A Novel Calmodulin-regulated Ca\textsuperscript{2+}-ATPase (ACA2) from Arabidopsis with an N-terminal Autoinhibitory Domain*  

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To study transporters involved in regulating intracellular Ca\textsuperscript{2+}, we isolated a full-length cDNA encoding a Ca\textsuperscript{2+}-ATPase from a model plant, Arabidopsis, and named it ACA2 (Arabidopsis Ca\textsuperscript{2+}-ATPase, isofrom 2). ACA2p is most similar to a “plasma membrane-type” Ca\textsuperscript{2+}-ATPase, but is smaller (110 kDa), contains a unique N-terminal domain, and is missing a long C-terminal calmodulin-binding regulatory domain. In addition, ACA2p is localized to an endomembrane system and not the plasma membrane, as shown by aqueous-two phase fractionation of microsomal membranes. ACA2p was expressed in yeast as both a full-length protein (ACA2-1p) and an N-terminal truncation mutant (ACA2-2p; Δ residues 2–80). Only the truncation mutant restored the growth on Ca\textsuperscript{2+}-depleted medium of a yeast mutant defective in both endogenous Ca\textsuperscript{2+} pumps, PMR1 and PMC1. Although basal Ca\textsuperscript{2+}-ATPase activity of the full-length protein was low, it was stimulated 5-fold by calmodulin (50% activation around 30 nM). In contrast, the truncated pump was fully active and insensitive to calmodulin. A calmodulin-binding sequence was identified within the first 36 residues of the N-terminal domain, as shown by calmodulin gel overlays on fusion proteins. Thus, ACA2 encodes a novel calmodulin-regulated Ca\textsuperscript{2+}-ATPase distinguished by a unique N-terminal regulatory domain and a non-plasma membrane localization.

Calcium (Ca\textsuperscript{2+}) appears to function as an important second messenger in all eukaryotes (1, 2). Ca\textsuperscript{2+} also plays an important role in regulating the processing of proteins in the secretory pathway (3, 4). Thus, cells require transport systems to carefully regulate Ca\textsuperscript{2+} concentrations in different cellular compartments. In plants, high affinity Ca\textsuperscript{2+}-translocating ATPases are thought to function in the endoplasmic reticulum, Golgi apparatus, tonoplast, plastid inner membrane, and plasma membrane (5–8). In addition, plants have low affinity Ca\textsuperscript{2+}/H\textsuperscript{+} antiporters (9). However, little is known at the molecular level about the genes which encode different Ca\textsuperscript{2+} transporters, or the specific functions of different Ca\textsuperscript{2+}-transport pathways.

Ca\textsuperscript{2+} pumps are members of a large superfamily of P-type ATPases and have been classified as endoplasmic reticulum (ER) or plasma membrane (PM)-type Ca\textsuperscript{2+}-ATPases (type IIA and IIB, respectively), based on enzymes first identified in animal systems (10). In plants, ECA1/ACA3 was the first cloned ER-type pump to be shown to have a localization and enzymatic activity analogous to an animal homolog (11). At present only two plant genes encoding homologs of “PM-type” Ca\textsuperscript{2+}-ATPases have been identified, ACA1 from Arabidopsis thaliana (12) and BCA1 from Brassica oleracea (13). Interestingly, these plant pumps differ from animal homologs not only in subcellular localization, but also in their structural arrangement. ACA1p is thought to be targeted to a plastid inner membrane (12), while BCA1p appears to be localized to the tonoplast (13). The enzyme activity of ACA1p has not been investigated. However, BCA1 appears to encode a calmodulin-stimulated Ca\textsuperscript{2+} pump, based on correspondence between the peptide sequence obtained from a Ca\textsuperscript{2+}-ATPase preparation and the predicted sequence of BCA1p (13, 14). A notable feature of both ACA1p and BCA1p is the absence of a long C-terminal regulatory domain, which is required for calmodulin activation in a typical PM-type Ca\textsuperscript{2+}-ATPase (15, 16). The structural divergence of these pumps raises two important questions: 1) does calmodulin still regulate members of this subfamily, and 2) how does calmodulin activation occur without a C-terminal calmodulin binding regulatory domain?

Here we report the predicted primary structure for ACA2p, a Ca\textsuperscript{2+}-ATPase most similar to ACA1p and BCA1p. We provide direct evidence that ACA2-like pumps constitute a novel subfamily of calmodulin-regulated Ca\textsuperscript{2+}-ATPases, distinguished by a unique N-terminal regulatory domain and their presence in non-plasma membrane locations. ACA2p is the first homolog of this family to be functionally expressed in yeast. We show that a truncation of the N-terminal domain results in a constitutively active pump that can complement a yeast strain harboring a disruption of its endogenous Ca\textsuperscript{2+} pumps. These biochemical and genetic studies are significant for two reasons. First, they show that P-type ATPases can be regulated by...
autoinhibitors at either the N- or C-terminal end. Second, they establish a yeast expression system to genetically dissect the autoinhibitory mechanism of a calmodulin-regulated P-type ATPase.

