Low Affinity Ca^{2+}-Binding Sites of Calcineurin B Mediate Conformational Changes in Calcineurin A

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ABSTRACT: Limited proteolysis of calcineurin in the presence of Ca^{2+} suggested that its calmodulin-binding domain, readily degraded by proteases, was unfolded while calcineurin B was compactly folded [Hubbard, M. J., and Klee, C. B. (1989) *Biochemistry 28*, 1868−1874]. Moreover, in the crystal structure of calcineurin, with the four Ca^{2+} sites of calcineurin B occupied, the calmodulin-binding domain is not visible in the electron density map [Kissinger, C. R., et al. (1995) *Nature 378*, 641−644]. Limited proteolysis of calcineurin in the presence of EGTA, shows that, when the low affinity sites of calcineurin B are not occupied, the calmodulin-binding domain is completely protected against proteolytic attack. Slow cleavages are, however, detected in the linker region between the calmodulin-binding and the autoinhibitory domains of calcineurin A. Upon prolonged exposure to the protease, selective cleavages in carboxyl-terminal end of the first helix and the central helix linker of calcineurin B and the calcineurin B-binding helix of calcineurin A are also detected. Thus, Ca^{2+} binding to the low-affinity sites of calcineurin B affects the conformation of calcineurin B and induces a conformational change of the regulatory domain of calcineurin A, resulting in the exposure of the calmodulin-binding domain. This conformational change is needed for the partial activation of the enzyme in the absence of calmodulin and its full activation by calmodulin. A synthetic peptide corresponding to the calmodulin-binding domain is shown to interact with a peptide corresponding to the calcineurin B-binding domain, and this interaction is prevented by calcineurin B in the presence but not the absence of Ca^{2+}. These observations provide a mechanism to explain the dependence on Ca^{2+} binding to calcineurin B for calmodulin activation and for the 10−20-fold increase in affinity of calcineurin for Ca^{2+} upon removal of the regulatory domain by limited proteolysis [Stemmer, P. M., and Klee, C. B. (1994) *Biochemistry 33*, 6859−6866].

Calcineurin[1] is the only calmodulin-regulated enzyme whose Ca^{2+} activation is dependent on Ca^{2+} binding to two structurally similar but functionally different Ca^{2+}-regulatory proteins: calcineurin B (CnB),[2] an integral subunit of the enzyme and calmodulin (CaM) (for recent reviews, see refs *4* and *5*). The activation by calmodulin is dependent on binding to the calmodulin-binding domain of the catalytic subunit, calcineurin A (CnA), that induces the displacement of an autoinhibitory domain (*1*, *7*). The identification of protease-sensitive regions in the structure of the catalytic subunit was a powerful tool for elucidating its domain organization and the mechanism of its activation by CaM (*1*, *6*). The structure of the Ca^{2+}-saturated form of calcineurin that was predicted by limited proteolysis has been confirmed by determination of the crystal structure of the recombinant α-isoform of human calcineurin (*2*) and of the proteolytic derivative of bovine brain calcineurin (*8*). In the recombinant enzyme, with four Ca^{2+} sites of CnB occupied, neither the CaM-binding domain nor a linker region between this domain and the autoinhibitory domain are visible on the electron density map indicating that the domain is flexible and readily accessible to calmodulin. The high-affinity Ca^{2+} sites of CnB remain occupied in the presence of EGTA ensuring the tight binding of CnB to CnA (*3*). Kinetic evidence, however, suggest that binding of Ca^{2+} to the moderate affinity sites of CnB is a prerequisite for CaM activation (*3*). Thus, Ca^{2+} binding to these sites may induce a conformational change of calcineurin that facilitates CaM binding and enzyme activation. We have used limited proteolysis of calcineurin in the presence of EGTA to identify the conformational changes accompanying Ca^{2+} binding to the moderate affinity sites of CnB and thereby to further our understanding of the role of CnB in the Ca^{2+} regulation of calcineurin.

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[1] The complex of the A and B subunits of calcineurin (also called protein phosphatase-2B) is referred to as calcineurin. To be consistent with the crystal structures of bovine calcineurin (*8*) and recombinant human calcineurin (*2*), the first residue of CnA is the initiator methionine, the residue numbers are those of CnAα, and the first residue of CnB is the myristoylated glycine.

[2] Abbreviations: CnB, calcineurin B; CnA, calcineurin A; CaM, calmodulin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; ATEE, *N*-acetyl-L-tyrosine ethyl ester; BAME, *N*-benzoyl-L-arginine methyl ester; TPCK, L-(tosylamido-2-phenyl)ethyl chloromethyl ketone; PVDF, polyvinylidene fluoride; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; FKBP, FK506-binding protein; NFAT, nuclear factor of activated T cells; IP_3, inositol 1,4,5-trisphosphate.

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EXPERIMENTAL PROCEDURES

Materials. Bovine brain calcineurin, purified as previously described (*9*), was freed of protease inhibitors by gel filtration on a Sephadex G-100 column equilibrated in 40 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT, and 10% glycerol. Recombinant human CnB (CnB^{AK}) with Cys^{11} and Cys^{153} replaced by Ala and Lys, respectively, was expressed in *Escherichia coli* and purified as previously described (*10*). A mutant of CnB^{AK} with a Glu^{73}/Gln substitution in the second Ca^{2+}-binding loop (CnBQ2) and biotinylated CaM were prepared as described (Gallagher et al., unpublished experiments, *11*). Stock solutions (1−2 mg/mL in 1 mM HCl) of L-(tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK)-treated trypsin and chymotrypsin, products of Worthington Biochemical Corp., were stored at −70 °C. *N*-Acetyl-L-tyrosine ethyl ester (ATEE), *N*-benzoyl-L-arginine methyl ester (BAME), TPCK, and soybean trypsin inhibitor were from Sigma. Polyvinylidene fluoride (PVDF) membranes were obtained from Millipore Co. (Immobilon 0.45 μm pore size). CaM−Sepharose was purchased from Amersham Pharmacia Biotech. Sequencing-grade reagents, glass fiber filters, and Biobrene were purchased from PE Applied Biosystems. Synthetic peptides, corresponding to the CaM-binding domain, ARKEIIRNKIRAIGKMARVFSVLR, and to the CnB-binding domain, DVFTWSLPFVGEKVTEMLVNVLNISSDDE,[3] of CnA were purchased from Peptide Technologies (Rockville, MD) and further purified by HPLC.

