Maize cap1 Encodes a Novel SERCA-type Calcium-ATPase with a Calmodulin-binding Domain*

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A cDNA (CAP1) isolated from maize roots shares sequence identity with genes encoding P-type Ca\textsuperscript{2+}-ATPases and restores the growth phenotype of yeast mutants defective in Ca\textsuperscript{2+}-pumps. CAP1 was transcribed and translated in the yeast mutant. Furthermore, the membrane-integrated product formed a Ca\textsuperscript{2+}-dependent phosphorylated intermediate and supported Ca\textsuperscript{2+} transport. Although CAP1 shares greater sequence identity with mammalian “endoplasmic reticulum-type” Ca\textsuperscript{2+}-pumps, it differs from these genes by having features of calmodulin (CaM)-regulated Ca\textsuperscript{2+}-pumps. CAP1 from yeast microsomes bound CaM, and the CAP1-dependent Ca\textsuperscript{2+} transport in yeast was stimulated by CaM. Peptides from the C terminus of CAP1 bound CaM. Anti-CAP1 antibodies specifically recognized a maize microsomal polypeptide that also bound CaM. A similar polypeptide also formed a Ca\textsuperscript{2+}-dependent phosphoenzyme. Our results suggest that cap1 encodes a novel form of CaM-regulated Ca\textsuperscript{2+}-ATPase in maize. CAP1 appears to be encoded by one or two genes in maize. CAP1 RNA is induced only during early anoxia, indicating that the Ca\textsuperscript{2+}-pump may play an important role in O\textsubscript{2}−-deprived maize cells.

Previous studies have shown that an elevation of cytosolic calcium ([Ca\textsuperscript{2+}]) precedes molecular and whole plant responses to oxygen deprivation in maize (1, 2). Furthermore, anoxia-induced [Ca\textsuperscript{2+}], elevation in maize cells falls into two distinct patterns, differing in their magnitude and kinetics (1). Tight regulation of [Ca\textsuperscript{2+}], is essential to the proposed role for Ca\textsuperscript{2+} as a cellular messenger (3). The maintenance of [Ca\textsuperscript{2+}], at submicromolar levels and regulation of spatio-temporal patterns of Ca\textsuperscript{2+} signals are mediated by various Ca\textsuperscript{2+} transporters, such as Ca\textsuperscript{2+}-pumps and proton-coupled antiporters (see e.g. Ref. 4). Hence, there has been a great interest in molecular cloning of Ca\textsuperscript{2+} transporters and characterizing their role in Ca\textsuperscript{2+}-mediated signaling pathways (5–9). As part of our analysis of the pathway and components of Ca\textsuperscript{2+}-mediated anoxia-signaling in maize, we initiated studies to identify Ca\textsuperscript{2+} stores (10) and transporters that may regulate the cytoplasmic Ca\textsuperscript{2+} signal. Here, we report the isolation and characterization of a cDNA clone (CAP1) that encodes a calmodulin-binding Ca\textsuperscript{2+}-ATPase in maize roots.

Ca\textsuperscript{2+}-ATPases belong to two distinct classes, differing in their size, sequence, cellular location, and regulation by calmodulin (CaM). The “ER-type” pump (located on the ER membranes) is a ~90-kDa polypeptide, lacks a CaM-binding domain, and is not dependent on CaM for its regulation. The “PM-type” pump (located on the plasma membrane) is a 138-kDa molecule, possesses a C-terminal CaM-binding region, and is regulated by CaM. Homologs of ER-type as well as PM-type Ca\textsuperscript{2+}-pumps (but with the CaM-binding domain at the N terminal) have been cloned from plants, recently (5–9). In maize, a calmodulin-regulated Ca\textsuperscript{2+}-pump, related in size and antigenicity to the animal PM-type Ca\textsuperscript{2+}-ATPase, was purified from endomembranes of young etiolated shoots (11–13). On the other hand, biochemical evidence was presented for the existence of an intriguing ER-type pump with CaM-binding properties, in the enriched ER membranes of young etiolated maize shoots (14). Our results indicate that cap1, by virtue of its sequence identity to ER-type Ca\textsuperscript{2+}-pumps and at the same time possessing a C-terminal CaM-binding domain characteristic to PM-type pumps, may encode a novel chimeric Ca\textsuperscript{2+}-ATPase such as the one implied by previous studies (14). In addition, we have functionally characterized the CAP1 protein using a yeast expression system and identified a putative cognate protein in maize microsomes. Furthermore, our expression analysis suggests that the abundance of CAP1 mRNA is low in maize roots and is mildly increased under anoxia.

EXPERIMENTAL PROCEDURES

Three-day-old dark-grown maize (Zea mays L., inbred B73Ht) seedlings were raised and anoxically treated, as described previously (2). Yeast (Saccharomyces cerevisiae) strains W303-1A (MAT\textalpha, leu2, his3, ade2, trp1, ura3, pkm1 A542 (MAT\alpha, pkm1: HIS3, ade2, trp1, ura3), pnc1 K605 (MAT\alpha, pnc1: TRP1, ade2, ura3), pnc1 K633 (MAT\alpha, pnc1: HIS3, ade2, trp1, ura3), and triple mutant K616 (MAT\textalpha, pkm1:: HIS3, pnc1:: TRP1, cml1: LEU2, ura3) were generously provided by Dr. Kyle Cunningham, The Johns Hopkins University. Wild type and mutant strains were grown in standard YPD (15) except that A542 and K616 were supplemented with 10 mM CaCl\textsubscript{2}. Transformation was carried out according to Ref. 16, and the transformants were selected on synthetic complete medium lacking uracil (SC-URA, Ref. 15).