EXPERIMENTAL PROCEDURES

A. thaliana Columbia was used for plant material. DNA cloning was done in Escherichia coli strain XL1-Blue (Stratagene) or DH10a (a derivative of DH5a, Stratagene). Unless otherwise noted, standard molecular techniques were performed according to Sambrook et al. (17). ACA2 cDNA Cloning—A Zap (Stratagene)-based cDNA library constructed from A. thaliana 3-day-old seedlings (18) was screened under moderate stringency (19) using a genomic fragment of ACA2 as a hybridization probe. This genomic fragment was generated by PCR using degenerate DNA oligomers (see "Results"). The "PEGL" 20-mer primer had the sequence dGC(G/C/T)GT(G//C/T)CG(G/C/T)GAGGG(G/ C/T)/CT/GCTTGCC one full-length DNA clone. The sequence at the fusion site between ACA2 and GFP is CAG GTC GAC ATG (first codon underlined, followed by the codon for M1). Two restriction sites were also introduced as silent mutations in Escherichia coli done in AT35S promoter. Second, they were used to modify ACA2 sequences in clones described below. PCR were used to modify ACA2 sequences in clones described below. PCR was performed using AmpliTaq (Perkin-Elmer) or Phusion DNA Polymerase (Finnzymes) according to the manufacturer's instructions.

DNA Sequencing—DNA sequencing was done in the Scripps Biochemistry Core Facility using automated ABI Prism 373XL sequencer. All cDNA sequence was confirmed by sequencing both strands.

Fusion Protein Purification—Fusion proteins were expressed in E. coli DH10a. Fusion proteins constructed as an affinity sandwich with an N-terminal GST or MBP tag and a C-terminal 6 histidine tag (Phe6-HGS* (GFP sequence shown in italic lowercase letters)). All deletion constructs except pGCC1(1–58) were made by an ExoIII nuclease deletion using the parent clone pGFP-ACA2-NL-1 (not described). pG10L1 encodes residues Val119–Phe161 of ACA2 as a fusion with an N-terminal maltose binding protein and a C-terminal 6 histidine tag (Phe6-MBP). It was constructed from a PCR-amplified fragment cloned into a modified vector pMAL-CR1. The insert was subcloned as a SalIPstI fragment.

pACA2-Ns encodes the same sequence (Val119–Phe161) encoded by pG10L1, except that it has an N-terminal glutathione S-transferase (GST) affinity tag instead of maltose-binding protein (MBP). It was derived by subcloning the SalIPst1 fragment from pG10L1 into the MstI site of pGEX-KG (ampicillin resistant gene was regained).

pGCC1(1–58) encodes residues Phe1–Val58 of ACA2 and the fusion region: CTT CG AAA CC TAT GAA GCC GCG GCG (first codon underlined, last codon is M1 from GFP). The sequence at the 3′ end of the GFP reads ATG GAT GAA CTA GAC CCG GGA ATG CAT CAC CAT CAC GAC GGA TCC TGA). It was constructed from a PCR-amplified fragment cloned into a modified version of vector pMAL-CR1. The insert is subcloned as a SalIPstI fragment.

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Fusion Protein Purification—Fusion proteins were expressed in E. coli DH10a. Fusion proteins constructed as an affinity sandwich with an N-terminal GST or MBP tag and a C-terminal 6 histidine tag were purified by sequential affinity purification for the 6 histidine tag then GST or MBP (22). Purifications based on single affinity tag selections were done with minor modifications of standard procedures (New England Biolabs and Invitrogen).

Antibodies—Anti-ACA2 (number 1471) rabbit polyclonal antiserum was produced at the Scripps Animal Resource Facility. Anti-ACA2 was raised against a fusion protein, encoded by the plasmid pACA2-Ns, containing residues Val119–Phe161. Purified fusion protein samples were injected into New Zealand White rabbits with RIBI adjuvant as recommended by the manufacturer (RIBI ImmunoChem Research).