Enzyme Assays. Protease activities were measured as described by Schwert and Tanaka (*12*). ATEE hydrolysis was monitored by the decreased absorbance at 237 nm (chymotrypsin), and BAME hydrolysis was monitored by the increased absorbance at 253 nm (trypsin) at 25 °C. One unit of chymotrypsin (1500 units/mg) catalyzes the hydrolysis of 0.1 μmol of ATEE/min, and one unit of trypsin (3300 units/mg) catalyzes the hydrolysis of 0.01 μmol of BAME/min under the standard assay conditions. The rates of hydrolysis were also determined under the conditions used for the limited proteolysis experiments to correct for the effect of $CaCl_2$ and EGTA on the activities of trypsin and chymotrypsin. The specific activities of trypsin and chymotrypsin in the presence of EGTA were 80% of those measured in the presence of $CaCl_2$ (*13*).

Limited Proteolysis of Calcineurin. Calcineurin (0.12 mg in 1.2 mL of 40 mM Tris, pH 7.5, containing 0.1 M NaCl, 1 mM $MgCl_2$, 1 mM DTT, and 10% glycerol and either 0.6 mM $CaCl_2$ or 2 mM EGTA) was preincubated for 1 min at 30 °C. Proteolysis was started by addition of 65 μL of a freshly diluted solution (20 μg/mL in 1 mM HCl) of chymotrypsin or trypsin to final concentrations of 1.5 or 3.5 units/mL, respectively. At the indicated times, 0.1-mL aliquots were added to tubes containing 5 μL of 0.2 mg/mL TPCK (chymotryptic digest) or 0.2 mg/mL soybean trypsin inhibitor (tryptic digest) to stop the reactions, and the samples were immediately placed in dry ice or stored at −70 °C.

SDS Gel Electrophoresis. Forty microliter aliquots of the digests (4 μg of calcineurin) mixed with 20 μL of denaturing solution (0.1 M Tris, pH 8, 1% SDS, 8 M urea, and 0.8 M DTT) were boiled for 1 min and immediately subjected to SDS−polyacrylamide gel electrophoresis on 7.5−15% linear acrylamide gradients containing 0.1% SDS according to Laemmli (*14*). The sizes of the proteolytic fragments were estimated by comparison with the R_f values of protein standards (phosphorylase *b*, 97 kDa; bovine serum albumin, 67 kDa; catalase, 58 kDa; fumarase, 48 kDa; actin, 42 kDa; lactate dehydrogenase, 36 kDa; and β-lactoglobulin, 17.5 kDa). The concentration of calcineurin fragments was determined by densitometric analysis of the stained bands using the public domain NIH Image 1.55 program for Macintosh computers written by Wayne Rasband (*15*) at the U.S. National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov. CnA and CnB were used as standards. The color responses of calcineurin derivatives are assumed to be proportional to their estimated sizes. The amount of calcineurin derivatives in the EGTA digests was corrected for the 20% decrease of the specific activities of the proteases under these conditions.

Biotinylated Calmodulin Overlay. The CaM-binding fragments of CnA transferred to a PVDF membrane were detected by CaM overlay using 0.5 μg/mL biotinylated CaM as described by Smith and Scott (*16*). CaM was detected with avidin and biotinylated horseradish peroxidase (Vectastain reagents from Vector Laboratories) and enhanced chemiluminescence (ECL) detection reagents from Amersham Pharmacia Biotech.

EDTA Binding to the CaM-Binding Peptide. EDTA binding to the CaM-binding peptide corresponding to the CaM-binding domain of the β-isoform of CnA was measured by the method of Hummel and Dreyer (*17*). A 0.25-mL aliquot of a peptide solution (0.5 mM in 40 mM Tris-HCl, pH 7.5, 0.1 M KCl, containing 1 mM [^{14}C] EDTA) was loaded on a Sephadex G-10 column (3.7 mL) equilibrated in the same buffer. Fractions (0.25 mL) were monitored for radioactivity (EDTA) and absorbance at 258 nm (peptide).

Microsequencing of Calcineurin Fragments. Amino terminal sequences of calcineurin and its proteolytic fragments were determined by microsequencing on an Applied Biosystems protein sequenator (model 477A) according to the manufacturer's instructions. CaM-binding fragments of calcineurin were isolated by affinity chromatography on a column of CaM−Sepharose, and their size was analyzed by SDS gel electrophoresis. Polypeptides with $M_r \geq 10\,000$ were transferred to PVDF membranes, and their amino-terminal sequences were determined as described by Matsudeira (*18*). The small fragments, not retained on the CaM−Sepharose, were purified by HPLC on a C_{18} μBondapak column (3.9 × 300 mm, 10 μm beads, from Waters Associates), with a 70-min, 0−50% linear gradient of acetonitrile in 0.1% trifluoroacetic acid. The flow rate was 1.5 mL/min, and the fraction size was 0.5 min. Peptide-containing fractions were pooled, flash evaporated, and dissolved in 20 μL of 30% acetonitrile in 0.1% TFA for sequencing.