Isolation and Analysis of cDNA Clones—A cDNA library constructed in λZAP vector (Stratagene) from the root tissue of 6-h anoxically

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AF099871.

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1 The abbreviations used are: CaM, calmodulin; ER, endoplasmic reticulum; PM, plasma membrane; PCR, polymerase chain reaction; SC-URA, synthetic complete medium lacking uracil; SERCA, sarco/ endoplasmic reticulum calcium-ATPase; RACE, rapid amplification of cDNA ends; HRP, horseradish peroxidase; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; TM, transmembrane; BTP, 1,3-bis[tris(hydroxymethyl)methylaminol]propane; Os, Oryza sativa L.
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Cloning and Sequencing of 5'-RACE-PCR Products—A 22-base primer derived from the 5'-end of CAP1 cDNA (20 bases inside) was labeled and used to reverse transcribe the missing 5'-end of CAP1 mRNA from maize root total RNA preparations. Precise products that could later be PCR-amplified were obtained only at priming temperatures >65 °C, using the thermostable reverse transcriptases (ThermoScript RT, Life Technologies, Inc.; or Carboxythermus hydrogenoformans polymerase, Roche Molecular Biochemicals). The primer extension products were separated on 6% acrylamide-urea sequencing gels. 32P-Labeled 10 and 100 base ladders were co-run to size the products. The 140-base-long product was eluted, PCR-amplified using a hot-start method (PLATINUM Taq polymerase, Life Technologies, Inc.), and cloned into Topo-TA vector (Invitrogen).

Expression of CAP1 Product in Yeast and Complementation of Yeast Mutants—The entire open reading frame of CAP1 and a part of the 3'-untranslated region were PCR-amplified from the original plasmid clone. An initiation codon at the 5'-prime and EcoRI sites at both termini were introduced during amplification. The product was ligated into the EcoRI site of a yeast expression vector p426Gal1 between the galactokinase (Gal1) promoter and CYC1 termination sequence. The junctions of the recombinant plasmid was sequenced to confirm the orientation. The plasmid with correct orientation of the insert was used to transform wild type and mutant yeast strains, and the transformants were selected for uracil prototrophy on SC-URA plates. The Ura+ colonies were used for complementation studies.

Overexpression of CAP1 Fragments in Escherichia coli and Purification of Recombinant Proteins—Two fragments of CAP1, one that encodes a major part of the central hydrophilic loop (i.e. residues 342–576, with a calculated molecular mass of 25 kDa) and the other from the C-terminal region comprising residues from 947 to 1038 (14 kDa), were PCR-amplified and cloned into an expression vector pQE-30 (Qiagen). The vector contains an N-terminal histidine tag downstream to the T5 promoter and the Lac operator. The chimeric protein, 10 nM [32P]ATP (10 μCi), 1 μM EDTA, 0.5 mM EGTA, 100 μM KCl, 25 mM HEPES-BTP, pH 6.0. The effects of Ca2+ and La3+ were tested by adding CaCl2 and LaCl3, to give a final free ion concentration of 100 and 50 μM, respectively, as estimated by the MAXCHELATOR program (21). The reaction was terminated, and proteins were resolved in acidic phosphate gels (22, 23).

Transport Assay with Yeast Membrane Vesicles—Membrane isolation from K616 cells transformed with vector alone or with pCAP1 and the measurement of 45Ca uptake were carried out, essentially as described by Liang and Sze (24). The transport buffer contained 25 mM succinate, 25 mM HEPES-BTP, pH 7.5, 10 mM KCl, 3 mM MgSO4, 0.4 mM sodium azide, 100 μM EGTA, 5 μM gramicidin, and 10 μM [32P]CaCl2 (ICN). The final specific activity was 2 μCi/mM CaCl2 per ml. Under the above assay conditions, the reaction was terminated using calcium chelating assay, the calculated free Ca2+ concentration varied between 10 and 50 μM (21). Membrane vesicles equivalent to 15 μg of protein were used in 0.25 ml of reaction mix. Uptake was initiated by the addition of 3 mM ATP and incubated at 25 °C for up to 20 min. The reaction mixture was spotted on pre-wet GS filters (Millipore) under vacuum, and the filters were washed in ice-cold rinse buffer containing 250 mM sucrose, 25 mM HEPES-BTP, pH 7.5, and 200 μM Ca2+. When used, the calmodulin inhibitor W7 was added at a final concentration of 100 μM and the Ca2+/ionophore A23187 at 1 μM.

Antibody Generation and Protein Gel Blot Assays—Monoclonal antisera were raised against the 24-kDa central hydrophilic loop expressed in E. coli. Calmodulin-binding proteins purified from maize microsomes were concentrated by trichloroacetic acid precipitation and resolved in 8 or 6–12% gradient SDS-acrylamide gels. Gels were stained in silver or Coomassie Blue. Proteins resolved in adjacent lanes were used for immunoblot analysis. Protein transfer onto polyvinylidene difluoride (Bio-Rad) and antibody incubations were done as described previously (25).