In some cases affinity-purified antibodies were used. Serum was first precipitated with 50% ammonium sulfate and redissolved in phosphate-buffered saline. Anti-GST antibodies were removed using a column with a GST-coupled protein. Anti-ACA2 antibodies were then allowed to bind to a column containing the fusion protein encoded by pACA2-Ns. Columns were made using cyanogen bromide-activated Sepharose 4B according to manufacturer (Pharmacia Biotech Inc.). Anti-ACA2 antibodies were eluted with 0.1 M glycine, pH 2.7, and immediately neutralized with 0.1 volume of 1 M Tris-HCl, pH 8.0, and dialyzed against phosphate-buffered saline. Control preimmune serum was purified over a pGEX-KG column (Pharmacia Biotech Inc.).

Total Protein Extracts—Leaves, flowers, and siliques were obtained from soil-grown plants grown in a growth room at 22 °C. Roots were obtained from 3-week-old plants grown in liquid culture consisting of Gamborg’s B5 medium (Life Technologies, Inc.) supplemented with 2% sucrose and 0.5% (w/v) MES, pH 5.7. Cultures were grown on a shaker at 125 rpm under constant illumination at 22 °C. Tissue samples were pulsed in liquid nitrogen with a mortar and pestle, and total protein was extracted in homogenization buffer (0.3 M sucrose, 10 mM EDTA, 2 mM EGTA, 100 mM Tris-HCl, pH 8.0, 35 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.5 μM leupeptin). The protein extract was clarified by a 5-min centrifugation in an Eppendorf microcentrifuge.

Aqueous Two-phase Partitioning—Plant microsomal membranes were prepared by modifications of a previously described procedure (23). All manipulations were conducted on ice or in a cold room, with prechilled buffers. Membranes were prepared from roots of plants grown in liquid culture. Roots were frozen in liquid nitrogen and pulsed with mortar and pestle or ground with dry ice in a coffee grinder. Pulverized tissue was suspended in an extraction buffer (290 mM sucrose, 25 mM EDTA, 250 mM Tris-HCl, pH 8.5, 2 mM phenylmethylsulfonyl fluoride, and 76 mM β-mercaptoethanol). Homogenates were filtered through cheesecloth to remove large debris and then centrifuged at 5,000 ×g for 20 min to remove intact organelles and cell walls. Supernatants were spun at greater than 40,000 ×g for at least 40 min to pellet microsomal membranes. The resulting supernatants were used as the soluble protein fraction. Membrane pellets were resuspended in...
resuspension buffer (0.33 mM sucrose, 4 mM KC1, 5 mM KH2PO4, pH 7.8). Plasma membranes were partially purified by aqueous two-phase partitioning (24). Each fractionation was conducted with 5–10 g of fresh weight tissue (liquid-grown plants) with yields around 75 µg of upper phase membrane protein.

**Western Blots**—Protein concentrations were determined by Bradford (25) assays using bovine serum albumin as a standard. For SDS-PAGE, samples were mixed with 3 x loading buffer (100 mM Tris, pH 6.8, 3.7% (w/v) SDS, 5% (w/v) dithiothreitol, 20% (w/v) sucrose or glycerol, 0.3% (w/v) bromophenol blue) and incubated for 15 min at 37 °C. After electrophoresis, proteins were transferred to nitrocellulose (Schleicher & Schuell), using a Bio-Rad transfer apparatus. Transfer buffer consisted of 192 mM glycine, 25 mM Tris-HCl, pH 8.3, 20% (v/v) methanol, and 0.02% (w/v) SDS.

Blots were blocked for at least 2 h in 20 mM Tris, pH 7.6, 137 mM NaCl, 0.5% (w/v) Tween-20 (TBS-T) with 5% (w/v) non-fat dry milk. Primary antisera were normally diluted in blocking buffer at the following concentrations: anti-ACA2 at 1:1000 and anti-CTF2 (26) at 1:5000. The secondary antibody used for immunodetection was a donkey anti-rabbit IgG conjugated with horseradish peroxidase (Amer sham Corp.), and was diluted at 1:5000 in blocking buffer. Primary and secondary antibody incubations were for 1 h at room temperature, and were followed by four 15-min washes in TBS-T. Secondary antibodies were detected by enhanced chemiluminescence (ECL, Amer sham Corp.) and exposure to x-ray film.

**Yeast Transformations**—Saccharomyces cerevisiae strains used for complementation studies were W303-1A (MATa, leu2, his3, ade2, ura3) and K616 (MATa, prr1:1,His5, prn1:1,TRP1, cbl1:1,LEU2, ura3) (27). For expression and ATPase assays we used strain G19 (MATa ura3, his3, leu2, trp1, ade2, ena1:1,His3:ena4a) (28) (generously provided by A. Rodriguez-Navarro and J. Schroeder).

