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A calmodulin-stimulated Ca^{2+}-ATPase from plant vacuolar membranes with a putative regulatory domain at its N-terminus

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Abstract A cDNA, ***BCA1***, **encoding a calmodulin-stimulated Ca^{2+}-ATPase in the vacuolar membrane of cauliflower (*Brassica oleracea*) was isolated based on the sequence of tryptic peptides derived from the purified protein. The *BCA1* cDNA shares sequence identity with animal plasma membrane Ca^{2+}-ATPases and *Arabidopsis thaliana ACA1*, that encodes a putative Ca^{2+} pump in the chloroplast envelope. In contrast to the plasma membrane Ca^{2+}-ATPases of animal cells, which have a calmodulin-binding domain situated in the carboxy-terminal end of the molecule, the calmodulin-binding domain of BCA1 is situated at the amino terminus of the enzyme.**

Key words: Signal transduction; Calcium; Pump; Vacuole

1. Introduction

Ca^{2+} plays a key role in the transduction of external signals through the cytoplasm of plant cells [1]. Ca^{2+} influx across the plasma membrane or release from intracellular stores occurs in response to diverse stimuli such as hormones, gravity, red light, salt stress, cold shock, touch and fungal elicitors. Elevated cytosolic free Ca^{2+} in turn triggers a variety of processes. Thus, in plants, transient changes in cytosolic Ca^{2+} levels are thought to participate in many signal transduction pathways, including regulation of enzyme or ion channel activities, cytoskeleton organization and gene expression [2].

Both in plants and in fungi, the vacuole is thought to play a critical role in the control of cellular Ca^{2+}. In addition to its function in space-filling and intracellular digestion, this membrane-bound compartment also serves as an intracellular Ca^{2+} reservoir. The vacuole contains millimolar levels of Ca^{2+} while the concentration of Ca^{2+} in the cytosol is submicromolar under resting conditions. This asymmetric distribution is believed to be achieved in concert by P-type Ca^{2+} pumping adenosine triphosphatases (ATPases) and Ca^{2+}/H^+ antiport systems, both of which are involved in the unidirectional removal of Ca^{2+} from the cytosol [1,3]. Ca^{2+} is released again during signal transduction events mediated by inositol 1,4,5-trisphosphate, cyclic ADP-ribose or changes in membrane potential [4].

In this work, we have isolated the cDNA (*BCA1*) for a calmodulin (CaM)-stimulated Ca^{2+}-ATPase in cauliflower which previously has been localized to the vacuolar membrane ([5]; P. Askerlund, submitted). Biochemical and molecular evidence indicates that *BCA1* encodes a unique Ca^{2+}-ATPase in terms of its intracellular location, CaM-binding ability and localization of the CaM-binding domain.

2. Materials and methods

2.1. Affinity purification of vacuolar Ca^{2+}-ATPase and sequencing of tryptic peptides

The 111 kDa Ca^{2+}-ATPase was purified by CaM-affinity chromatography from low-density intracellular membranes of cauliflower inflorescences as described by Askerlund [5]. The properties of the purified enzyme have been described [5,6]. Trypsin digestion and sequencing of the resulting peptides were carried out by Bo Ek, Uppsala Genetic Center, Swedish University of Agricultural Sciences. The 111 kDa Ca^{2+}-ATPase, the only visible polypeptide in this size range [5], was cut out from the gel and washed repeatedly with deionized H_2O. Gel pieces were incubated with trypsin (Promega), essentially as described by Rosenfeld et al. [7]. The eluted peptides were separated using a SMART chromatography station equipped with an mRPC SC C2/C18 2.1/10 column (Pharmacia). The gradient was from 0.1% trifluoroacetic acid (TFA) in water to 50% acetonitrile/0.085% TFA for 75 min with a flow of 100 μl/min. Peaks were monitored at 214 and 280 nm, and automated peak collection was used. Amino acid sequencing was performed on a sequencer according to the manufacturer's instructions (model ABI 476A; Applied Biosystems/Perkin-Elmer). Trypsin treatment yielded fragments from which six sequences, ranging in size between 5 and 13 amino acids, were obtained (Fig. 1).

