fragment. Alternatively, calcineurin may contain more than one calmodulin-binding site. (3) The affinity and Ca^{2+} dependence of calmodulin binding to the 14- and 8-kDa fragments are similar to those of native calcineurin.[6] Unlike the smaller calmodulin-binding peptides isolated from myosin light chain kinases and erythrocyte ATPase, which require stringent conditions such as denaturing agents or high ionic strength for effective dissociation of calmodulin (Klevit et al., 1985; James et al., 1988), complete and rapid ($t_{0.5}$ = 15 s) dissociation of calmodulin from the 8-kDa calcineurin peptide occurs upon chelation of Ca^{2+} with EGTA.[6]

In the absence of calmodulin, clostripain rapidly removes both the inhibitory and the calmodulin-binding domains from calcineurin. Consistent with the removal of the inhibitory domain, the resulting enzyme is as active in the absence of Ca^{2+} and calmodulin as is the Ca^{2+}/calmodulin-stimulated native calcineurin. Moreover, addition of Ca^{2+} increases the *p*-nitrophenylphosphatase activity 3–4 times over the Ca^{2+}/calmodulin-stimulated level of the native enzyme. Thus, calmodulin stimulation is only partial, and the calmodulin-binding domain may exert some inhibition on its own. Removal of the calmodulin-binding domain unmasks the Ca^{2+}-dependent activation of calcineurin, presumably modulated by the high-affinity Ca^{2+}-binding subunit, calcineurin B. Previous reports of the Ca^{2+} regulation of the phosphatase in the absence of calmodulin revealed only a modest 2–3-fold Ca^{2+} stimulation over basal activity (Stewart et al., 1982). The extreme lability of the inhibitory and calmodulin-binding domains of calcineurin to clostripain, trypsin, and chymotrypsin[3] (Manalan & Klee, 1983; Tallant et al., 1984; Li & Chan, 1984) raises the possibility of phosphatase regulation through removal of one or both of these domains. It is currently unknown whether active, processed, forms of calcineurin exist in vivo but the endogenous Ca^{2+}-regulated neutral protease calpain has been shown to activate calcineurin in vitro in a manner analogous to other proteases (Tallant et al., 1988). Calmodulin stimulation is only observed when the inhibitory domain is intact. Although not influenced by calmodulin, the core enzyme (which lacks both inhibitory and calmodulin-binding domains) is still regulated by Ca^{2+}.

The stepwise proteolysis of calcineurin A by clostripain results from large differences in the rates of proteolysis at the susceptible cleavage sites. When proteolysis is carried out in the presence of calmodulin, the first cleavage occurs between the inhibitory domain and the calmodulin-binding domain, near the carboxyl terminus, and yields, within 1 min, a relatively stable M_r 57 000 intermediate. The rate of proteolysis at the second site, about 20 residues away from the amino terminus, is approximately one-tenth that at the first site. Exposure of the second site requires the presence of calmodulin and presumably reflects a calmodulin-induced conformational change in calcineurin A. Cleavage at the third site, between the calcineurin B binding domain and the calmodulin-binding domain, is slow when calmodulin is present. Its occurrence does not seem to depend absolutely on prior cleavages which yield the 57- and 55-kDa proteins since faint bands corresponding to intermediate species (M_r 48 000 and 44 000) are

[5] The calcineurin used for these studies contained 0.2–0.25 mol of P_i/mol (M. H. Krinks, unpublished results).

[6] M. J. Hubbard and C. B. Klee, manuscript in preparation.

clearly detectable and small amounts of the 14-kDa fragment are detected prior to disappearance of the 55-kDa species (Figure 3). In contrast, the third site is the first one to be recognized by clostripain in the absence of calmodulin, directly yielding the 43-kDa fragment.

Calcineurin A appears, therefore, to be structured as a series of distinct domains connected by mobile, protease-sensitive, hinge regions as illustrated in Figure 6. Three domains, catalytic, inhibitory, and calmodulin-binding, lie, in whole or in part, at the ends of the calcineurin A polypeptide and are readily removed by proteolysis. The calcineurin B binding site, which remains intact whether calmodulin is present or not, must be located in the central protease-resistant core of the molecule. The presence of calcineurin B may indeed be responsible for this resistance to proteolysis. It is anticipated that the further characterization of the inhibitory and calmodulin-binding domains, currently in progress, will help in the design of specific inhibitors of the basal and calmodulin-stimulated phosphatase activities of calcineurin.

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