For transformations W303-1A and K616 were grown in standard YPD media supplemented with 10 mM CaCl2 for K616 cells; G19 cells were grown in synthetic media minus histidine. Yeast were transformed with either pYX112 vector alone, pYX-ACA2-1, or pYX-ACA2-2 by the LiOac/PEG method (29, 30) and selected for uracil prototrophy by plating on synthetic medium minus uracil (SC-URA): 6.7 g/liter yeast nitrogen base without amino acids, 2 g/liter of drop-out mix without uracil, 2% glucose or sucrose as a carbon source, and 1.5% agar (31). The Ura+ colonies were picked and grown for 2–3 days on SC-URA agar plates. For complementation studies, colonies were streaked again on SC-URA agar plates containing 10 mM EGTA, pH 6.0, and incubated for 2–3 days at 30 °C as described previously (11).

**Isolation of Yeast Membranes**—Yeast cells were homogenized and fractionated on a continuous gradient of 20–60% sucrose with modifications of Villalba et al. (32). A 500-ml culture of yeast was grown to log phase with shaking at 30 °C, harvested, washed with cold H2O, and processed for a single sucrose gradient. Twelve 1-ml fractions were collected from the top, frozen in liquid N2, and stored at −70 °C until use.

**ATPase Assays**—ATPase assays were conducted with modifications of Baginsky et al. (33). Samples (10–15 µl) were assayed at 22 °C in a buffer of 20 mM MOPS, 8 mM MgSO4, 50 mM KNO3, 0.25 mM K2MoO4, 5 mM NaN3, and 1 mM EGTA (to chelate free Ca2+). Ca2+ stimulation was tested by the addition of 1.1 mM CaCl2. Vanadate was added to some reactions at 0.1 mM. Calmodulin (bovine brain or spinach) was obtained from Sigma.

**RESULTS**

**Isolation of ACA2 cDNA**—ACA2 was identified as a full-length cDNA in a λ-Zap-based cDNA library (18) and subcloned as a plasmid pACA2 (Fig. 2). To identify this cDNA we used a partial genomic fragment of ACA2 as a hybridization probe. This genomic fragment was obtained by two sequential rounds of PCR amplification (i.e. nested PCR) using degenerate primers. In both reactions the 3′ primer corresponded to a sequence conserved in all P-type ATPases (MTGDDGVNDA) (primer number 1025) (34). In the first reaction the 5′ primer (PEGL) corresponded to a sequence conserved in Na+ and Ca2+-pumps (A/IV/PEGLP). In the second reaction we used a nested 5′ primer corresponding to a more generally conserved sequence (CSDKTGTLT) (34). A critical feature of our strategy was the PEGL primer which biased reactions into amplifying putative Ca2+-pumps. Without such a bias, the primary reaction products were fragments of protein pump genes, perhaps reflecting the large size of this gene family in Arabidopsis (34).

**Primary Structure Shows Unique N-terminal Domain**—Fig. 3 shows the predicted 110-kDa protein encoded by ACA2. The cDNA sequence contained a single long open reading frame with an in-frame stop codon upstream of the predicted start codon. ACA2p shows greatest identity to a subfamily of PM-type Ca2+-ATPases, including ACA1p/PEA1p from Arabidopsis (12) (78% identity), BCA1p from Brassica (13) (62% identity) (shown aligned in Fig. 3). ACA2p shows approximately 44% identity to a mammalian PM-type Ca2+-ATPase, but less than 32% identity to ER-type Ca2+-ATPases, including the rabbit SERCA1p (35) or Arabidopsis ACA3p/ECAlp (11).

Ten transmembrane domains are predicted for ACA2p based on programs predicting hydrophathy and topology (Predict protein and PSORT programs) (36, 37). The N- and C-terminal ends of ACA2p are predicted on the cytoplasmic side of the membrane. However, the N-terminal domain contains an additional 19-residue hydrophobic segment from Ile63 to Ser82, which may provide an 11th transmembrane domain. The C-terminal end does not show a putative ER retention motif (KXXK) (38, 39) as seen for the plant ER-type Ca2+-ATPases. In comparison to animal homologs the most distinct feature of ACA2p is the absence of a long C-terminal regulatory domain. Instead, there is a unique, relatively long N-terminal domain of 160 residues, compared with 94 residues for a typical mammalian pump (e.g. hPMCA2p) (40). It is possible to align ACA2p with hPMCA2p starting around position Glu87, but the identity up to the first transmembrane remains poor (22%).

**ACA2p Is Most Abundant in Roots and Flowers**—To determine the relative tissue distribution of ACA2p, we produced a polyclonal antibody (anti-ACA2) against a 43-residue sequence in the N-terminal domain (Val112–Phe161). This region shows high variability, with only 56% identity to ACA1p. This variable region was used in hopes of generating an isomeric specific antibody.