2.2. Amplification of Ca^{2+}-ATPase cDNA

Based on the sequence of one of the peptides (VAFYVQK) and on the sequence of the highly conserved region of the phosphorylation site in P-type ATPases (DKTGTL), corresponding degenerate oligonucleotides were designed. A 1.2 kb fragment of the Ca^{2+}-ATPase gene was obtained by polymerase chain reaction (PCR) with a combination of two oligonucleotide primers (5′-GTNGCNTTY-TAYGTNCARAAR-3′ and 5′-NARNGTNCCNGTYTTRTC-3′; N = A, C, G and T; Y = C and T; R = A and G). Randomly primed first strand cDNA from cauliflower inflorescences was used as template. The 1.2 kb fragment was subcloned into PCR-Script (Stratagene). To obtain the 3′-end of the gene, a double stranded cDNA library was synthesized from poly(A)$^+$ cauliflower RNA with a lock-docking oligo(dT) primer and ligated to cDNA adaptors following the protocol of the Marathon cDNA Amplification Kit (Clontech). The library was diluted 250 times, and used as template for RACE-PCR with an adaptor primer and a forward gene specific primer (5′-ACT-GATGAGGTCAGACAAGCG-3′) from the 5′ region of the 1.2 kb clone. The resulting products were diluted 50 times, and 5 μl was used as template for nested PCR using a nested forward gene specific primer (5′-GGATTTCACGTGGAAGCTGATGAAC-3′) derived from the 5′ region of the 1.2 kb clone. The major band of 3 kb

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Abbreviations: CaM, calmodulin.

The sequence reported in this paper has been deposited in the GenBank, EMBL and DDBJ data bases (accession no. X99972).

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was cut out from a preparative agarose gel, purified with QIAquick gel extraction kit (Qiagen) and sequenced directly.

To obtain the 5′ end of the gene, the same adaptor-ligated cDNA library was used as template in RACE-PCR with an adaptor primer and a reversed gene specific primer (5′-ATGATCTGGCTGGTTTCT-CA-3′) derived from the 5′ region of the 1.2 kb clone. The PCR products were diluted 50 times, and 5 μl subjected to a second round of PCR, with an adaptor primer and a nested reverse primer (5′-TTAGTGAGGCTCCTTGTGTCGTG-3′), likewise derived from the 5′ region of the 1.2 kb clone. A combination of Hot Start PCR and modified Touchdown PCR [8,9] was used to minimize mispriming. The obtained fragment of 600 bp was sequenced directly after gel purification as above.

2.3. Sequencing and analysis of PCR fragments

The two isolated PCR fragments corresponding to the 5′ and 3′ ends of the gene and the 1.2 kb cDNA fragment corresponding to the central part of the gene were sequenced on an ABI PRISM automated sequencer model 377 and 310, using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq FS DNA Polymerase according to the manufacturer's instructions (Perkin-Elmer). Sequences were analyzed using the GCG program package (Program Manual for the Wisconsin Package, Version 8, Sept. 1994, Genetics Computer Group, Madison, WI, USA), including BLAST and TFASTA for homology searches, and the program GeneWorks (IntelliGenetics, Mountain View, CA).

The PCR fragments from at least two independent reactions were completely sequenced in both directions. No deviations between the sequences were found. This ruled out the possibility that mutations were introduced during the PCR reaction.

2.4. Peptide synthesis

The peptide from BCA1 (A^{19}RQRWRSSVSIVKNRARRFRMIS-NL^{43}) was synthesized (Biomolecular Core Facilities, Lund University) on an automated solid-phase peptide synthesizer (Applied Biosystems model 430A) using the Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) program provided by the manufacturer. The identity and purity of the peptide were verified by mass spectrometry and HPLC. The purity was 91%. The peptide from the *Arabidopsis* plasma membrane H^+-ATPase AHA2 (E^{869}REAQWALAQRTLHGLQPK^{887}) was a gift from Prof. Christer Larsson, Department of Plant Biochemistry, Lund University.