UV Difference Spectroscopy. A Cary model 118 C spectrophotometer connected to an Olis Spectroscopy Operating system was used for data collection, and the data were analyzed with the Dataplot program (*19*) on a Silicon Graphics work station. Stock solutions of the CnB-binding peptide in the presence or absence of CnB (50 μM each) and of the CaM-binding and control peptides (50 μM) in 20

[3] To increase the solubility of the synthetic CnB-binding peptide, the three carboxyl-terminal residues (DDE), not required for interaction, were included and Cys^{372} was substituted by Ser to prevent disulfide formation and dimerization.

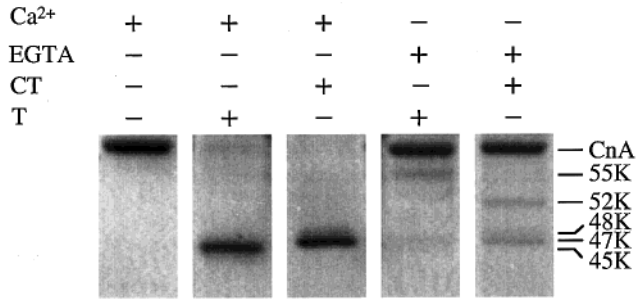

FIGURE 1: Limited proteolysis of calcineurin. Calcineurin was digested with chymotrypsin (CT) or trypsin (T) in the presence of Ca^{2+} or EGTA as described under Experimental Procedures. After 5-min incubation at 30 °C, the digests were analyzed by SDS gel electrophoresis and stained with Coomassie blue. The M_r of the protein standards is on the right.

mM Hepes, pH 7.5, containing 0.1 M KCl and 2 mM EGTA or 0.1 mM Ca^{2+} were used to measure the spectra of a 1:1 mixture of the two solutions or the sum of the individual spectra in double-compartment cells as described (*20*).

The protein concentrations were determined spectrophotometrically using the extinction coefficients $\epsilon^{1\%}_{280\ nm} = 7.4$ for calcineurin (C. Klee & P. Stemmer, unpublished observations), $\epsilon^{1\%}_{276\ nm} = 2.41$ for CnB (*10*), $\epsilon_{258\ nm} = 195$ for the CaM-binding peptide, and $\epsilon_{280\ nm} = 5560$ for the CnB-binding peptide based on their tryptophan and phenylalanine contents.

RESULTS

Ca^{2+} Binding to CnB Exposes the Regulatory Domain of CnA to Proteolysis. As previously reported, CnA is rapidly converted to a 45 000 M_r derivative by limited proteolysis with trypsin in the presence of Ca^{2+} (*6*). Similarly, limited proteolysis with chymotrypsin rapidly yields a 47 000 M_r derivative (Figure 1). In contrast, in the presence of EGTA only small amounts of 52 000 and 48 000 M_r derivatives are detected in the chymotryptic digests, and a 55 000 M_r derivative is found in the tryptic digest. Thus, the CaM-binding domain, which is readily severed from the rest of the molecule by these two proteases in the presence of Ca^{2+}, is protected against proteolysis in the presence of EGTA suggesting that Ca^{2+} binding to CnB induces a large conformational change in the regulatory domain of CnA thereby exposing its CaM-binding domain to proteases.

A comparison of the time courses of calcineurin proteolysis by chymotrypsin in the presence of Ca^{2+} and EGTA is shown in Figure 2. In the presence of Ca^{2+}, CnA is rapidly converted to the 47 000 M_r derivative and is not degraded further for up to 20 min, while CnB is resistant to proteolysis. In the presence of EGTA, CnA is slowly converted to 52 000 and 48 000 M_r fragments. The rates of appearance of these two fragments suggest the presence of two cleavage sites almost equally susceptible to proteolysis. After a lag phase, CnB is slowly degraded with only 40% of the initial protein remaining after 20-min digestion. When the concentration of chymotrypsin and the incubation time were increased, the 52 000 and 48 000 M_r derivatives did not accumulate but were slowly converted to 47 000 and 42 000 M_r fragments. In the presence of Ca^{2+}, neither the 47 000 M_r fragment nor CnB was further degraded.

The proteolytic fragments of CnA were tested for their ability to bind calmodulin by biotinylated calmodulin overlay.

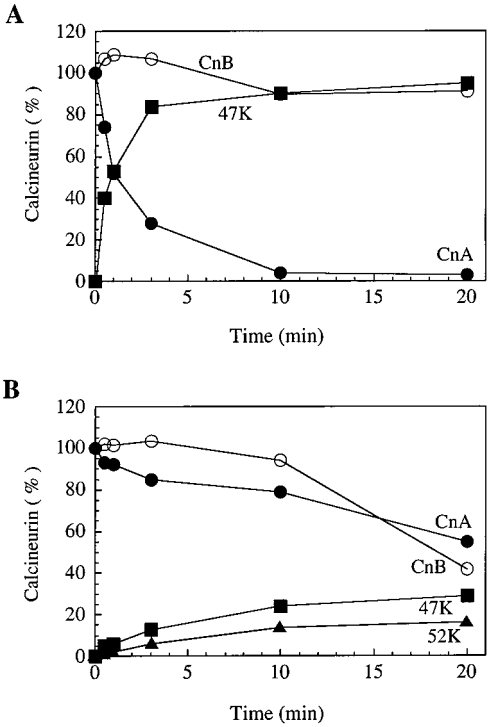

FIGURE 2: Time course of the proteolysis of calcineurin with chymotrypsin. Calcineurin was digested at 30 °C as described in Experimental Procedures in the presence of Ca^{2+} (A) or EGTA (B). At the times indicated, aliquots were removed, the reactions were stopped with TPCK, and the samples were subjected to SDS gel electrophoresis. After staining with Coomassie blue, the calcineurin fragments were quantitated as described in Experimental Procedures. The results are expressed as mol % of the value obtained with the original amount of calcineurin in the digest.