Fig. 4 shows a Western blot analysis of total proteins extracted from different tissues. Anti-ACA2 specifically detected a 110-kDa protein, most abundant in roots and flowers. Cross-

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**FIG. 2. Diagram of clone pACA2-wt.** Diagnostic and useful restriction enzyme sites within the cDNA sequence are shown above. Restriction sites from the vector polylinker are shown below. During the deletion of the 5′-untranslated sequence, *Bat*FI and *Sac*II sites were introduced in the coding region as silent mutations. *S81* marks the penultimate residue in the N-terminal deletion mutant encoded by pACA2-2. The cross-hatched region corresponds to the position of the peptide sequence used to generate ACA2 antisense.
reaction of anti-ACA2 with other Arabidopsis isoforms (e.g. ACA1p) has not been tested. However, no cross-reaction was observed with proteins extracted from tobacco or onions (data not shown), suggesting that this antiserum is highly specific.

ACA2p Is an Endomembrane Pump—To test the hypothesis that ACA2p is a plasma membrane pump, we fractionated microsomal membranes by an aqueous two-phase partitioning procedure (Fig. 5). Plasma membranes partitioned to the upper phase, as shown by immunodetection of a plasma membrane H^+\text{-ATPase} marker. In contrast, ACA2p partitioned to the lower phase, indicating an endomembrane localization. In controls, an ER-type Ca^{2+}-ATPase (ACA3p/ECA1p) also partitioned to the lower phase (not shown). Endomembrane localization was confirmed by showing immunodecoration of subcellular structures using confocal microscopy on root tip cells (not shown).

ACA2-2p Complements a Disruption of Yeast Ca^{2+} Pumps—To genetically test for Ca^{2+} pump activity, ACA2p was expressed in a mutant yeast strain, K616. K616 (pmr1 pmc1 cnb1) has a disruption of three genes involved in Ca^{2+} homeostasis, PMR1, PMC1, and CNB1. PMR1 and PMC1 encode Ca^{2+}-ATPases in the yeast vacuole and Golgi apparatus, respectively (41, 42), and CNB1 encodes a Ca^{2+}-dependent phosphatase (calcineurin), which regulates a H^+\text{/Ca^{2+}}-exchanger (43). Due to the disruption of the yeast Ca^{2+} pumps, this strain displays poor growth on Ca^{2+}-depleted medium (+10 mM EGTA, pH 6.0). ACA2p was expressed as a full-length protein, ACA2-1p (construct pYX-ACA2-1), and an N-terminally truncated mutant, ACA2-2p (construct pYX-ACA2-2). Both ACA2-1 and -2 were cloned in a yeast vector (pYX112) to provide a moderate level of constitutive expression from a microsomal membranes by an aqueous two-phase partitioning procedure (Fig. 5). Plasma membranes partitioned to the upper phase, as shown by immunodetection of a plasma membrane H^+\text{-ATPase} marker. In contrast, ACA2p partitioned to the lower phase, indicating an endomembrane localization. In controls, an ER-type Ca^{2+}-ATPase (ACA3p/ECA1p) also partitioned to the lower phase (not shown). Endomembrane localization was confirmed by showing immunodecoration of subcellular structures using confocal microscopy on root tip cells (not shown).

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triose phosphate isomerase promoter (Novagen).

In controls, wild type yeast transformed with vector alone grew on Ca\(^{2+}\)-depleted medium, while the K616 mutant transformed with the vector alone did not (Fig. 6). When the K616 host was transformed with pYX-ACA2-1 or -2, only the truncation mutant was able to restore growth. The expression levels for both ACA2-1p and ACA2-2p were comparable, as indicated by immunodetection of a 110- and 102-kDa polypeptide, respectively, using anti-ACA2 antisera (not shown).

ACA2-1p and ACA2-2p Have Ca\(^{2+}\)-ATPase Activity—To biochemically determine the activity of ACA2p, we used sucrose gradients to fractionate yeast microsomal membranes containing either ACA2-1p or ACA2-2p. As a control we fractionated membranes from yeast transformed with a vector only. Sucrose gradients were analyzed for ATPase activities in four separate experiments with equivalent results. Approximately equal amounts of protein were loaded on each gradient.

Results from a typical set of fractionations are shown in Fig. 7. All gradients showed a peak of vanadate-sensitive ATPase activity (Ca\(^{2+}\)-independent) at approximately 48% sucrose, consistent with the expected position of the plasma membrane \(H^+\)-ATPase (23). In the vector-only control, there was very little Ca\(^{2+}\)- or Ca\(^{2+}\)/calmodulin-dependent ATPase, consistent with the reported low levels of endogenous Ca\(^{2+}\) pump activity in yeast (41).

