

A C-terminal, calmodulin-like regulatory domain from the plasma membrane Ca^{2+} -pumping ATPase

(inhibitory domain/immunochromogenic screening/sarcoplasmic reticulum Ca^{2+} -ATPase)

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ABSTRACT A cDNA that encodes what appears to be the inhibitory domain of the plasma membrane calcium-pumping ATPase (Ca^{2+} -ATPase) has been isolated by screening a λ gt11 bovine brain cDNA library with antibodies prepared against the human erythrocyte membrane Ca^{2+} -ATPase. This screening resulted in isolation of a bacteriophage containing a 1.5-kilobase cDNA insert encoding a 71-residue polypeptide, the remainder being a large 3' terminal noncoding region. A portion of this deduced peptide sequence was identical to that of a peptide isolated from a V8 protease digest of the human erythrocyte Ca^{2+} -ATPase except for 1 residue. Antibodies purified by immunoabsorption to the fusion protein containing this cDNA-encoded polypeptide reacted only with those fragments of a limited trypsin digest of the human erythrocyte Ca^{2+} -ATPase that contain the inhibitory domain. Moreover, these antibodies were able to partially stimulate basal enzyme activity and block further activation by calmodulin. The encoded polypeptide bears homology to the glutamic acid-rich regions N-terminal to the Ca^{2+} -binding loops of calmodulin and to a lesser extent with the loops themselves. This encoded polypeptide also represents the C terminus of the Ca^{2+} -ATPase. Portions of the isolated cDNA were homologous to the 3' noncoding region of the sarcoplasmic reticulum Ca^{2+} -ATPase cDNA, indicating a possible mechanism for the evolution of these distinct membrane Ca^{2+} pumps.

Many responses of eukaryotic cells to external stimuli proceed through a calcium-mediated "second messenger" pathway. The target proteins of this pathway are affected either by direct binding of calcium or by interaction with the calcium-binding protein calmodulin (CaM). CaM is a small, acidic protein that possesses four Ca^{2+} -binding sites identifiable as related domains (I, II, III, and IV) throughout the linear sequence (1). In the three-dimensional structure, these four domains appear to be arranged in two pairs at each end of the molecule connected by a long central α -helix (2).

Despite detailed knowledge of the structure of CaM and its four Ca^{2+} -binding sites, information concerning the structures through which CaM and target enzymes interact remains limited. It is clear that distinct regions of the CaM molecule are used to interact with different target enzymes. Myosin light chain kinase and 3',5'-cyclic nucleotide phosphodiesterase appear to interact with the N-terminal portion of the central helix (3, 4), whereas the phosphoprotein phosphatase, calcineurin (5), and the plasma membrane Ca^{2+} -pumping ATPase (Ca^{2+} -ATPase) (4) preferentially recognize the domain III region of CaM. To date, only the CaM-regulatory region of myosin light chain kinase has been characterized in detail (6–9).

The 138-kDa plasma membrane Ca^{2+} -ATPase is the only ion-transport ATPase and only one of two integral mem-

brane enzymes known to be regulated by CaM binding. Limited proteolysis and chemical labeling studies suggest that the human erythrocyte enzyme contains discrete domains involved in membrane association (30–35 kDa), catalysis (81 kDa), and CaM regulation (8–10 kDa). Attempts to isolate and characterize these domains, particularly that containing the CaM-regulatory region, by conventional biochemical techniques have been uniformly unsuccessful in this and other laboratories. Therefore, cloning of the Ca^{2+} -ATPase employing oligo(dT)-primed cDNA libraries was chosen as an alternative strategy for study of the latter, owing to the tentative placement of this CaM-regulatory region at the C terminus of the enzyme (10, 11).