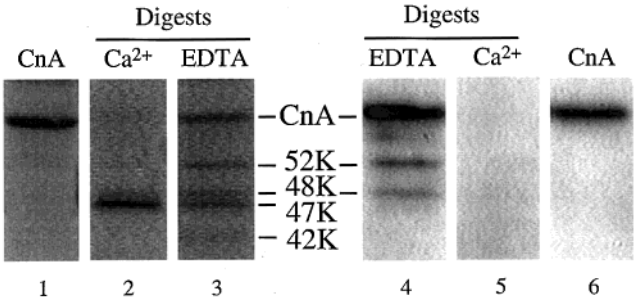

FIGURE 3: Calmodulin binding to proteolytic fragments of calcineurin A. Calcineurin was digested with chymotrypsin (2.4 units/mL) for 10 min, and the calcineurin fragments were separated by SDS gel electrophoresis, transferred to PVDF membranes, and either stained with Coomassie blue (lanes 1−3, 4 μg/lane) or tested for their ability to bind biotinylated calmodulin [0.6 μg (lane 4), 0.1 μg (lanes 5 and 6)] as described in Experimental Procedures. The amount of digest applied to lane 4 was increased to compensate for the low concentration of CnA fragments.

As shown in Figure 3 (lane 6), CnA strongly binds calmodulin. Under these conditions, the 47 000 M_r fragments in the Ca^{2+} and EDTA digests as well as the 42 000 M_r fragment detected after prolonged incubation with chymotrypsin lost their ability to bind calmodulin (lanes 4 and 5). Although the 52 000 and 48 000 M_r fragments in the EDTA digest retained their ability to bind calmodulin (lane 4), their affinity for calmodulin was reduced significantly. Only 40% and 20% as much calmodulin was bound to the 52 000 and 48 000 fragments, respectively, as to CnA (Table 1).

Table 1: Calmodulin Binding to Chymotryptic Fragments of Calcineurin A[a]

CnA polypeptides (M_r)	protein[b] (pmol)	biotinylated-CaM[c] (units/pmol)
	EDTA Digest	
CnA	25	1.0
52K	10	0.4 ± 0.1
48K	8	0.2 ± 0.1
47K	19	nd[d]
	Ca^{2+} Digest	
47K	85	nd

[a] Calcineurin (0.1 mg/mL) was digested with chymotrypsin (2.4 units/mL) for 10 min in the presence of Ca^{2+} or EDTA. [b] Cn fragments were quantitated on the basis of direct proportionality between size and staining intensity. [c] One unit is arbitrarily defined as that amount of biotinylated-CaM bound to 1 pmol of CnA. [d] nd, not detectable.

EDTA Does Not Bind to the CaM-Binding Peptide. To rule out the possibility that binding of negatively charged EGTA to the positively charged CaM-binding domain of CnA was responsible for the protective effect of EGTA or EDTA, the binding of EDTA to the CaM-binding peptide was measured by the Hummel−Dreyer technique (*17*). No significant binding of EDTA to the CaM-binding peptide was observed in the presence of 1 mM EDTA, a concentration sufficient to protect calcineurin against proteolysis (data not shown).

Identification of the Chymotrypsin-Sensitive Sites. The cleavage sites of calcineurin were identified in order to probe further the nature of the conformational change accompanying Ca^{2+} binding to CnB. The large calcineurin fragments were purified by affinity chromatography on CaM−Sepharose, and small fragments were purified by HPLC. All CnA fragments with a $M_r > 47\,000$ were quantitatively recovered in the bound fraction (EGTA eluate), including a 57 000 M_r derivative detected only after prolonged proteolysis (Figure 4A). Most of the 47 000 M_r fragment, whose affinity for CaM was too low to be detected by CaM gel overlay (Figure 3, lane 4), was also bound to CaM−Sepharose. The 42 000 species did not bind to the column [the 42 000 band detected in the bound fraction (Figure 4A, lane 2) has not been definitively identified]. Like native CnA, none of the fragments with $M_r \geq 42\,000$ shown in panel A (lanes 2 and 3) had detectable amino termini suggesting that they all resulted from cleavages in the carboxyl-terminal regulatory domain of CnA. Thus, except for the 42 000 M_r fragment, all large CnA fragments retained at least part of the CaM-binding domain (*5*).

The Coomassie blue pattern of CnB and its proteolytic fragments, identified by Western blotting (Figure 4C), is shown in Figure 4B. Most of the residual CnB recovered in the bound fraction must remain tightly bound to CnA or its fragments since CnB does not bind to CaM−Sepharose. The small amount of CnB detected in the flow through may be dissociated from the 42 000 M_r derivative of CnA resulting from a cleavage in the CnB-binding domain (see below). The 14 000 and 10 000 M_r CnB fragments[4] in the bound fraction were identified by microsequencing as the carboxyl-terminal fragments (residues 27−167 and 82−167, respectively) whereas the 14 000 and 10 000 M_r fragments not bound to CaM−Sepharose (flow through), with no detectable end groups, were amino-terminal fragments (Table 2). The 14 000 M_r fragment in the flow through was not identified but was assumed to be the result of a nonidentified cleavage in the carboxyl-terminal lobe of CnB. Thus, once cleaved by chymotrypsin the amino-terminal fragments of CnB no longer bind to CnA whereas the carboxyl-terminal fragments,