2.5. Iodination of CaM and ^{125}I-CaM overlays

CaM (Sigma catalog no. P2277) was iodinated as described by Chafouleas et al. [10] using Bolter and Hunter reagent (Amersham). Peak fractions containing ^{125}I-CaM (~300 pmol, 0.3 MBq in 2 ml) were mixed with an equal volume of 10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.05% (w/v) Tween 20 (TNT) containing 1% (w/v) BSA and either 0.2 mM $CaCl_2$ or 2 mM EGTA (pH adjusted to 8.0). The synthetic peptides were dissolved in 10 mM Tris-HCl, pH 7.4, 15 mM NaCl (TBS) and dot-blotted on Immobilon polyvinylidene difluoride (Millipore). The blots were air dried, coated with 3% (w/v) BSA in TBS on a shaker for 1 h, and incubated with ^{125}I-CaM in the presence of $CaCl_2$ or EGTA for 10 h at 22°C on a shaker. Blots incubated with ^{125}I-CaM in the presence of $CaCl_2$ or EGTA were washed with TNT plus 0.2 mM $CaCl_2$ or TNT plus 2 mM EGTA, respectively, for 3 h with several changes. Finally, blots were exposed for 3.5 h at 22°C with Hyperfilm-βmax (Amersham) in the presence of a Hyperscreen intensifier screen (Amersham).

3. Results

The cauliflower vacuolar membrane Ca^{2+}-ATPase gene *BCA1* was isolated as three overlapping PCR fragments. Randomly primed first strand cDNA was used as template. The combination of one primer based on a tryptic fragment (VAFYVQK) and one primer based on a conserved region (DKTGTL; Fig. 1) resulted in a 1.2 kb PCR product. This clone was used as a probe to screen two different cauliflower cDNA libraries cloned in λZAP. Since no clones containing this region could be found in any of the libraries, the 5′ and 3′

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MSNLLKDFQVEAKNPSLEARQRWRSSVSIVKNRARRFRMISNLEKLAENE      50
KKRCQIQEKIRVAFYVQKAALQFIDAGTRREYKLTDEVRQAGFHVEADEL     100
ASMVRNHDTRSLTKSGGAEGIAQKLSVSLTEGVRSNDLDIREKIYGANRY     150
AEKPARSFLTFVWEALQDVTLIILMVCAVVSIGVGVATEGFPKGMYDGTG     200
                          M1
ILLSIILVVMVTAISDYRQSLQFRDLDREKKKINIQVTRDGNRQEVSIDD     250
  M2
LVVGDVVHLSIGDRVPADGVFISGYNLEIDESSLSGESEPSHVNKEKPFL     300
LSGTKVQNGSAKMLVTTVGMRTEWGKLMETLSEGGEDETPLQVKLNGVAT     350
IIGKIGLGFAVLTFVVLCVRFVIGKAAAGGISEWSSEDALTLLDYFAIAV     400
     M3
TIIVVAVPEGLPLAVTLSLAFAMKQLMKDRALVRHLAACETMGSSTCICT     450
  M4
DKTGTLTTNHMVVNKVWICENIKERREENFELNLSEQVKNILIQAIFQNT     500
GSEVVKDKEGKTQILGSPTERAILEFGLLLGGDVEMQGREHKILKIEPFN     550
SDKKKMSVLTSHSGGKVRAFCKGASEIVLRMCEKVVDSSGKSVPLSEEKI     600
AAVSEVIEGFASEALRTLCLVYTDLDEAPSGDLPDGGYTLVAVVGIKDPV     650
RPGVRKAVQTCQNAGITVRMVTGDNISTAKAIAKECGILTAGGVAIEGSE     700
FRNLPPHEMRAILPKIQVMARSLPLDKHTLVNNLRKIGEVVAVTGDGTND     750
APALHESDIGLAMGIAGTEVAKENADVIIMDDNFATIVNVARWGRAVYIN     800
IQKFVQFQLTVNVVALIINFVSACITGSAPLTAVHLLWVNMIMDTLGALA     850
     M5                        M6
LATEPPNEGLMKRQPIGRTASFITRAMWRNIIGQSIYQLIVLGILNFYGK     900
                                M7
QILDLNGPDSTAVLNTIIFNSFVFCQVFNEVNSREIEKINVFAGMFSSWV     950
          M8
FVAVMTATTGFQLIIVELLGAFASTVPLSWQHWLLCIVIGSISMILAVGL    1000
     M9                        M10
KCIPVESNSHHDGYSLLPSGPSDSA                              1025
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Fig. 1. Amino acid sequence of the BCA1 protein as deduced from the nucleotide sequence of the cDNA. Peptide sequences obtained from the purified protein and found to belong to BCA1 are indicated by boxes. The amino acid sequences used for the design of degenerate oligonucleotide primers are designated by solid lines with arrows. A putative CaM-binding domain is denoted by a double line. Putative transmembrane segments are underlined and numbered consecutively. Conserved amino acids in all P-type ATPases are in bold. The complete nucleotide sequence including the 5′ and 3′ untranscribed regions has been deposited in the GenBank, EMBL and DDBJ databases under accession no. X99972.