Preparation of Horseradish Peroxidase-coupled CaM (HRP-CaM) and CaM Overlay Assays—Maize recombinant calmodulin was conjugated to activated horseradish peroxidase (Pierce) by primary amine coupling and was used to probe CaM-target interactions. The eluant from CaM-Sepharose columns was concentrated using a Centricon concentrator. For use in phosphoenzyme essays, 0.05% Tween 20 and standard protocols (27). Fragments from three different regions of CAP1 clone, namely the 5'-end, sequence coding for the central hydrophilic loop, and the 3'-end, were used as probes. The hybridization and washings were done at moderate or high stringency. Yeast mutant K616 cells transformed with p426Gal1 or pCAP1 in SC-Ura medium containing glucose or galactose as the carbon source. CaCl2 at 5 mM was added to support growth of pGal1 transformed cells. Total RNA was isolated from 250-ml cultures grown for 20 h. RNA gel blot analysis was carried out as described previously (2).
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RESULTS

cap1 Encodes a Putative P-type Ca\textsuperscript{2+}-ATPase—The clone, CAP1, was isolated by screening a cDNA library made from the root tissue of anoxic maize seedlings, with a tomato cDNA encoding an ER-type Ca\textsuperscript{2+}-ATPase (LCA1, 9). As shown in Fig. 1, the full-length CAP1 sequence can be translated into a polypeptide of 1,049 amino acids with a calculated molecular mass of 113,099 Da (GenBank accession number AF096871). The deduced protein is 63% identical to the ER-type Ca\textsuperscript{2+}-ATPase reported from rice (5) (Fig. 1). The sequence identity was 46% with SERCAs (sarcoplasmic/endooplasmic reticulum calcium-ATPases, see Ref. 28) and 56–64% with plant homologs from tomato and Arabidopsis (7, 9). The sequence identity is greater than 75% in the essential sequence motifs (see below). The CAP1 protein includes all the domains highly conserved in P-type Ca\textsuperscript{2+}-ATPases. The 10 transmembrane domains predicted by hydropathy analysis are analogous to those in SERCA-pumps (TM1 to TM10 in Fig. 1). A hydrophilic domain between TM4 and TM5 of CAP1 contains a potential aspartyl phosphorylation site within the CSDK motif and two ATP-binding sites characteristic of all P-type ion pumps (29). Six residues within TM4, -5, -6, and -8 required for high affinity Ca\textsuperscript{2+} transport (28) are all conserved in the maize clone (Fig. 1). The transmembrane domains in CAP1 have different degrees of identity to the animal SERCAs and the plant Ca\textsuperscript{2+}-pumps reported thus far. TM5 and TM6 of CAP1 are 80 and 88% identical to those of SERCAs, 92–96% with those in tomato LCA1 and Arabidopsis ECA1 products, and 100% with those in rice Os-Ca-ATPase. However, CAP1 differs in its sequence and structure (30% sequence identity) from the PM-type Ca\textsuperscript{2+}-pumps including those recently reported from cauliflower (8) and Arabidopsis (6).

The sequence shown in Fig. 1 is compiled from the original cDNA, CAP1 (which is short only by 19 amino acids from the complete sequence), and an extension product of the 5'-sequence by rapid amplification of cDNA ends (RACE)-PCR reaction. CAP1 is shorter at the N terminus by 25–32 residues than its plant homologs from rice, tomato, and Arabidopsis, but it shows a longer C-terminal tail (by ~40 residues) relative to these SERCA-type pumps.

The putative Ca\textsuperscript{2+}-pumping function of CAP1 was tested by expressing the clone in yeast mutants that lack the Golgi-Ca\textsuperscript{2+}-pump (pmr1) or both Golgi- and vacuole-located Ca\textsuperscript{2+}-ATPases as well as calcineurin (pmr1 pmc1 cnb1). As shown in Fig. 2, A and B, wild type yeast with functional endogenous Ca\textsuperscript{2+}-pumps grew on plates containing 10 mM EGTA or MnCl\textsubscript{2}. However, mutants in the Golgi-located Ca\textsuperscript{2+}-pumps (pmr1; Fig. 2, A and B) or triple mutants lacking both the endomembrane Ca\textsuperscript{2+}-pumps (pmr1 pmc1 cnb1, also known as K616; Fig. 2A, data not shown) failed to grow on EGTA or 3 mM MnCl\textsubscript{2}, as reported earlier (7, 30). CAP1 restored the growth of both pmr1 and triple mutants on EGTA plates (Fig. 2A). The growth complementation was observed only in the presence of galactose (Fig. 2A), the inducer of Gal1 promoter under whose control the CAP1 cDNA was inserted. This suggested that CAP1 was transcribed as well as translated, and the product was assembled as a fully functional Ca\textsuperscript{2+}-pumping enzyme in yeast membranes. The transformants could not grow on MnCl\textsubscript{2} (Fig. 2B, data not shown for K616), indicating that CAP1 cannot transport Mn\textsuperscript{2+}. Rabbit SERCA-pump was also specific for Ca\textsuperscript{2+} transport and failed to catalyze Mn\textsuperscript{2+} transport (31). However, yeast PMR1 or Arabidopsis ECA1 products restored the growth of pmr1 on Mn\textsuperscript{2+} (7, 32). The specificity of CAP1 protein for Ca\textsuperscript{2+} was further indicated by its inability to restore the growth of the pmr2 mutant (deficient in Na\textsuperscript{+}/Li\textsuperscript{+} efflux activity) on high lithium-containing medium (Fig. 2C).