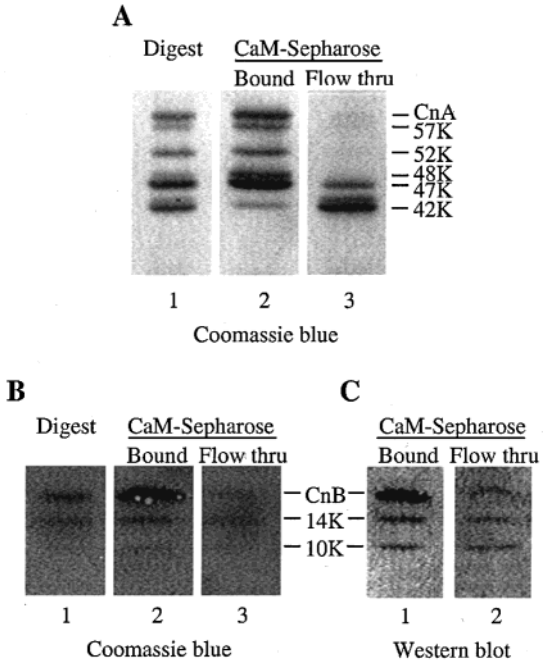

FIGURE 4: Isolation of calcineurin fragments by affinity chromatography on calmodulin−Sepharose. A chymotryptic digest of calcineurin in EGTA (0.2 mg in 0.5 mL incubated with 0.75 unit of chymotrypsin for 40 min) was applied to a 0.2-mL column of calmodulin−Sepharose. The protein fragments not retained on calmodulin−Sepharose were recovered in the flow through and a 0.2-mL wash (Flow thru), and the fragments bound to the column were eluted with EGTA. The protein fragments were separated by SDS gel electrophoresis, transferred to a PVDF membrane, and stained with Coomassie blue. (A) Calcineurin A fragments. (B) Calcineurin B fragments (samples equivalent to 5, 40, and 30 μg of calcineurin present in the digest were applied to lanes 1−3, respectively). (C) Western blots of calcineurin B fragments (0.17 and 0.5 μg equivalents of calcineurin were applied to lanes 1 and 2, respectively).

Table 2: Identification of Chymotrypsin-Sensitive Sites

calcineurin fragments		
M_r	amino-terminal sequences	cleavage sites
	Flow Through	
1 800 (CnA)	TLKGLTPTGMLPSGVL[a]	Leu^{421}, Leu^{437}
(CnA)	SVLREESESV[b]...	Phe^{410}
13 000 (CnA)	SPQ-KITSFAE...	Phe^{461}
14 000 (CnA)	SGGKQTLQTL...	Leu^{437}
10 000 (CnB)	nd[c]	Phe^{81}
14 000 (CnB)	nd	
	Bound	
(CnA)	VGEKVTEMLV[b]...	Phe^{356}
10 000 (CnB)	SVKGDKEQKL ...	Phe^{81}
14 000 (CnB)	KKLDLSNSGS...	Phe^{26}

[a] Amino acid sequence of the single peptide, recovered in the flow through of calmodulin−Sepharose after a short digestion with chymotrypsin and purified by HPLC as described in Experimental Procedures. [b] Amino-terminal sequences of calcineurin peptides detected in the flow through and EGTA eluate of the calmodulin−Sepharose illustrated in Figure 4. [c] nd, no end group detected.

[4] As previously shown for CnB, the M_r of CnB fragments may not accurately correspond to their MW (*36*).

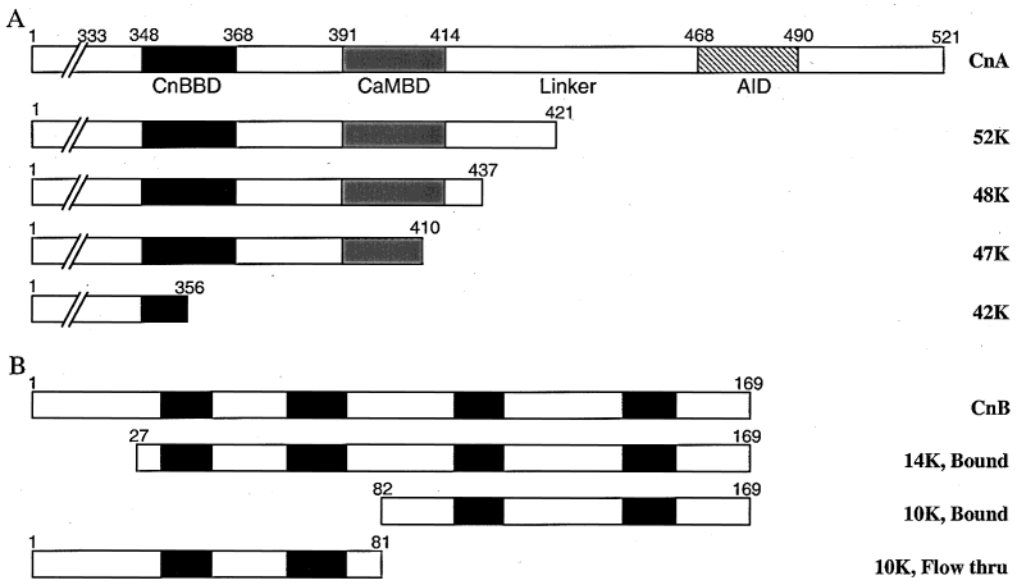

FIGURE 5: Schematic representation of the chymotrypsin sensitive sites in the regulatory domain of calcineurin A (A) and calcineurin B (B) in the presence of EGTA.

with the two high-affinity Ca^{2+} sites (*21*; Gallagher et al., unpublished observations) remaining bound to CnA.