We have recently shown that the plasma membrane Ca^{2+} -ATPase is abundant in neuronal cells of mammalian brain tissue in a form indistinguishable from that in human erythrocytes (12). Therefore, we chose to screen a bovine brain λ gt11 cDNA library by using a polyclonal rabbit antibody raised against the purified human erythrocyte enzyme. As reported here, one clone isolated by this procedure encodes a C-terminal portion of the enzyme that appears to contain an inhibitory domain of the Ca^{2+} -ATPase.‡

METHODS

General Methods. Ca^{2+} -ATPase was purified from outdated human erythrocytes by the method of Niggli *et al.* (13). Limited trypsin digestion of this enzyme was performed essentially as described by Zurini *et al.* (10). Digestion with V8 protease, purification, and sequence determination of the resultant fragments will be described elsewhere (M.Z. and T.C.V., unpublished data). CaM was purified from bovine testis by the method of Jamieson and Vanaman (14). The λ gt11 cDNA library was constructed from frozen bovine brain as described by Neve *et al.* (15). DNA was sequenced by the dideoxy chain-termination method (16). ^{125}I -labeled CaM (^{125}I -CaM) overlay experiments were performed as described (17).

Preparation of Affinity-Purified Antibodies. Polyclonal anti- Ca^{2+} -ATPase antibodies were prepared against human erythrocyte Ca^{2+} -ATPase as described by Verma *et al.* (18). These antibodies were purified by absorption of the antiserum to a column of immobilized antigen. The latter was prepared by loading a small amount of CaM-Sepharose (19) with 400 μg of purified erythrocyte Ca^{2+} -ATPase and cross-linking the resulting complex by treatment with 1 mM disuccinimidyl suberate in 50 mM sodium borate (pH 8.0) for 20 min at 4°C. Unreacted reagent was destroyed with excess

Abbreviations: Ca^{2+} -ATPase, calcium-pumping ATPase; CaM, calmodulin; SR, sarcoplasmic reticulum.

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‡The sequence reported in this paper is being deposited in the EMBL/GenBank data base (Intelligenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03649).

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ammonium acetate and the resin was extensively washed with an EGTA-containing buffer. Five milliliters of immune serum was diluted 1:1 with phosphate-buffered saline (PBS; 0.1 M NaPO₄/0.15 M NaCl, pH 7.5) containing 0.2% (wt/vol) sodium azide and passed through a CaM-Sepharose column. This material was then passed over the immobilized antigen column. After extensive washing with PBS/azide, the antibodies were eluted with 50 mM glycine (pH 2.3) containing 0.5 M NaCl. The eluted fraction was immediately neutralized with 1 M Tris base, brought to 4 mg/ml in bovine serum albumin, and dialyzed overnight against 4 liters of PBS/azide. This material was stored in 0.5-ml aliquots at -80°C.

Immunochromogenic Screening of λ gt11 Bovine Brain cDNA Library. For screening, the λ gt11 bovine brain cDNA library was plated, at a density of 50,000 plaque-forming units (pfu) per 150-mm plate, on *Escherichia coli* Y1090 as described (20). Plates were incubated at 42°C for \approx 8 hr, covered with nitrocellulose filters saturated with 10 mM isopropyl β -D-thiogalactopyranoside, and further incubated at 37°C for 3–4 hr. The filters were removed and placed in 250 ml of PBS containing 5% (vol/vol) horse serum and 0.02% (wt/vol) sodium azide for 15 min; this was followed by incubation in 250 ml of PBS containing 20% (vol/vol) horse serum and 5% (wt/vol) milk powder for 1 hr. These blocked filters were placed in heat-sealing bags (two per bag), containing 10 ml of a 1:100 dilution of affinity-purified anti-Ca²⁺-ATPase antibodies (\approx 5 μ g) in PBS and incubated for 2 hr with gentle agitation. The filters were washed three times, 10 min each, with 250 ml of PBS, washed once for 10 min with 250 ml of PBS containing 1% (vol/vol) horse serum, and washed once for 1 hr with PBS containing 1% (vol/vol) horse serum and goat anti-rabbit IgG conjugated to horseradish peroxidase (Zymed, San Francisco, CA or Bio-Rad) at the manufacturer's recommended dilution. The filters were washed five times for 5 min each with 250 ml of PBS and then color developed by adding a PBS solution containing 400 μ g CoCl₂ per ml, 500 μ g of diaminobenzidine per ml, and a 1/1000 vol of 30% (vol/vol) hydrogen peroxide (added just before use).