In contrast to the vector-only control, gradients containing ACA2-1p and -2p always showed a broad peak of Ca\(^{2+}\)–stimulated activity between 30 and 45% sucrose (fractions 4–7). The Ca\(^{2+}\)-stimulated activity was consistently higher (3–7-fold) in gradients containing the truncation mutant ACA2-2p, compared with the full-length ACA2-1p. However, only the full-length protein displayed significant calmodulin stimulation (around 5-fold).

To confirm that ACA2-1p could be activated by Ca\(^{2+}\)/calmodulin, samples from fraction 5 were assayed with increasing concentrations of calmodulin (Fig. 8). Calmodulin activated ACA2-1p in a dose-dependent fashion, with a half-maximal activation around 30 nM. Calmodulin activation was Ca\(^{2+}\)-dependent and vanadate-sensitive, as shown by controls assayed in the presence of 100 nM calmodulin with 1 mM EGTA or 0.1 mM vanadate, respectively (not shown). In parallel, the addition of up to 100 nM Ca\(^{2+}\)/calmodulin failed to stimulate activity from the truncation mutant or vector only control.

In the detailed analysis shown in Fig. 8, the truncation mutant showed a higher specific activity than the full-length protein, in the presence or absence of calmodulin. In this assay there was less truncation mutant protein than full-length enzyme, as determined by immunodetection on three independent Western blot analyses (e.g. see Fig. 9A). Therefore, the truncation mutant appears more active than the full-length protein on a per mole of enzyme basis. This higher relative activity was observed in all four sucrose gradient fractionations.

To confirm that a deletion of the N-terminal end in ACA2-2p did not dramatically alter its subcellular localization in yeast, a mixture of microsomal membranes containing ACA2-1p and ACA2-2p were fractionated on the same gradient, and a Western blot analysis was used to analyze their fractionation profile. A control shown in Fig. 9A demonstrates that ACA2-1p and ACA2-2p can be mixed and still resolved as separate bands by SDS-PAGE. Fig. 9B shows that the two proteins cofractionate when run as a mixture on the same gradient. Cofractionation was tested in two separate experiments and is consistent with results obtained from each enzyme fractionated separately. The peak fraction for both ACA2-1p and ACA2-2p was
between 31 and 45% sucrose, consistent with the broad peak determined by ATPase activity measurements.

The N-terminal Domain Contains a Calmodulin-binding Sequence—To determine if the N-terminal domain contained a calmodulin-binding sequence, we performed binding assays on a series of fusion proteins (e.g. MC2(1–72)p) (Fig. 10). In Fig. 11, a calmodulin gel overlay analysis shows that calmodulin binds to a control MBP or GST, nor to a fusion protein containing residues 1–19. This suggests that the calmodulin binding sequence lies within the first 36 residues, and probably downstream of residue 19.

FIG. 8. Calmodulin activation of ATPase activity for ACA2-1p. Samples from fraction 5 were analyzed in detail for calmodulin-stimulated activity. Relative amounts of ACA2-1p and ACA2-2p in each assay are shown in Fig. 9A, as detected on a Western blot by anti-ACA2 serum. ATPase assays included 100 μM free Ca2+. Calmodulin was varied from 0 to 2000 nM. A dotted line indicates the estimated 50% activation point. Assays were done with 1–7 μg of membrane protein with no significant difference observed for the estimated 50% activation point. 50% activation occurred between 20 and 50 nM calmodulin with three different enzyme preparations tested. We estimated ACA2-1p in our assays to be significantly less than 2 nM (based on the observation that ACA2-1p appears to represent less than 1% of the membrane protein, as indicated by the failure to detect ACA2-1p in a Coomassie-stained SDS-PAGE gel).

DISCUSSION

Two lines of evidence indicate that ACA2 encodes a Ca2+-ATPase most similar to a PM-type Ca2+-ATPase (type IIB). First, ACA2p has 44% sequence identity with PM-type Ca2+-ATPases and only 32% identity with an ER-type Ca2+-ATPase. Second, ACA2p displayed calmodulin-stimulated activity, a biochemical hallmark of PM-type Ca2+-ATPases. Nevertheless, we show here that ACA2p is fundamentally distinct in two respects. First, ACA2p is localized to an endomembrane system (i.e. not the plasma membrane), as shown by aqueous two-phase partitioning of microsomal membranes (Fig. 5). Second, ACA2p is regulated by an autoinhibitor located in the N-terminal instead of C-terminal domain, as shown by (i) genetic complementation studies in yeast (Fig. 6), (ii) ATPase activity assays on full-length and truncated pumps (Fig. 8), and (iii) identification of a calmodulin-binding sequence in the N-terminal instead of C-terminal domain (Figs. 10 and 11). Thus, our data provide strong support for the hypothesis (13) that ACA2-like pumps constitute a unique subfamily of calmodulin-regulated Ca2+-ATPases, distinguished by their novel structural arrangement and their presence in non-plasma membrane locations.