ends of the gene were likewise amplified by PCR employing primers derived from the 1.2 kb clone. Sequencing of the overlapping regions indicated that the three clones correspond to the same Ca^{2+}-ATPase gene.

From the sequences of three overlapping PCR clones, the sequence of the entire cauliflower Ca^{2+}-ATPase was constructed (Fig. 1). The PCR amplified cDNA encodes a protein of 1025 amino acids, which we named BCA1. When we compared the amino acid sequence with the sequences of the tryptic fragments, all of the six sequenced peptides belonged to BCA1. This confirmed the identity of *BCA1* as a cDNA encoding the CaM-binding Ca^{2+}-ATPase characterized at the biochemical level [5]. Hydrophobicity analysis [11] indicated the presence of up to 10 transmembrane helices (Fig. 2) with large extramembrane domains between the second and third and between the fourth and fifth putative transmembrane domains, reminiscent of the canonical structure of the P-type ion transporters. In addition to the conserved sequence DKTGTL, two diagnostic sequences (SGES and GDGTNDAP) present in all P-type ATPases [12] are present

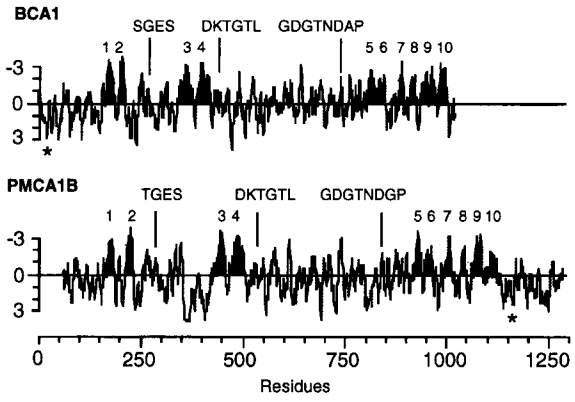

Fig. 2. Comparison of the hydropathy profiles [11] for plant BCA1 and plasma membrane Ca^{2+}-ATPase (PMCA1B [35]). Putative transmembrane segments are numbered. Asterisks denote the position of putative CaM-binding domains.

in BCA1, thus confirming the enzyme as a member of this family. The N- and C-terminal ends of P-type ATPases, as well as the two extramembrane domains are oriented on the cytosolic side of the membrane [13], which implies that the extracytosolic domains of the cauliflower ATPase are very small. No glycosylation motifs are present in any of these regions; this finding is consistent with the close correspondence between the apparent molecular size of the ATPase estimated by SDS-PAGE (111 kDa [5]) and the molecular size calculated from the derived sequence (111 750 Da).

A DNA gel blot of genomic DNA digested with *Xho*I and *Bgl*II, alone and in combination, and probed with the 1.2 kb cDNA clone at high stringency showed only single bands, indicating that BCA1 is the product of a single gene containing a *Xho*I site and a *Bgl*II site (data not shown). The presence of these sites could be confirmed by analysis of the nucleotide sequence for *BCA1*.