CAP1 Is Transcribed and Translated in the Yeast Mutant K616—We have determined whether the phenotypic complementation of yeast mutants by CAP1 was indeed due to the expression of CAP1 cDNA in a galactose-dependent manner. Total RNA preparations from K616 transformants grown in the presence of glucose or galactose were probed with the 5'-end of CAP1 cDNA. The results, presented in Fig. 3A, show that CAP1 transcripts are detectable only in pCAP1-transformed mutant
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**FIG. 2.** Complementation of yeast mutants lacking either Golgi Ca\(^{2+}\)-pumps (pmr1, AA542) or both Golgi and vacuolar Ca\(^{2+}\)-pumps (pmr1 pmc1; K616) by CAP1 expression. A, CAP1 restores the growth of AA542 and K616 yeast mutants on low Ca\(^{2+}\) medium. Wild type (W303), pmr1, and pmr1 pmc1 (K616) were transformed with the vector (p426) alone or the chimeric plasmid containing the maize CAP1 clone driven by Gal1 promoter. Cells were streaked on SC-Ura plates containing 10 mM EGTA + glucose or galactose and incubated at 30 °C for 4 days. B, CAP1 failed to restore the growth of yeast mutants lacking Ca\(^{2+}\)-pumps on Mn\(^{2+}\). Wild type (W303) and pmr1 cells transformed with p426 alone or the vector containing the CAP1 were grown on SC-Ura plates containing 3 mM MnCl\(_{2}\) + glucose or galactose for 5 days at 30 °C. C, CAP1 failed to restore the growth of yeast pmr2 mutant (defective in Na\(^{+}/\)Li\(^{+}\) efflux pump) on high Li\(^{+}\)-containing medium. Wild type (W303) and pmr2 cells were transformed with the vector alone or the chimeric plasmid containing the CAP1. Cells were grown on SC-Ura plates containing glucose or galactose supplemented with 150 mM LiCl for 5 days at 30 °C.

**FIG. 3.** CAP1 is transcribed and translated in the yeast mutant K616. A, gel blot hybridization of yeast RNA with CAP1. 7.5 μg of total RNA from glucose (Glu)- or galactose (Gal)-grown K616 cells either transformed with the vector alone (p426) or the vector containing CAP1 (pCAP1) is blotted after separation in a 1% agarose-formaldehyde gel and probed with random-primed CAP1 and actin cDNA. B, protein gel blot analysis of yeast microsomal proteins. Microsomes were prepared from K616 cells transformed either with the vector alone (p426) or with p426 containing the CAP1 clone (pCAP1) and probed with monoclonal anti-24-kDa CAP1 protein antisera. 5 μg of protein was loaded per lane. Probing an identical blot with preimmune serum or anti-histidine antibody did not give any signal. Size markers are shown on the left side of the panel.

The CAP1 Product Forms a Ca\(^{2+}\)-dependent Phosphorylated Intermediate Characteristic of P-type ATPases—We asked whether the CAP1 product expressed in yeast mutants can form a phosphorylated intermediate (E-P) characteristic of Ca\(^{2+}\)-ATPases, i.e. a hydroxylamine-sensitive and Ca\(^{2+}\)-dependent acyl-phosphate intermediate. As shown in Fig. 4, the membranes from K616 cells expressing CAP1 showed one 32P-labeled band (~110 kDa) in the presence of Ca\(^{2+}\). Furthermore, no signals were seen in membranes from yeast cells transformed with vector alone (Fig. 4), indicating that the ~110-kDa phosphoprotein is most likely identical to the CAP1 product. The labeling of the 110-kDa band was dependent on a short incubation time (~15 s, data not shown), which is consistent with the rapid turnover rate of the phosphorylation intermediate common for all P-type ATPases (33). Incubating the reaction product with hydroxylamine completely removed the label (Fig. 4), confirming that the phosphorylation was by an acyl phosphate-linkage characteristic to P-type ATPases. The phosphate labeling was dependent on the presence of Ca\(^{2+}\) in the reaction mixture and enhanced by lanthanum (Fig. 4), consistent with the slow turnover rate of the intermediate in the presence of lanthanum. Thus, the E-P assay further confirmed that the CAP1 product may be a functional Ca\(^{2+}\)-ATPase.

**CAP1 Expressed in Yeast Mutant Binds CaM-Sepharose in a**
**Ca\(^{2+}\)**-dependent Manner—Since the CAP1 product appeared to be divergent from the known ER-type Ca\(^{2+}\)-ATPases by possessing an extended C-terminal tail rich in positively charged residues interspersed by hydrophobic amino acids, it is possible that the CAP1 product may be a CaM-regulated Ca\(^{2+}\)-ATPase. The CaM binding nature of the CAP1 protein expressed in the yeast mutant K616 was assessed using CaM-affinity chromatography. Detergent-solubilized microsomal proteins (0.75–1 mg) were loaded on a 2-ml column of CaM-Sepharose in the presence of Ca\(^{2+}\). The column was washed with excess column buffer and eluted with 10 mM EGTA. The second and third column fractions of the eluent showed enrichment of polyacrylamide-precipitated proteins were solubilized in the presence of hydroxyamine. Size markers are shown on the left side of the figure.