Putative positive plaques were isolated and rescreened twice, as above, at a density of \approx 300 pfu per 90-mm plate.

Production of Fusion Protein and Purification of Antigen-Specific Antibodies. Cultures to produce the protein product of the *lacZ*-cDNA fusion gene followed established procedures (20), with the exception that *E. coli* Y1090 was used instead of Y1089 and cells were grown at 37°C for \approx 1 hr after induction. The cell extract was resolved on a 7.5% NaDodSO₄/polyacrylamide gel (21) and electrophoretically transferred to a nitrocellulose membrane (22). The membrane was then immunostained with affinity-purified polyclonal anti-human erythrocyte Ca²⁺-ATPase antibodies as described for the screening of the λ gt11 library, with the exception that alkaline phosphatase was conjugated to the secondary antibody (Zymed). The immunoreactive band was excised from the membrane and used to purify antibodies specific for the fusion protein by the method of Smith and Fisher (23).

Antibodies corresponding to specific regions of the human erythrocyte Ca²⁺-ATPase were isolated essentially as described above by using transfers of fragments from a limited trypsin digest of the purified Ca²⁺-ATPase resolved by NaDodSO₄/polyacrylamide gel electrophoresis on a 12.5% gel.

Measurement of ATPase Activity in the Presence of Different Antibodies. ATPase activity was measured spectrophotometrically with a coupled enzyme assay. The assay mixture contained 30 mM Hepes (pH 7.35), 130 mM NaCl, 3 mM MgCl₂, 10 μ M CaCl₂, 1 mM ATP, 0.2 mM NADH, 0.5 mM phosphoenolpyruvate, 1 international unit of pyruvate kinase per ml, and 1 international unit of lactate dehydroge-

nase per ml. The reaction was started by the addition of 1–2 μ g of purified ATPase and ATP hydrolysis was followed at 37°C as the decrease in absorbance at 366 nm due to NADH consumption.

For activity measurements in the presence of different antibodies, a 2-fold concentrated assay mixture was used. Routinely, 490 μ l of affinity-purified antibodies and 10 μ l of purified ATPase (1–2 μ g) were incubated for 20 min at room temperature. Activity was then measured by adding 500 μ l of the 2-fold concentrated assay mixture as described above. CaM stimulation was determined by adding CaM to the assay mixture at 1 μ g/ml.

RESULTS

Screening of the λ gt11 Library. Primary screening of 300,000 plaques from the bovine brain cDNA library with anti-human erythrocyte Ca²⁺-ATPase antibodies resulted in the detection of six positive plaques. Secondary screening of these plaques resulted in one isolate being identified as positive. Subsequent tertiary and quaternary screenings demonstrated that this isolate was clonally pure. This bacteriophage was designated λ CAATP1.

Expression of β -Galactosidase Fusion Protein. When lysates from λ CAATP1-infected *E. coli* cultures, appropriately induced to express the *lacZ*-cDNA fusion gene of λ CAATP1, were analyzed on a NaDodSO₄/polyacrylamide gel and stained with affinity-purified anti-human erythrocyte Ca²⁺-ATPase antibodies, a single band at about 126 kDa was observed. This polypeptide was identified as a product of the *lacZ*-cDNA gene containing the λ CAATP1 insert based on the following considerations (data not shown): (i) it reacted specifically with affinity-purified anti-ATPase antibodies; (ii) it appeared only when the phage was induced to be lytic by elevated temperature and the *lacZ* gene was derepressed by the addition of isopropyl β -D-thiogalactopyranoside; and (iii) it had the anticipated molecular mass for a fusion protein of the polypeptide encoded by the λ CAATP1 cDNA sequence (Fig. 4) and β -galactosidase (112 kDa). As