The amino-terminal sequences of small peptides recovered in the flow through and the EGTA eluate (Table 2) were used to identify the sites of cleavage of CnA and CnB illustrated in Figure 5. A single peptide corresponding to residues 422−437 detected in the HPLC profile of the flow through fraction of the short digest of calcineurin illustrated in Figure 1 identified Leu^{421} and Leu^{437} as the sites of cleavage yielding the 52 000 and 48 000 M_r fragments of CnA. Upon further proteolysis, an additional cleavage at Phe^{410} accompanied the appearance of the 47 000 M_r fragment while cleavage at Phe^{461} may yield the 57 000 M_r fragment detected upon prolonged proteolysis (Figure 4). A trace of peptide resulting from a cleavage at Phe^{356} suggested that the 42 000 M_r fragment is the result of a cleavage in the CnB-binding domain. No cleavage was detected in the CaM-binding domain between residues 391 and 410.

Interaction between the CaM-Binding and the CnB-Binding Peptides. The protection of the CaM-binding domain of calcineurin against proteolysis in the presence of EGTA suggested that this domain may interact with CnB or the CnB-binding domain of CnA when the low-affinity sites of CnB are not occupied. To test this possibility, the interaction of the CaM-binding peptide with CnB and with the CnB-binding peptide was monitored by UV difference spectroscopy. The UV spectrum of CnB mixed with the CaM-binding peptide in the presence of EGTA was identical to the sum of their individual UV spectra in agreement with previous reports, indicating that CnB does not bind to CaM-binding peptides in the presence or absence of Ca^{2+} (*10*, *11*). Increased light scattering was previously observed upon mixing the CaM-binding peptide (Ala^{391}−Arg^{414}) with a CnB-binding peptide (Phe^{334}−Val^{361}), suggesting that the CaM-binding and CnB-binding domains of CnA interact with each other (P. M. Stemmer and C. B. Klee, unpublished observations). This interaction is specific for the CaM-binding peptide of CnA. A Ca^{2+}-independent increase in light scattering was observed upon mixing the CnB-binding peptide used in these studies with the CaM-binding peptide

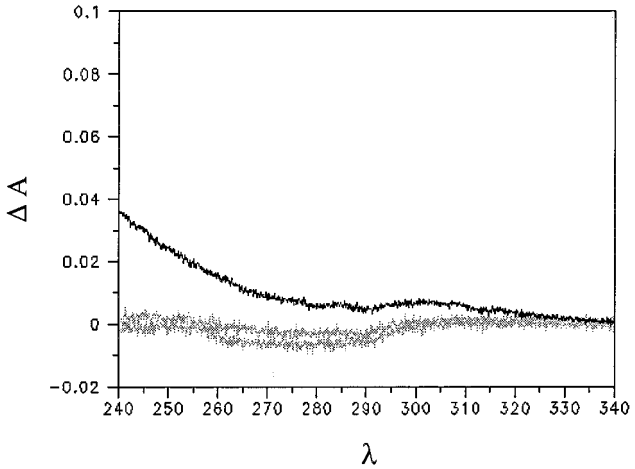

FIGURE 6: Specific interaction of the CnB-binding peptide with the CaM-binding peptide of CnA. UV difference spectra measured as described in Experimental Procedures in the presence of 2 mM EGTA of a 50 μM solution of the CnB-binding peptide mixed with an equal volume of 50 μM solutions of CaM-binding peptide (solid line) or of control peptides corresponding to Arg^{401}−Lys^{424} and Ser^{411}−Lys^{441} of CnA (dotted lines). A similar pattern was observed in the presence of Ca^{2+}.

but not with peptides corresponding to residues Arg^{401}−Lys^{424} and Ser^{411}−Lys^{441} (Figure 6). As shown in Figure 7A,B, a time-dependent increase in light scattering was observed when a solution containing an equimolar mixture of CnB and the CnB-binding peptide was mixed with the CaM-binding peptide in the presence of EGTA but not in the presence of Ca^{2+} (a subsequent decrease of light scattering, observed upon prolonged incubation, was due to the sedimentation of aggregated proteins). Moreover, when CnB was replaced by a mutant of CnB, whose affinity for Ca^{2+} at the two low-affinity sites is greatly reduced (Gallagher et al., unpublished experiments), light scattering was still observed in the presence of Ca^{2+} (Figure 7C). Thus, addition of CnB confers Ca^{2+} dependence to the interaction between the CaM-binding peptide and the CnB-binding peptide.