A search of the non-redundant group of databases at the EMBL indicated little similarity between the cauliflower ATPase and the endomembrane Ca^{2+}-ATPases of the sarco(endo)plasmic reticulum Ca^{2+}-ATPase subfamily of P-type ATPases. This latter subfamily includes a putative Ca^{2+}-ATPase cloned from tomato [14]. The most homologous protein is ACA1 (62% identity at the amino acid level), a presumed Ca^{2+}-ATPase from *Arabidopsis thaliana* of likely chloroplast inner envelope origin [15]. By using the BLAST programme to search the GenBank EST Division, three expressed sequence tags of *A. thaliana* (N65090, T41650 and R65015) were identified that encode BCA1 like enzymes (> 80% identity). These all show higher homology to *BCA1* than they do to *ACA1* (data not shown). This suggests that in this organism at least two Ca^{2+}-ATPase genes are present. A phylogenetic tree constructed on the basis of eucaryotic Ca^{2+}-ATPase sequences is presented in Fig. 3. The sequences of both BCA1 and ACA1 are very similar to the predicted protein products of the CaM-stimulated (plasma membrane) Ca^{2+}-ATPases in animals. CaM binds to plasma membrane Ca^{2+}-ATPases at a regulatory domain situated in a C-terminal extension following the last transmembrane stretch [16]. However, as suggested by the hydropathy analysis, the BCA1 and ACA1 Ca^{2+}-ATPases have a very short C-terminal tail and in this region no obvious CaM-binding domain could be observed.

Analysis of the deduced amino acid sequence in the N-terminal region of BCA1 showed a potential CaM-binding domain (residues 19–43; ARQRWRSSVSIVKNRARRFRMISNL; Fig. 4C) which, in the α-helical wheel representation, shows a segregation of basic and polar residues on one side and hydrophobic amino acids on the other (Fig. 4B). This feature is typical for CaM-binding domains [17–19]. To determine whether this region is in fact involved in CaM binding, we synthesized a peptide comprising this putative CaM-binding site and tested the peptide for its ability to bind to CaM. The peptide bound CaM in the presence as well as in the absence of Ca^{2+} (i.e. in the presence of excess EGTA) (Fig. 4A). Half-maximal binding was observed at approx. 75 nM CaM, in both the presence and absence of Ca^{2+} (data not shown). An unrelated peptide derived from the C-terminus of the plasma membrane H^+-ATPase did not bind CaM at all (Fig. 4A). In addition, a CaM agarose affinity column bound the peptide derived from BCA1 strongly both in the absence and in the presence of EGTA (data not shown). Application of EGTA to the CaM affinity column, however, eluted a small fraction of the peptide (data not shown), indicating that the affinity of CaM for the peptide was lowered somehow in the absence of Ca^{2+}.

4. Discussion

Previous work has pointed to the presence of a Ca^{2+}/CaM-regulated Ca^{2+} pump in internal membranes of plant cells (for review, see [3]). Here we have identified a gene, *BCA1*, encoding the major vacuolar Ca^{2+}-ATPase found to be a direct target of CaM. Two plant genes, *ACA1* isolated from *Arabidopsis* [15] and *LCA1* from tomato [14], were previously suggested to encode Ca^{2+}-ATPases based on their homology to animal Ca^{2+}-ATPases. Like ACA1, BCA1 is related to mammalian plasma membrane Ca^{2+}-ATPases, whereas LCA1 is