Yeast Complementation—The expression of ACA2 in yeast (mutant K616) provides genetic evidence that ACA2 encodes a Ca2+-pump. Expression of ACA2-2p (N-terminal truncation mutant) allowed the K616 strain to grow on Ca2+-depleted medium. K616 (pnc1 pnr1 cnb1) contains a disruption of the endogenous Ca2+-pumps, PMC1 and PMR1, as well as calbinurin (CNB1), a Ca2+-stimulated phosphatase (43). K616 cells fail to grow on Ca2+-depleted medium, presumably because they cannot scavenge sufficient Ca2+ into an endomembrane compartment. Thus, the ability of ACA2-2p to restore growth on Ca2+-depleted medium is consistent with the hypothesis that it can function as a high affinity endomembrane Ca2+-pump.

Interestingly, complementation of the K616 conditional growth phenotype was observed only with the truncation mutant, and not the full-length enzyme. We offer two explanations. First, the truncated pump may provide more activity in vivo than the full-length pump. This hypothesis is consistent with in vitro ATPase assays showing that the truncated pump is comparatively more active, even when the full-length pump is fully stimulated by calmodulin. In addition, it is likely that the full-length enzyme is normally autoinhibited in vivo, since its activation by Ca2+/calmodulin would require continuously high levels of cytosolic Ca2+, a condition not expected for K616 cells grown on Ca2+-depleted medium. However, even if high concentrations of cytosolic Ca2+ occur, the full-length pump may still remain unactivated if (i) levels of the endogenous yeast calmodulin are too low, or (ii) the yeast calmodulin cannot functionally interact with the plant pump. Our second alternative explanation is that only the truncated pump may be targeted to the proper functional location to permit complementation. Although a targeting problem remains a formal possibility, both truncated and full-length pumps co-fractionated to similar densities on the same sucrose gradient, suggesting a similar subcellular localization. Nevertheless, the sensitivity of this fractionation assay may not have detected a small, but functionally important difference in their subcellular localization.

Ca2+-ATPase Activity—Biochemical ATPase assays on full-length ACA2-1p demonstrate that ACA2 encodes a calmodulin-regulated Ca2+-ATPase. Using yeast membranes fractionated by a sucrose gradient, the activity associated with the full-length ACA2-1p was shown to be Ca2+/calmodulin-dependent, displaying half-maximal activation around 30 nM calmodulin.
Ca\textsuperscript{2+}-ATPase Isoform ACA2

**Fig. 10.** N-terminal domain fusion proteins used to show a calmodulin-binding sequence within the first 36 residues. Fusion proteins diagrammed here were tested for calmodulin binding as shown in Fig. 11. A portion of the protein sequence is shown for two fusions, MC2(1–72)p and GC2(1–58)p. MC2(1–72)p stands for Maltose-binding protein fusion with Ca\textsuperscript{2+}-pump ACA2 residues 1–72. In GC2(1–58)p the G denotes a glutathione S-transferase fusion protein. Markings on top of the sequences (left) indicate the positions of factor X and thrombin protease sites, respectively. A series of deletions were made to delineate the minimal sequence required for calmodulin binding. The truncation points in GC2-deletion constructs are indicated by a number under GC2(1–58)p. These fusions have an ACA2p sequence sandwiched between an N-terminal GST and a C-terminal GFP. They were made by an ExoIII deletion strategy which resulted in slightly different linkers between the ACA2p sequence and GFP, as shown at the right (GFP sequence shown in italic lowercase letters). Black filling, fusion proteins displayed strong Ca\textsuperscript{2+}-dependent calmodulin-binding; no filling, no calmodulin binding. The shortest construct to bind calmodulin was GC2(1–36). A putative binding site is marked by a bracket on top of the MC2(1–72)p sequence.