**Microsomes from the CAP1-expressing Yeast Mutant Mediate Calmodulin-stimulated Ca\(^{2+}\) Transport**—To determine further whether the CAP1 complementation of yeast mutants defective in Ca\(^{2+}\)-pumps was indeed resulted by the restoration of calcium transport, we have tested the Ca\(^{2+}\) transport activity of microsomes isolated from the triple mutant K616 expressing CAP1. The mutant transformed with vector alone showed back-transport activity of the microsomes in the presence of Ca\(^{2+}\). The results, presented in Fig. 6, show up to a 2-fold stimulation of Ca\(^{2+}\) transport activity by an external addition of CaM, indicating that the CAP1 product may be associate with endogenous CaM. With supplemental CaM in the assay buffer, W7 caused a very high background retention of Ca\(^{2+}\) on the filters. Therefore, its effect on the Ca\(^{2+}\) transport activity of the microsomes in the presence of CaM could not be reliably ascertained (data not shown). In summary, these results provide further evidence that CAP1 is a calmodulin-regulated Ca\(^{2+}\)-ATPase.

**Peptides from CAP1 C Terminus Bind Ca\(^{2+}\)/Calmodulin**—Given the CaM binding nature of recombinant CAP1 and CaM stimulation of its activity, we expected to find a potential CaM-binding domain in its sequence. Taking analogy to the animal calmodulin-regulated pumps and the presence of an extended C terminus into consideration, we searched for a putative CaM-binding domain at the C terminus of CAP1. Although not conserved in their primary sequence, CaM-binding domains most commonly form basic amphipathic \(\alpha\)-helices (35). The C terminus of CAP1 showed helical structures with clusters of positively charged amino acids. Three overlapping regions (between 995 and 1049 residues, Fig. 7A) were selected based on the properties of their helical wheel projections. Synthetic peptides were made corresponding to these sequences and tested for their ability to shift the mobility of calmodulin (recombinant or purified from roots) in native acrylamide gels (19). Of the three peptides tested, only the two distal peptides (peptides P2 and P3, Fig. 7A) with an overlapping sequence of KQKASSER-RLTFD bound CaM and shifted its mobility in a Ca\(^{2+}\)-dependent manner (Fig. 7, B–D). Between P2 and P3, the latter caused a greater retardation in the CaM mobility in the presence of urea (Fig. 7B). However, electrophoresis of CaM-peptide complexes in the absence of urea led to a complete shift of CaM by P2 at equimolar concentrations (Fig. 7C). The two peptides (P2 and P3) that caused a mobility shift were highly basic in their.

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**Fig. 4.** The CAP1 product from yeast microsomes forms a Ca\(^{2+}\)-dependent phosphorylated intermediate characteristic of P-type ATPases. Microsomes were prepared from K616 cells transformed either with the vector alone (p426) or with p426 containing the CAP1 clone (pCAP1) and used for the phosphoenzyme assay (5–7.5 \(\mu\)g of protein per lane). Microsomes were incubated with \(\gamma^{32P}\)ATP in the presence of CaCl\(_2\) (Ca), EGTA, or LaCl\(_3\) (La) as described under "Experimental Procedures." In the lane labeled NH\(_{3}\)OH, the trichloroacetic acid-precipitated proteins were solubilized in the presence of hydroxyamine. Size markers are shown on the left side of the figure.

**Fig. 5.** CAP1 expressed in yeast mutant binds CaM-Sepharose in a \(\alpha\)-dependent manner. Protein gel blot analysis of root microsomal CaM-binding proteins. Monoclonal antisera raised against the purified 24-kDa CAP1 product were used to probe a gel blot of the CaM-binding fraction of maize root microsomal proteins (5 \(\mu\)g). Probing an identical blot with preimmune serum or anti-histidine antisera did not give any signal.

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Ca\(^{2+}\)-dependence and other factors affecting the transport of Ca\(^{2+}\) across membranes, including the presence of CaM, ATP, and other modulators, are discussed in detail in "Results and Discussion."
represent results of three separate membrane preparations. ATP. Values are means of duplicate samples from a single assay and the absence of ATP was subtracted from each corresponding assay with calmodulin. Bovine serum albumin was substituted for CaM to provide effect of supplemental calmodulin was tested using recombinant maize CAP1 in yeast microsomes.

Overall charge (pI = 12) in contrast to the P1 peptide (pI = 6.36) that failed to induce a shift. However, P2 and P3 did not bind to other acidic proteins such as soybean trypsin inhibitor or bovine serum albumin (data not shown), under identical conditions. This indicated that the two CAP1 peptides bound calmodulin by specific hydrophobic interactions and not due to electrostatic attraction.