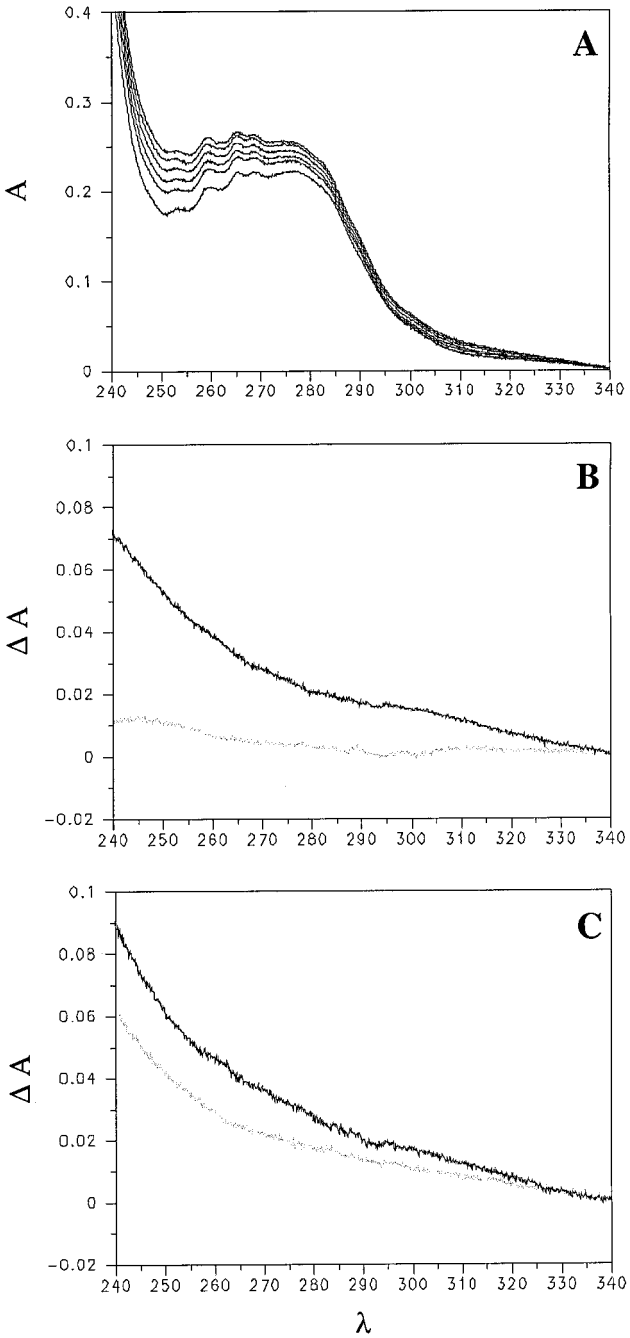

FIGURE 7: Ca^{2+} dependence of the interaction of the CaM-binding peptide with the CnB-binding peptide in the presence of CnB. (A) UV absorbance spectra of a solution containing 50 μM CnB and 50 μM CnB-binding peptide mixed with an equal volume of a 50 μM solution of CaM-binding peptide in the presence of 2 mM EGTA collected immediately after mixing and at 40-min intervals. (B) UV difference spectra of the mixed peptide solution in 2 mM EGTA as described above (solid line) or in the presence 0.1 mM Ca^{2+} (dotted line) taken immediately after mixing against the sum of the individual spectra of the two solutions in the corresponding buffers. (C) Same as panel B, but CnB was replaced by the Glu73/Gln CnB mutant, CnBQ2. The data were analyzed as described in Experimental Procedures.

DISCUSSION

The limited proteolysis experiments described above were designed to identify the nature of the conformational change accompanying Ca^{2+} binding to calcineurin in order to further our understanding of the role of CnB in the regulation of the protein phosphatase activity of calcineurin. The conformational change of CnA induced by Ca^{2+} binding to the moderate affinity sites of CnB, predicted on the basis of these experiments, is illustrated in Figure 8.

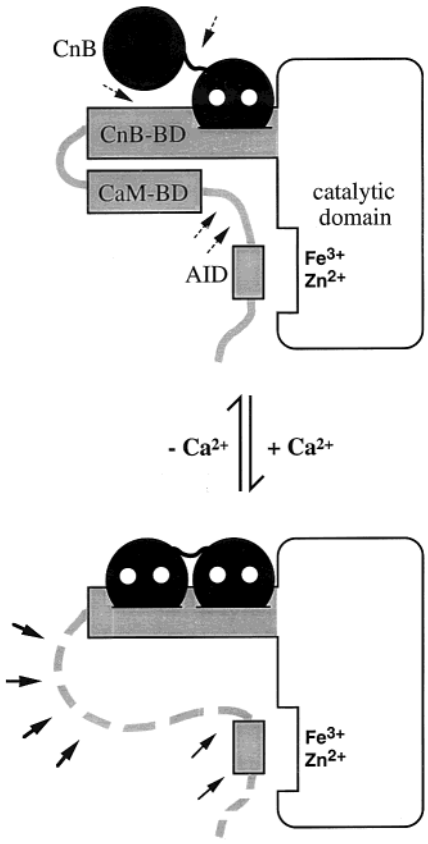

FIGURE 8: Schematic representation of the conformational change of CnA induced by Ca^{2+} binding to CnB proposed on the basis of limited proteolysis experiments. The regulatory domain of CnA is shown in gray. The dotted lines indicate a flexible region in the crystal structure of recombinant calcineurin (*2*). The arrows indicate the cleavage sites, and the filled Ca^{2+} sites are shown as white circles. CnB-BD, CnB binding-domain; CaM-BD, CaM-binding domain; AID, autoinhibitory domain.

In the crystal structure of calcineurin, with the four Ca^{2+} sites of CnB occupied, the CaM-binding domain is flexible and not visible in the electron density map (*2*). Accordingly, in solution, this domain is rapidly proteolyzed in the presence of Ca^{2+} (*1*, *6*, *22*−*26*) and is readily accessible to calmodulin. Limited proteolysis of calcineurin in the presence of EGTA shows that the CaM-binding domain is resistant to proteolysis and may not be available for calmodulin binding. In the presence of EGTA, the high-affinity site(s) of CnB are not depleted and CnB remains bound to CnA (*3*). The two high-affinity sites of CnB have been identified as the EF3 and EF4 hands in the carboxyl-terminal lobe (*21*). It is likely that occupancy of these two sites is required to promote the interaction of the carboxyl-terminal lobe of CnB with the amino-terminal CnB-binding helix of CnA predicted by site-directed mutagenesis (*27*) and confirmed by the determination of the crystal structure of calcineurin (*2*, *8*). In the presence of EGTA, the EF1 and EF2 hands with a moderate affinity for Ca^{2+} are not occupied, and kinetic evidence suggested that Ca^{2+} binding to these sites is required for calmodulin activation (*3*). Protection of the CaM-binding domain against proteolysis, when these sites are not occupied, was observed with two proteases with different substrate specificities. It is therefore likely that this protective effect

is due to a conformational change of the regulatory domain of CnA induced by Ca^{2+} dissociation from the EF1 and EF2 hands of CnB and that Ca^{2+} binding to these two sites results in the exposure of the CaM-binding domain of CnA allowing activation of the enzyme.