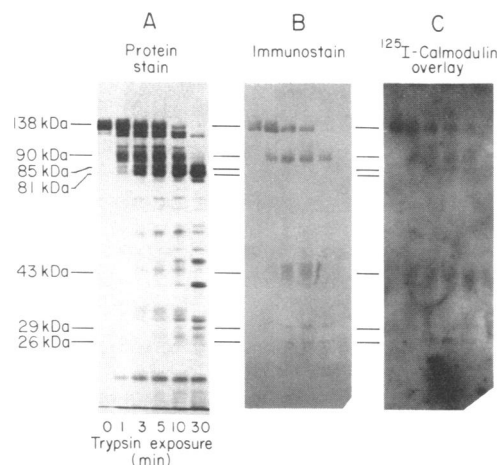


FIG. 1. (A) Silver stain of fragments generated by limited trypsin digestion of human erythrocyte Ca²⁺-ATPase. Erythrocyte Ca²⁺-ATPase was subjected to limited trypsin digestion for various times to yield fragments of defined activity (10). Aliquots of the digests were resolved on a 10% NaDodSO₄/polyacrylamide gel and visualized by silver staining (24). (B) Immunostaining of Ca²⁺-ATPase tryptic fragments. The material in a gel identical to A was electrophoretically transferred to a nitrocellulose membrane and immunostained by using antibodies purified with the fusion protein. Goat anti-rabbit IgG coupled to horseradish peroxidase was the secondary antibody. (C) A nitrocellulose transfer of this gel also was treated with ¹²⁵I-CaM and autoradiographed as a control for the accurate determination of CaM-binding fragments.

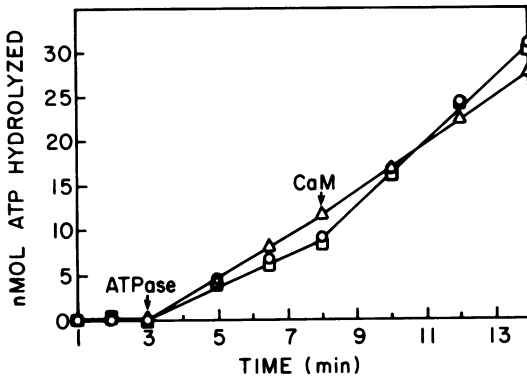


FIG. 2. ATP hydrolysis by human erythrocyte Ca^{2+} -ATPase in the presence of fusion protein-specific antibodies. ATP hydrolysis was determined in the presence of CaM with control antibodies present (\square), with no antibody present (\circ), or when the Ca^{2+} -ATPase was preincubated with antifusion protein antibodies (\triangle). CaM was added at the time indicated to a concentration of 1 $\mu\text{g}/\text{ml}$.

further confirmation, antifusion protein antibodies purified from the polyclonal anti-ATPase antiserum were shown to cross-react with a specific region of the human erythrocyte Ca^{2+} -ATPase as discussed below.

Interaction of Fusion Protein-Specific Antibodies with Ca^{2+} -ATPase. Antibodies specific for the fusion protein were selected from the polyclonal anti-human erythrocyte Ca^{2+} -ATPase population by affinity purification (see *Methods*). Fig. 1B shows the reaction of these antibodies with the products of a limited tryptic digestion of the intact enzyme. Fragments of 90, ≈ 43 , 29, and 26 kDa were observed to react with fusion protein-specific antibodies. These fragments also bound ^{125}I -CaM (Fig. 1C). The 90-kDa fragment has been shown to retain full enzymatic activity and CaM stimulation (10), whereas the 81-kDa tryptic fragment is fully active but neither binds to nor is stimulated by CaM. For this reason, it is believed to lack the CaM-binding domain (10). As shown