The binding of CaM to its targets induces structural changes in CaM as well as the target peptides/proteins. Binding is accomplished by a change in conformation of the central a-helix of CaM to a random coil allowing the globular lobes of CaM to engulf the target peptide (36). In turn, the target peptides may attain a greater helicity upon CaM binding. Hence, spectropolarimetry has been a useful tool to follow the helicity changes in CaM-target complexes (19). CAP1 peptide and CaM interactions were monitored using CD spectra. Calmodulin displayed a CD spectrum typical of helical proteins with minima at 222 and 208 nm and a maximum at 190 nm (data not shown). The peptides themselves showed random conformation as indicated by the spectra of uncomplexed peptides (data not shown). The peptides showed random conformation as indicated by the spectra of uncomplexed peptides (data not shown). Fig. 8A presents the difference spectra resulting after the subtraction of the spectrum of individual peptide-CaM complex from that of CaM alone. When co-incubated with calmodulin, peptides P2 and P3 induced a change in the molar ellipticity of CaM at 208 and 222 nm, whereas P1 induced nonspecific changes (Fig. 8A). The changes induced by P2 and P3 indicate a decreased helicity of CaM and an increase in the helicity of the two peptides (19). Furthermore, the interaction was dependent on Ca2+, as these conformational changes were abolished by EGTA (Fig. 8B). The absence of a helicity increase in P1 confirmed that CAP1 residues included in this peptide are not involved in the interaction of the pump with CaM, in accordance with our results from CaM-gel shift assays (data not shown). Furthermore, CD spectral analysis also showed that P3 caused greater decrease in the helicity of CaM than P2 did (Fig. 8B), confirming that in addition to KQKASSER-RLTFD other residues in the P3 peptide are important for the interaction of the pump with calmodulin. A recombinant peptide homologous to the C terminus that encompasses the CaM-binding peptides was expressed in E. coli. This longer peptide can be purified to homogeneity by CaM-affinity chromatography and interacts with CaM in filter binding assays (data not shown). Since this peptide is toxic to E. coli and could not be overexpressed, synthetic peptides will be used to fine map the CaM-binding domain.

Antibodies Against a Recombinant CAP1 Polypeptide Recognize a Single Maize Microsomal Protein Purified by CaM-Affinity Chromatography—We attempted to identify and characterize the CAP1 cognate protein from maize root tissue, using CAP1 antisera. Although most of the monoclonal antisera gave a reactivity at high titer with the recombinant polypeptide expressed in E. coli (>16,000-fold dilution) or yeast (1:4000), none of the clones recognized any polypeptide in total maize microsomes or after fractionation on sucrose gradients (data not shown). This indicated that the cognate protein, if present, is in very low abundance in maize tissues. Since our results with the CAP1 product suggested that it is a CaM-binding protein (Figs. 5–8), we enriched maize root microsomal proteins on CaM-affinity chromatography and then probed with CAP1 antisera. Microsomal CaM-binding preparations showed a major polypeptide of -110 kDa and 5–6 additional bands of varying sizes (Fig. 9A). The CAP1 antibodies cross-reacted only with the 110-kDa polypeptide (Fig. 9B), indicating that the CAP1 cDNA encodes a single microsomal protein that binds CaM by itself or through interaction with a calmodulin-binding protein.

A 110-kDa Protein from the CaM-Sepharose Binding Fraction of Maize Microsomes Directly Interacts with Ca2+/Calmodulin—The ability of putative CAP1 cognate protein to in-

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**Fig. 6.** Characterization of Ca2+ transport activity driven by CAP1 in yeast microsomes. A, yeast microsomes from vector or CAP1-transformed cells were isolated and assayed for ATP-dependent Ca2+ transport activity as described under “Experimental Procedures.” At the end of 5 min, 1 μM of the ionophore A23187 was added in a replicate assay. Vector-transformed cells showed no ATP-stimulated activity in the presence of gramicidin (routinely added at 50 μM to the assay medium). The calculated free Ca2+ in this assay was 57 nM. However, the linearity of transport was maintained only for the first 5 min, even at higher free Ca2+ concentrations tested (1 or 5 μM). B, Ca2+- dependence of 45Ca transport driven by CAP1. CaCl2/EGTA mixtures were used to buffer free Ca2+ as described under “Experimental Procedures.”

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A 110-kDa Protein from the CaM-Sepharose Binding Fraction of Maize Microsomes Directly Interacts with Ca2+/Calmodulin—The ability of putative CAP1 cognate protein to in-

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A 110-kDa Protein from the CaM-Sepharose Binding Fraction of Maize Microsomes Directly Interacts with Ca2+/Calmodulin—The ability of putative CAP1 cognate protein to in-
Peptides from the C terminus of CAP1 bind to calmodulin. A, synthetic peptides (P1, P2, and P3) identical in their sequence to the C terminus of deduced CAP1 were used to test the affinity of CAP1 product to calmodulin. The location of each peptide in the CAP1 sequence is shown by the number of start and end residues. The sequence overlap within the peptides is indicated by an underline. B, interaction of CAP1 peptides with calmodulin as studied by native urea-acrylamide gel electrophoresis. 150, 300, 750, and 1500 pmol of P1 (lanes 1–5), P2 (lanes 6–9), or P3 (lanes 10–13) were incubated with 300 pmol of calmodulin in the presence of 4 M urea and 100 μM CaCl₂ for 1 h. The peptide-protein complexes were analyzed in native 4 M urea, 12.5% acrylamide gel as described by Erickson-Viitanen and DeGrado (19). Lanes 1 and 14 show the migration of free CaM. Electrophoresis of free peptides in the same gel did not result in any bands. C, interaction of CAP1 peptides with calmodulin as studied in native gels lacking urea. 150 and 300 pmol of P1 (lanes 2 and 3), P2 (lanes 4 and 5), or P3 (lanes 6 and 7) were incubated with 300 pmol of calmodulin in the presence of 100 μM CaCl₂ for 1 h. The peptide-protein complexes were analyzed in native acrylamide gels (19). Lane 1 shows the migration of free CaM. D, Ca²⁺ dependence of the CaM interaction with CAP1 peptides. The protein and peptide incubations were as in lanes 1–5 of B, except that Ca²⁺ was replaced by 2 mM EGTA in the binding and gel running buffers (to lower the free Ca²⁺ concentration to near zero). Results are shown for P2 and were similar with P1 and P3 peptides. Lane 1 is free CaM and lanes 2–5 are 150, 300, 750, and 1500 pmol of peptide incubated with 300 pmol of CaM.