**Fig. 11.** Calmodulin gel overlays show calmodulin binding to a sequence in the N-terminal domain of ACA2p. Fusion proteins were purified and subjected to SDS-PAGE (10% gel), transferred to nitrocellulose, and probed with 60 nM biotinylated calmodulin (52). Bound calmodulin was detected by ECL and exposure to x-ray film. The top panel shows a Coomassie-stained gel of the fusion proteins. Calmodulin-probed blots are shown below. No binding was observed in the absence of Ca\textsuperscript{2+}. A description for each fusion is provided in Fig. 10. Although the MC2(1–72)p fusion is smaller than the GC2 fusions, it actually contains the longest N-terminal sequence. Together these data indicate that the N-terminal domain contains a calmodulin-regulated autoinhibitor and its removal results in a constitutively active enzyme. We speculate that the deregulated activity associated with ACA2-2p allowed the K616 yeast to grow on Ca\textsuperscript{2+}-depleted medium. Activation of ACA2p by an N-terminal truncation mutation appears to be functionally analogous to a C-terminal truncation of hPMCA4p (47), a typical PM-type Ca\textsuperscript{2+} pump. Both truncations produced highly active pumps which were calmodulin-independent. The development of the ACA2-2 truncation mutant provides the potential to constitutively activate a specific Ca\textsuperscript{2+}-transport pathway in transgenic plants. An interesting question is whether such a dominant mutant would alter Ca\textsuperscript{2+} signal transduction, or disrupt the function of a specific organelle.

**N-terminal Calmodulin Binding Autoinhibitor**—Calmodulin binding assays demonstrate that the N-terminal domain of ACA2p contains a calmodulin-binding sequence. Calmodulin binding to a fusion protein containing only the first 36 residues of ACA2p was Ca\textsuperscript{2+}-dependent. Within this region there is one sequence (Val\textsuperscript{20}–Val\textsuperscript{31}) with a reasonable potential to form an amphipathic basic helix, typical of many calmodulin-binding sequences (48). However, a complete binding sequence may include additional downstream residues. In the N-terminal domain of BCA1p, an analogous binding site has been proposed. However, the BCA1p sequence displayed a Ca\textsuperscript{2+}-independent interaction with calmodulin, as detected using a 25-residue peptide spotted on nitrocellulose (13). Although such Ca\textsuperscript{2+}-independent binding may be an artifact of using a short peptide, it may also reflect a functional difference between the putative binding sequences in BCA1p and ACA2p. In plants, evidence for pumps with both low and high affinity calmodulin-binding sequences have been reported (14, 46, 49).

Our findings demonstrate that P-type ATPases can be regulated by autoinhibitors located at either N or C termini. In general, autoinhibitors may function by: 1) directly binding to an active site on the enzyme and thereby sterically blocking access of a substrate (intrasteric inhibition), or 2) binding or altering the structure of a region removed from the active site and locking the enzyme in an inactive conformation. In a typical PM-type Ca\textsuperscript{2+}-ATPase the C-terminal autoinhibitor is thought to interact with multiple regions, including the central cytoplasmic loop (15). However, this need not be the case for the ACA2 N-terminal autoinhibitor. If a portion of the N-terminal domain is also part of the core enzyme structure, then an N-terminal autoinhibitor may directly regulate that compo-
Ca²⁺-ATPase Isoform ACA2

For example, the N-terminal domain of many ATPases is thought to control access of ions to the channel domain (50). Thus, it might be possible to regulate the enzyme activity by converting this N-terminal structure into a regulated ion gate. This raises the question of whether ACA2p employs a novel strategy of autoinhibition, made possible by its unique structural arrangement.

In a comparison of primary structures, we failed to identify any significant identities between the N-terminal domain of ACA2p and the C-terminal regulatory domain of a true PM-type Ca²⁺-ATPase. Thus, it is unclear if the two regulatory domains evolved independently or diverged after a domain swapping event in a common ancestor. With ACA2 as a possible exception, there has been no other evidence for “domain shuffling” in P-type ATPases (51). Regardless of origin, both domains function as calmodulin-regulated autoinhibitors, a comparative investigation into their structure and function should provide valuable insights into the regulation of calmodulin-dependent Ca²⁺ pumps.

Function—The biological functions of ACA2p are not known. Since ACA2p is activated by calmodulin, it may provide a Ca²⁺-regulated feedback mechanism to control Ca²⁺ levels in the cytoplasm. It may regulate Ca²⁺ levels within a specific organelle, such as a plastid, Golgi apparatus, or vacuole. In contrast to animal cells, plant cells appear to have calmodulin-regulated organelles, such as a plastid, Golgi apparatus, or vacuole. In addition to the plasma membrane (7). Whether the plant plasma membrane-localized pumps are also members of this family remains an open question whether plant cells function with two distinct types of calmodulin regulated isoforms.

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A Novel Calmodulin-regulated Ca\(^{2+}\)-ATPase (ACA2) from Arabidopsis with an N-terminal Autoinhibitory Domain
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