The next question to be addressed was how can Ca^{2+} binding to the moderate affinity sites of CnB induce the exposure of the CaM-binding domain of CnA? In the presence of Ca^{2+}, CnB is completely resistant to proteolysis (*1*, *6*) whereas in the presence of EGTA, when its moderate affinity sites are depleted, CnB is cleaved at Phe^{26} and at Phe^{81} in the central, flexible, helix. By analogy with CaM, it is likely that Ca^{2+} binding to CnB protects it against proteolysis while exposing two hydrophobic patches, each formed by a pair of EF hands, that are responsible for the binding of CnB to its binding domain. In the structure of calcineurin the two lobes of CnB, each containing two occupied Ca^{2+} sites, are aligned along the hydrophobic surface of the CnB-binding helix of CnA (*2*, *8*). Thus, Ca^{2+} binding to the two moderate affinity sites of CnB may be necessary for the binding of the N-terminal lobe of CnB to the CnB-binding helix of CnA. This is consistent with the dissociation of the fragment 1−81 of CnB from CnA observed upon proteolysis in the presence of EGTA while the C-terminal fragments 27−169 and 82−169 (containing at least one high-affinity site occupied) are still bound to CnA.

We next tried to understand how the Ca^{2+}-dependent binding of the amino-terminal lobe of CnB to the CnB-binding helix could induce the exposure of the CaM-binding domain. Although the exposure of part of the CnB-binding helix in the presence of EGTA should render the CnB-binding helix susceptible to proteolysis, the proteolytic cleavage at Phe^{356}, a peptide bond resistant to proteolysis in the presence of Ca^{2+}, is also very slow in the presence of EGTA, suggesting that neither the CnB-binding helix nor the CaM-binding domain is readily accessible. Interaction between the two domains could explain their resistance to proteolysis. The increased light scattering observed upon mixing the CaM-binding peptide and the CnB-binding peptide in the presence of CnB confirmed that the two peptides interact with each other. As predicted, this interaction was prevented by the addition of Ca^{2+} that is required for CnB binding to its binding domain (*10*, *28*). Moreover a CnB mutant (CnBQ2) deficient in Ca^{2+} binding to one of the moderate affinity sites failed to prevent the aggregation of the peptides upon addition of Ca^{2+}. A similar CnB mutant with a Glu^{73}/Lys substitution also failed to yield a fully active enzyme when coexpressed with CnA (*21*). In the intact protein, CnB remains bound to CnA in the presence of EGTA (with its high-affinity sites occupied). Depletion of the low-affinity sites may be required to expose the C-terminal half of the CnB-binding helix and thereby allow its interaction with the CaM-binding domain.

We previously reported that the phosphatase activity of a truncated form of calcineurin lacking the C-terminal regulatory domain (residues 392−521) is calmodulin independent but still depends on Ca^{2+} binding to CnB (*3*). The 10−20-fold increased affinity for Ca^{2+} of this truncated enzyme suggested that the regulatory domain exerts a negative control on the affinity of calcineurin for Ca^{2+}. Although removal of the inhibitory domain (Gly^{460}−Gln^{521}) is sufficient to render the *p*-nitrophenyl but not the protein phosphatase activity of calcineurin independent of calmodulin (*1*, *29*−*31*), the increased affinity for Ca^{2+} was only observed upon removal of residues 392−451 (*3*) and was later shown to require the deletion of residues 421−457 (*29*, *30*). The increased affinity of calcineurin for Ca^{2+} upon removal of the regulatory domain could be explained by the preferential interaction of this regulatory domain with CnB or the CnB-binding helix of CnA when the moderate affinity sites of CnB are not occupied. When the interaction of overlapping synthetic peptides corresponding to the CnA regulatory domain (residues 391−441) with the CnB-binding peptide was tested by UV difference spectroscopy, the only peptide shown to interact with the CnB-binding peptide was the CaM-binding peptide (residues 391−414). The presence of two protease-sensitive bonds in the 420−457 linker between the CaM-binding and inhibitory domain of Ca^{2+}-depleted calcineurin also suggests that this linker domain, accessible to proteolysis, does not interact with CnB or its binding helix. Since the 420−457 linker does not include the CaM-binding domain, it is possible that its deletion increases the affinity of calcineurin for Ca^{2+} by facilitating the dissociation of the CaM-binding domain from the CnB-binding helix which, as described below, should result in an increased affinity of calcineurin for Ca^{2+}.

In the absence of Ca^{2+}, the binding of the CaM-binding domain to the polar surface of the CnB-binding helix may also interfere with the binding of the immunosuppressive drugs, FK506 and cyclosporin A, complexed with their binding proteins to the hydrophobic cleft formed by the bottom surface of the CnB-binding helix, and the linker between the third and fourth EF hands of CnB (*8*, *27*). A Ca^{2+}-induced displacement of the CaM-binding domain could then explain why Ca^{2+} binding to CnB is required for the Ca^{2+}-dependent interaction of calcineurin with these inhibitory complexes (*32*, *33*). The Ca^{2+}-dependent interaction of calcineurin with the transcription factor, NFAT (*34*), or the FKBP-dependent interaction of calcineurin with the IP_3 receptor (*35*) could also depend on the Ca^{2+}-induced exposure of the CaM-binding domain.

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