Fig. 7. Circular dichroism spectra of calmodulin and its complexes with CAP1 peptides. A, difference spectra obtained by subtracting the spectrum of 3 μM each peptide + 3 μM CaM from that of 3 μM CaM alone; symbols are as follows: circles, P1; squares, P2; diamonds, P3. The binding buffer contained 0.5 mM CaCl₂. The mean residue ellipticity is expressed in deg cm²/dmol of amino acid residues in the peptide. B, Ca²⁺ dependence of helicity changes in the CaM-peptide complexes. The difference spectrum for P3 peptide, indicated by circles, was obtained in the presence of CaCl₂ (as described in A) and the one indicated by squares was obtained in the absence of CaCl₂ i.e. in the presence of 2 mM EGTA.

The CaM-Sepharose Fraction Has a 110-kDa Polypeptide That Forms a Ca²⁺-dependent Phosphorylated Intermediate—We also investigated if the 110-kDa CaM-binding protein of maize microsomes forms Ca²⁺- dependent acyl-phosphate intermediate. As shown in Fig. 10C, the CaM-binding preparation showed one ³²P-labeled band in the presence of Ca²⁺. The phosphoprotein was similar in its molecular size (~110 kDa) to that of the anti-CAP1 cross-reactive polypeptide. The labeling of the polypeptide was also dependent on a short incubation time, sensitive to hydroxylamine dependent on the presence of Ca²⁺ in the reaction mixture and enhanced in the presence of lanthanum (Fig. 10C). These results suggest that the putative CAP1 cognate protein in maize membrane may be a functional Ca²⁺-ATPase.
CAP1 Transcripts Are Low Abundant and Induced by Anoxia in Maize Roots—Our interest is to elucidate the role of this chimeric Ca\textsuperscript{2+}-pump in intracellular signaling, particularly in the Ca\textsuperscript{2+}-mediated signaling of anoxia. As a first step toward this goal, we investigated the abundance and induction patterns of CAP1 message in maize roots by different environmental stresses. Gel blot hybridization and RT-PCR experiments indicated that that CAP1 transcripts are of very low abundance in maize tissues, as was the concentration of its cognate protein in the microsomes. Consistent detection of signals in the RNA gel blots required the purification of poly(A) RNA. Specific hybridization signals could often be detected by loading >40 μg of total RNA per lane (data not shown). There was only one single transcript class of about 6 kilobase pairs long indicating that there could be a processing regulation of the primary transcript. Despite the low abundance of its transcripts in maize roots, the CAP1 cDNA was isolated by screening left side of the figures.

In mammalian cells, molecular and biochemical evidence shows that the CaM-regulated Ca\textsuperscript{2+}-pump is located on the plasma membrane and is divergent in its sequence and structural features from the SERCA-type Ca\textsuperscript{2+}-pumps. Evidence has accumulated that there are calmodulin-regulated Ca\textsuperscript{2+}-ATPase activities in plant cells (39). However, unlike in animal cells, such activities have been reported both from purified plasma membrane preparations (40–42) as well as enriched endomembranes (e.g., Refs. 13, 22, and 42–44). The presence of multiple calmodulin-regulated Ca\textsuperscript{2+}-pumping activities and their distribution to different cellular compartments indicate a crucial role for these enzymes in plant cell signaling. Isolation and functional characterization of genes encoding these multiple Ca\textsuperscript{2+}-pumps would facilitate the unraveling of mechanisms of cellular Ca\textsuperscript{2+} homeostasis and attendant pathways of cellular communication. Recently, Malmstrom et al. (8) and Harper et al. (6) have cloned cDNAs for CaM-regulated Ca\textsuperscript{2+}-pumps from cauliflower and Arabidopsis (BCA1 and ACA2, respectively). These clones are related to the mammalian plasma membrane Ca\textsuperscript{2+}-ATPases, although the CaM-binding domain in the plant clones is located at the N terminus. CAP1 presents yet another novel type of CaM-binding Ca\textsuperscript{2+}-pump in plants. Sequence homology to SERCAs, growth and functional complementation of yeast mutants deficient in Ca\textsuperscript{2+}-ATPases, binding affinity of synthetic peptides and transgenic CAP1 protein to calmodulin, CaM-stimulated Ca\textsuperscript{2+} transport activity in yeast microsomes, antigenic identity of CAP1 product with a Ca\textsuperscript{2+}- and CaM-binding microsomal protein from maize roots, properties of the phosphoenzyme formed by cognate proteins, provides strong support to our proposal that CAP1 encodes a calmodulin-binding Ca\textsuperscript{2+}-ATPase in maize. It differs from the canonical mammalian CaM-regulated Ca\textsuperscript{2+}-pumps in that it has greater overall sequence identity with the SERCA-type Ca\textsuperscript{2+}-ATPases (e.g. Refs. 7, 29, and 45). At the same time, it is similar to the animal PM-type pumps in having a CaM-binding domain in the C-terminal tail. Thus, CAP1 shares features of both the PM- and ER-type Ca\textsuperscript{2+}-pumps of mammalian systems. However, it is very divergent from BCA1 or ACA2 (the plant homologs of PM-type pumps) in the overall sequence as well as in the location of its CaM-binding domain. Thus, the presence of multiple CaM-regulated activities distributed on more than one cellular membrane and novel type of genes encoding these activities indicate that plant signaling pathways or components involved may not always fit the animal paradigm.