in Fig. 1B, this fragment also did not bind the fusion protein-specific antibodies, suggesting that these antibodies recognize a sequence near the CaM-binding domain. Interestingly, the 85-kDa tryptic fragment (Fig. 1A and C) also did not bind the antibodies (Fig. 1B). This fragment has been reported to retain enzymatic activity and binds CaM (Fig. 1C) but is not stimulated by it (25). This suggests that the fusion protein-specific antibodies bind to an inhibitory domain of the Ca^{2+} -ATPase lacking this fragment (25).

The effects of fusion protein-specific antibodies on the activity of human erythrocyte Ca^{2+} -ATPase are shown in Fig. 2. The basal ATP hydrolysis activity of the enzyme alone was found to be 1.8 nmol/min. Addition of CaM stimulated this activity to 3.75 nmol/min. Preincubation of the Ca^{2+} -ATPase with fusion protein-specific antibodies resulted in partial stimulation of the enzyme activity to 2.4 nmol/min. Addition of CaM to this assay mixture did not increase in enzyme activity. These effects of the antifusion protein antibodies do not appear to be a nonspecific effect of antibody binding. Antibodies specific to the 81-kDa catalytically active fragment of the ATPase, purified by immunoadsorption as described above, neither stimulated ATP hydrolysis nor blocked CaM activation (Fig. 2).

Coding and Noncoding Nucleotide Sequences in λCAATP1 . The complete sequence of the λCAATP1 cDNA insert was established by dideoxy sequencing of both strands of restriction fragments and sequential deletions of the intact insert. The orientation of the λCAATP1 insert, relative to the *lacZ* gene, was determined by a double digest of λCAATP1 with the restriction enzymes *Bgl* II and *Kpn* I (data not shown).

As shown in Fig. 3, the λCAATP1 cDNA insert contains 1451 nucleotides, 213 of which are in an open reading frame from the 5' terminus ending with the single termination codon UGA. This open reading frame codes for a 71-residue peptide whose deduced sequence also is shown in Fig. 3. That this sequence represents a region of the plasma membrane Ca^{2+} -ATPase is further confirmed by the fact that positions 5–18 are nearly identical to the partial sequence of