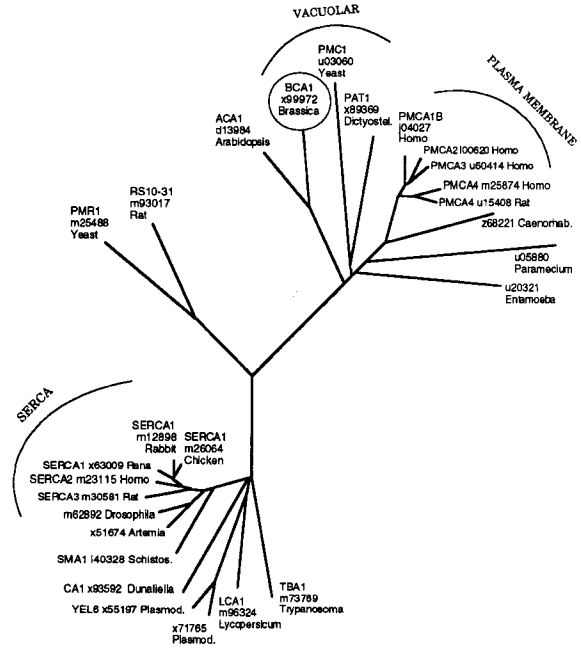

Fig. 3. Most parsimonious unrooted tree found for an alignment of eucaryotic Ca^{2+}-ATPase sequences. The *Brassica* Ca^{2+}-ATPase (BCA1) is circled. Data bank accession numbers and species are indicated.

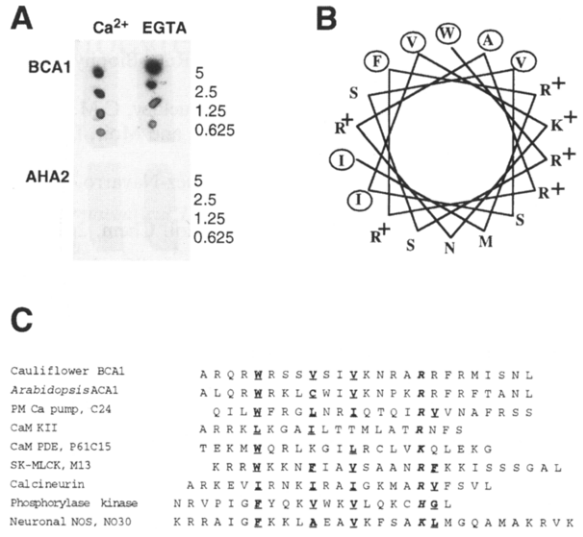

Fig. 4. CaM-binding domain in the N-terminus of BCA1. (A) Binding of peptide to ^{125}I-CaM. A 25 amino acid peptide representing residues 19–43 of BCA1 was tested for its ability to bind CaM. As a control, an unrelated peptide of 19 amino acid derived from the C terminus of the H^+-ATPase AHA2 (residues 869–887) was used. The dissolved synthetic peptides were dot-blotted on a polyvinylidene difluoride membrane and incubated with ^{125}I-CaM in the presence of 0.1 mM $CaCl_2$ (left column) or 1 mM EGTA (right column) as described in Section 2. Numbers (right) indicate the applicated amount of peptide in μg. (B) α-Helical wheel diagram of amino acid residues (19–43) in the CaM-binding domain of BCA1. Positively charged amino acids are denoted with a $^+$ superscript. Hydrophobic amino acids are circled. (C) Sequence alignment of some known and putative CaM-binding domains of protein and peptide CaM targets. The putative CaM-binding domain of the BCA1 Ca^{2+}-ATPase (this paper; X99972; residues 19–43) and the homologous region in the ACA1 Ca^{2+}-ATPase (D13984; residues 21–45) have been aligned to the binding domains of plasma membrane Ca^{2+}-ATPase (P20020; residues 1104–1127), CaM kinase II (P11275; residues 295–314), CaM-dependent phosphodiesterase (P14100; residues 23–44), myosin light chain kinase (P20689; residues 579–604), calcineurin (P20652; residues 391–413), phosphorylase *b* kinase (P18688; residues 1079–1099) and neuronal NO-synthase (P29476; residues 725–754). Conserved hydrophobic residues are underlined. Conserved basic residues are in italic. Database accession numbers are indicated. For references see [19].

more related to the subfamily of animal sarco(endo)plasmic reticulum Ca^{2+}-ATPases. Deduction of ion specificity of P-type ATPases from their primary structure has many pitfalls. Thus, *PMR2*, a yeast gene with striking homology to animal Ca^{2+}-ATPases [20] was later shown to encode sodium specificity [21]. Likewise, the sodium pump and the proton pump of gastric mucosa show very little difference in primary structure [22]. BCA1 represents the first plant Ca^{2+}-ATPase gene cloned so far for which biochemical evidence exists for the Ca^{2+}-pumping nature of its gene product.