Previously, evidence was presented for the presence of CaM-regulated Ca\textsuperscript{2+}-ATPases in young maize seedling shoots (11, 13, 14). However, the polypeptides that were attributed to belong to Ca\textsuperscript{2+}-pump were of two different size ranges. Briars et al. (11) purified an enzyme on CaM-affinity columns, and this preparation showed a 140-kDa polypeptide that cross-reacted with antisera for the mammalian CaM-binding Ca\textsuperscript{2+}-ATPase. Later, this polypeptide was confirmed to be a Ca\textsuperscript{2+}-pump by reconstitution studies as well as phosphoenzyme analysis (12, 13). On the other hand, Logan and Venis (14) identified a 102-kDa polypeptide of maize microsomes as CaM-binding Ca\textsuperscript{2+}-ATPase based on its cross-reactivity with anti-SERCa antisera as well as binding to CaM. However, no further studies have been reported on this protein. The product of CAP1 clone has a predicted mass of 113 kDa, which is also in the size range of the polypeptide recognized by CAP1 antisera in maize. This is much smaller than the 140-kDa Ca\textsuperscript{2+}-pump purified by Theodoulou et al. (13). The cross-reactivity of the 140-kDa protein with the antisera for mammalian PM Ca\textsuperscript{2+}-pump further indicates that these two pumps are most likely divergent even in their sequence. In contrast, the 102-kDa
CaM-binding putative Ca^{2+}-ATPase reported by Logan and Venis (14) is in the size range of the CAP1 product. Furthermore, these authors showed that a similar polypeptide cross-reacted with antibodies for a region conserved in all SERCA-type pumps, including the CAP1 (Refs. 5, 7, 9, and 46; Fig. 1).

Our studies further indicate that the CAP1 protein possesses its CaM-binding domain at the C-terminus, and the evidence is 3-fold. CaM-affinity chromatography indicated that a recombinant polypeptide corresponding to the last 100 residues of the C-terminus bound calmodulin in the presence of Ca^{2+} (data not shown). CaM mobility shift assays demonstrated that shorter peptides within this C-terminal tail bound to CaM in calcium-dependent manner and retarded its mobility in acrylamide gels (Fig. 7). Furthermore, co-incubation of CaM with CAP1 peptides resulted in characteristic conformational changes typical to CaM-target complexes, as revealed by the CD spectra (Fig. 8). These studies were also indicative of the amino acid residues involved in the interaction of CAP1 with CaM. Of the three peptides tested, only P2 and P3 were effective in binding to CaM in gel shift as well as spectropolarimetric assays. The overlapping sequence KQKASSERRLTFD appears to be critical for CaM binding, although CaM gel
 retardation assays and CD spectra indicate additional residues in the extreme C terminus may enhance the interaction.

A high degree of sequence similarity between CAP1 and SERCAs indicates that the CAP1 protein may be localized on the ER membranes. However, the tomato LCA1 product, despite its similarity to the mammalian ER pumps, is not ER-localized but is distributed on the tonoplast and the plasma membrane (45). This has also been the case with the cauliflower calmodulin-regulated BCA1 product, which is a PM-type pump (42). It is not known if the CAP1 product is also localized on more than one membrane in maize cells. Nevertheless, the products of ECA1 and ACA2 from Arabidopsis are confined to the ER/intracellular membranes. In fact, biochemical evidence suggests that the CaM-regulated Ca\(^{2+}\) pumping activity in maize seedlings is predominantly distributed on the endomembranes, either the ER or the tonoplast (Refs. 13, 14, 47, and 48; but also see Ref. 40).

The low abundance of the CAP1 protein and transcripts in maize tissues indicates a tight regulation of CAP1 expression. Furthermore, regulation of the Ca\(^{2+}\) transport activity by calmodulin suggests the involvement of CAP1 product in a feedback attenuation of cytosolic Ca\(^{2+}\) concentration during cell stimulation. A stringent regulation of Ca\(^{2+}\) sequestration from the cytosol should allow the Ca\(^{2+}\)-dependent signaling processes to continue without the cell attaining cytotoxic levels of free Ca\(^{2+}\). Induction of CAP1 transcripts in maize roots only during the early hours of anoxia indicates such a regulation of Ca\(^{2+}\) homeostasis in the O\(_2\)-deprived maize cells.

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