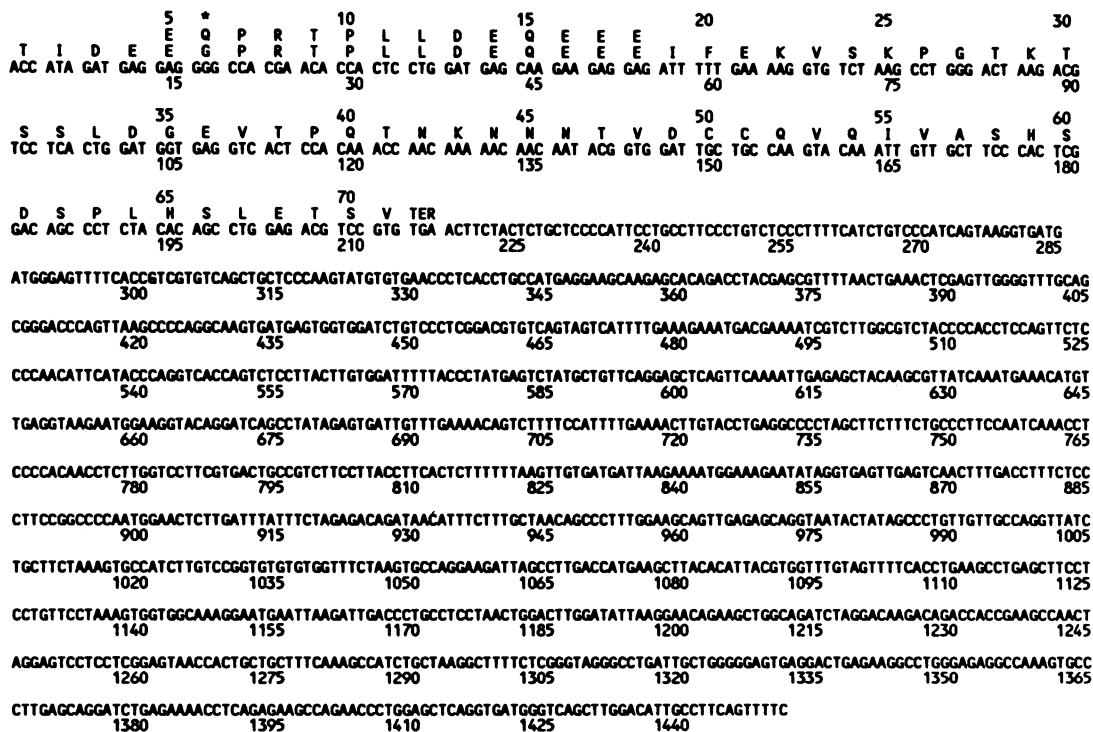


FIG. 3. Complete nucleotide sequence of the λCAATP1 cDNA insert and the deduced protein sequence. The sequence of a peptide isolated from a V8 protease digest of human erythrocyte Ca^{2+} -ATPase is shown above the deduced polypeptide sequence with a single residue mismatch denoted by an asterisk.

UPPPER SEQUENCE - 3' NONCODING REGION OF THE SARCOPLASMIC RETICULUM CALCIUM ATPASE
 LOWER SEQUENCE - PLASMA MEMBRANE CALCIUM ATPASE

Homology Region 1

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3270  GCTCCACATTTTC-TCTGTGCTGTCAGCTTATCCAGATG-TGC-ACTGTTGA--GTTGTAATG
***** ** * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
244   GCTCC-CTTTTCATCTGTCCCATCAG--TAAGTGATGATGGGAGTTTCCACCGTCG---G

3328  -CATGCCTTCAGCTCAAGTAGCCAGCGTT-----TC-CCTGCAGTGACCTTGAACCTGCTAC
** * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
301   TCA-GC-TGCT-CCCAAGTA---TGTGTGAACCCCTCACCTGCCATGAGGAAGCAAGA-GC-AC
    
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Homology Region 2

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3486  TCTGA-GTGTGCAGACAGCTATTTTGCAGTATTAATG
** * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
448   TCGGACGTGT-CAGT-AGTCAATTTTG-AAAG----AAATG
    
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Homology Region 3

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3555  GATGTT--GGTGC--AATACAAACATGTGTGATGCATTTATC--TTA
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *
582   GCTGTTCCAGGAGCTCAGTCAAAA-ATTGAGA-GCTACAAGCGTTA
    
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FIG. 4. Regions of homology between the 3' noncoding region of the SR Ca²⁺-ATPase and the 3' end of the plasma membrane Ca²⁺-ATPase cDNA isolated from bovine brain. Three regions of highest homology between the SR and plasma membrane Ca²⁺-ATPase sequences are shown. Asterisks denote identities. Numbering of the plasma membrane Ca²⁺-ATPase sequence is according to Fig. 3 and of the SR Ca²⁺-ATPase is as in ref. 26.

a V8 protease fragment isolated from the human erythrocyte protein. The only exception is residue 6, which is a glycine to glutamine substitution. This single replacement is not unexpected since the sequences being compared are from different species and tissues.

No strong homologies were revealed when the deduced protein sequence was compared to those contained in the National Biomedical Research Foundation protein sequence library. The 1238-base-pair (bp) noncoding sequence contained neither a poly(A) tail nor a sequence resembling the consensus poly(A) signal AATAAA, suggesting that the complete 3' terminus of the mRNA is not represented. No striking homologies were found when the nucleotide sequence was compared to sequences in the GenBank (release 40.1) nucleotide sequence library.