The presence of Ca^{2+}-ATPases in the vacuolar membrane has been reported previously [3,23,24]. This would imply the presence of at least two Ca^{2+}-transport systems in this membrane: one is the BCA1 Ca^{2+}-ATPase, and the other is a Ca^{2+}/H^+ antiporter [25]. Similarly, yeast vacuolar membranes contain a Ca^{2+}-ATPase (Pmc1p) as well as a Ca^{2+}/H^+ antiporter (Vcx1p), both of which are characterized at the molecular level [26,27]. Thermodynamic considerations argue that Ca^{2+}-ATPases are necessary to account for the extremely low concentrations of Ca^{2+} in the cytosol [28]. Thus, increased expression of Ca^{2+}-ATPases would benefit the cell in high-Ca^{2+} conditions to offset increased Ca^{2+} influx. PMC1 [26,27] and PAT1 [29], homologues of animal plasma membrane Ca^{2+}-ATPases in *Saccharomyces cerevisiae* and *Dictyostelium discoideum*, respectively, are also vacuolar membrane proteins. This suggests that a vacuolar localization of members of this subfamily of Ca^{2+}-ATPases may be a more general phenomenon.

Plant CaM-stimulated Ca^{2+}-ATPases, however, are not restricted to the vacuolar membrane. Thus, a specific CaM-stimulated Ca^{2+}-ATPase may be present in the plasma membrane (for a review, see [3]). In addition, at least in some plant species, Ca^{2+}-ATPases with homology to animal SERCA Ca^{2+}-ATPases seem to be present in both the vacuolar membrane and the plasma membrane [30].

Trypsination of membrane vesicles harbouring the vacuolar membrane CaM stimulated Ca^{2+}-ATPase resulted in activation of Ca^{2+} pumping and loss of CaM sensitivity [5]. Immunoblotting experiments showed that the trypsin activation was accompanied by decrease in the amount of intact Ca^{2+}-ATPase (111 kDa) and accumulation of a 102 kDa band which did not bind CaM, suggesting that 80–90 terminal amino acids had been lost from the intact ATPase. The CaM-binding domain would have to be situated within this stretch(es) of amino acids. Approx. 168 amino acids in the N-terminus precede the first putative membrane-spanning segment. In contrast, the predicted C-terminal tail following the last transmembrane helix is not longer than 22 amino acids. Based on a hydropathy analysis of the BCA1 sequence, amino acids located in position 80–90 upstream from the C-terminus are in a hydrophilic region between the putative eighth and ninth membrane-spanning segments, and may therefore be accessible for tryptic digest. Thus, we cannot completely rule out the possibility that part of the C-terminus was removed by trypsin.

Two lines of evidence suggest that CaM indeed binds to the N-terminus of BCA1. First, a stretch of N-terminal amino acids situated between Ala-19 and Leu-43 is predicted to fold as an amphipathic helix with characteristics typical for CaM-binding regions (Fig. 4B,C). Such a region is not found at the C-terminus. Second, a synthetic peptide corresponding to this region bound strongly to CaM. Whereas binding of the native enzyme to CaM is strictly dependent on Ca^{2+} [6], the synthetic peptide bound to CaM in both the presence and absence of Ca^{2+} (Fig. 4A). It has been observed before that peptide CaM targets bind CaM even in the absence of Ca^{2+} [31–33]. It has been suggested that peptide targets may have higher affinity for CaM than the corresponding regions in the native enzyme which are surrounded by other, often negatively charged, amino acid residues [31].

Very little is known about the function of the N-terminus in P-type ATPases. Our results suggest that the N-terminus of at least BCA1 can have a regulatory function. A homologous domain is present in the N-terminus of the ACA1 polypeptide (Fig. 4). The function of the N-terminal domain in these Ca^{2+}-ATPases may be analogous to those of the C-terminal regulatory domains in plasma membrane Ca^{2+}-ATPases [16] and plasma membrane H^+-ATPases [34].

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