Comparison with the Sarcoplasmic Reticulum (SR) Ca²⁺-ATPase. When the 3' noncoding region of the SR Ca²⁺-ATPase cDNA sequence for the slow-twitch muscle enzyme (26) was compared with the cDNA sequence for the 3' end of the plasma membrane Ca²⁺-ATPase presented here, homology between the two was observed over limited regions (Fig.

4). The 3' noncoding region of the cDNA for the fast-twitch muscle SR enzyme (27) did not share this homology with the plasma membrane Ca²⁺-ATPase cDNA sequence. The 3' noncoding region of the Ca²⁺-ATPase (1238 bp) is also substantially longer than the 3' noncoding region for either slow-twitch (603 bp) (26) or fast-twitch (237 bp) (27) muscle SR Ca²⁺-ATPase cDNAs.

DISCUSSION

This study presents the cDNA and deduced polypeptide sequence for the C-terminal 71 residues of the plasma membrane Ca²⁺-ATPase that we believe represents a portion of its CaM-regulatory domain. This conclusion is based on a combination of homology with direct peptide sequence derived from the human erythrocyte Ca²⁺-ATPase, cDNA expression, antibody binding, and enzymatic assays. It has been shown that limited trypsin digestion of human erythrocyte membrane Ca²⁺-ATPase yields discrete fragments corresponding to functional domains of the enzyme (10, 25) as set forth in *Results*. Those studies have shown that CaM binding and inhibition of ATPase activity are specified by two discrete segments of the protein sequence apparently located within 9 kDa of the C terminus (10, 11, 25). Furthermore, the CaM-binding domain appears to be located at the N-terminal end of this region and the inhibitory domain at its C terminus (25).

Since the deduced polypeptide sequence presented here represents only the C-terminal 7.7 kDa of the enzyme, it is possible that some or all of the CaM-binding segment is lacking in this sequence. Attempts to demonstrate CaM binding to the CAATP1 fusion protein by gel overlay or photoaffinity labeling with ¹²⁵I-CaM derivatives have been uniformly unsuccessful (data not shown), further supporting this conclusion.

As shown in Fig. 1B, antibodies specific for the fusion protein encoded by CAATP1 do not cross-react with the 85-kDa trypsin fragment that contains the CaM-binding domain but lacks the inhibitory domain. However, they react with the native Ca²⁺-ATPase and, more importantly, with the 90-kDa fragment that is known to contain the inhibitory domain and from which the 85-kDa fragment is derived. Furthermore, antibodies specific for this sequence not only antagonize CaM activation but also partially activate the enzyme. These data strongly support the conclusion that CAATP1 encodes most or all of the inhibitory region of the Ca²⁺-ATPase.

Comparison of the amino acid sequence encoded by CAATP1 to the proposed CaM-binding region of smooth (28) and skeletal (6) muscle myosin light chain kinase, phosphorylase b kinase, or CaM kinase II (29) showed no detectable homology. This is not particularly surprising as

	HELIX	TURN	LOOP	TURN
CAATP1 residues 12 - 49 :	L D E Q	E E E I F E	K V S K P G T K T S S L D G E V T P Q T N K N N T V D	
CaM Domain III residues 78 - 112 :	D T D S	E E E I R E	- - - A F R V F D K D G N G Y I S A A E L R H V M T N L	
CaM Domain IV residues 114 - 148 :	E K L T	D E E V D E	- - - M I R E A N I D G D G Q V N Y E E F V Q H M T A K	
CaM Domain II residues 41 - 75 :	Q N P T	E A E L Q D	- - - M I N E V D A D G N G T I D F P E F L T H M A R K	
CaM Domain I residues 2 - 39 :	D Q L T	E E Q I A E	F K E A F S L F D K D G D G T I T T K E L G T V M R S L	
	HELIX	LOOP	HELIX	

FIG. 5. Homology between the plasma membrane Ca²⁺-ATPase inhibitory domain encoded by CAATP1 and the Ca²⁺-binding loops of bovine brain CaM (1, 30). Residues of CaM coordinated with Ca²⁺ are indicated by asterisks. The invariant glycol residue in the sixth position of the Ca²⁺-binding loops is indicated by a +. The conserved glutamic acid-rich regions N-terminal to the loop structures are boxed. The secondary structure of the Ca²⁺-ATPase sequence predicted by the method of Garnier *et al.* (31) is shown at the top. The observed secondary structure of the CaM Ca²⁺-binding loops (2) is shown at the bottom.

the CaM-binding region of myosin light chain kinase appears to be an analog of the normal peptide substrate (28) and presumably acts by occluding the kinase active site. It has been proposed that CaM binding displaces this inhibitory peptide segment resulting in kinase activation.

However, CaM-regulated enzymes, such as the Ca²⁺-ATPase, whose catalytic activity does not require peptide substrate recognition, most likely employ an alternative mechanism for regulation. As shown in Fig. 5, residues 16–43 of the amino acid sequence of the inhibitory region of the Ca²⁺-ATPase, encoded by CAATP1, bear similarity to regions of CaM itself. In this alignment, the homology of the Ca²⁺-ATPase sequence Glu-Glu-Glu-Ile-Phe-Glu to that of the sequence Glu-Glu-Glu-Ile-Arg-Glu in CaM is particularly intriguing. This sequence is strongly predicted to be α -helical and is the region of strongest homology to CaM. Replacement of the Glu-Glu-Glu sequence with Lys-Lys-Lys by site-directed mutagenesis has been shown to result in loss of CaM activation of myosin light chain kinase and NAD kinase but not 3',5'-cyclic nucleotide phosphodiesterase (32). Similarly, chemical modification of lysine-94 decreases CaM's apparent affinity for the Ca²⁺-ATPase but not for 3',5'-cyclic nucleotide phosphodiesterase (4). Thus, this region of CaM has been implicated as a region for interaction with the Ca²⁺-ATPase and other target enzymes.

The CAATP1-encoded sequence contains the requisite spacing of oxygen side chain containing amino acids at each potential Ca²⁺-ligand position (*), the invariant glycyl residue at the top of the loop (+), and a conserved hydrophobic residue between the fourth and fifth potential ligand contributing residues. However, the replacement of essential COOH side chains (aspartic acid and glutamic acid) with hydroxyl groups (serine and threonine) suggests this loop structure may not bind Ca²⁺ with significant affinity.

The apparent homology between CaM and the inhibitory segment of the Ca²⁺-ATPase suggests a possible mechanism for regulation in which the inhibitory segment of the Ca²⁺-ATPase interacts with its own CaM-binding domain. Displacement of this inhibitory segment on CaM binding could cause conformational changes elsewhere in the molecule leading to activation.

It is interesting to note that a phenothiazine-binding site exists within the C terminal 9 kDa of the Ca²⁺-ATPase (10). This further suggests the existence of a CaM-like structure in this region.

The limited DNA sequence homology between the plasma membrane and SR Ca²⁺-ATPases noted in Fig. 4 also is intriguing. The SR enzyme neither binds to nor is activated by CaM. The fact that portions of the 3' noncoding region of the SR enzyme are homologous to portions of the sequence encoded by CAATP1 suggests that the SR enzyme lacks the necessary CaM-regulatory domains. This may have resulted from a frameshift or point mutation in the plasma membrane Ca²⁺-ATPase gene that converted a sense codon to a stop codon upstream of the CaM-regulatory domain coding sequence. Alternatively, the plasma membrane Ca²⁺-ATPase may have evolved from the SR enzyme by the insertion of a coding sequence for the CaM-regulatory domain. In either case, the divergence must have been a comparatively recent event as there is no obvious selective pressure to maintain the high degree of homology observed in these two DNA sequences.

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