Structural Determinants of Ca\(^{2+}\) Transport in the *Arabidopsis* H\(^{+}/Ca^{2+}\) Antiporter CAX1*

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Ca\(^{2+}\) levels in plants, fungi, and bacteria are controlled in part by H\(^{+}/Ca^{2+}\) exchangers; however, the relationship between primary sequence and biological activity of these transporters has not been reported. The *Arabidopsis* H\(^{+}/\)cation exchangers, CAX1 and CAX2, were identified by their ability to suppress yeast mutants defective in vacuolar Ca\(^{2+}\) transport. CAX1 has a much higher capacity for Ca\(^{2+}\) transport than CAX2. An *Arabidopsis thaliana* homolog of CAX1, CAX3, is 77% identical (93% similar) and, when expressed in yeast, localized to the vacuole but did not suppress yeast mutants defective in vacuolar Ca\(^{2+}\) transport. Chimeric constructs and site-directed mutagenesis showed that CAX3 could suppress yeast vacuolar Ca\(^{2+}\) transport mutants if a nine-amino acid region of CAX1 was inserted into CAX3 (CAX3-9). Biochemical analysis in yeast showed CAX3-9 had 36% of the H\(^+/Ca^{2+}\) exchange activity as compared with CAX1; however, CAX3-9 and CAX1 appear to differ in their transport of other ions. Exchanging the nine-amino acid region of CAX1 into CAX2 doubled yeast vacuolar Ca\(^{2+}\) transport, but did not appear to alter the transport of other ions. This nine-amino acid region is highly variable among the plant CAX-like transporters. These findings suggest that this region is involved in CAX-mediated Ca\(^{2+}\) specificity.

Modulation of cytosolic Ca\(^{2+}\) levels is essential for adapted physiological responses (1–3). The transient Ca\(^{2+}\) signal is determined by two opposing fluxes, Ca\(^{2+}\) influx via channels and Ca\(^{2+}\) efflux via active transporters (4). The mechanisms of Ca\(^{2+}\) channel function and Ca\(^{2+}\) pumps that are directly energized by ATP hydrolysis (Ca\(^{2+}\)-ATPase) have been previously investigated (5, 6); however, almost no work has been focused on the mechanisms of H\(^{+}\)-coupled Ca\(^{2+}\) antiport, which is driven by a proton electrochemical gradient (H\(^{+}\)/Ca\(^{2+}\) exchange).

H\(^{+}/Ca^{2+}\) antiporters have been cloned from bacteria, fungi, and plants (7–9). H\(^{+}/Ca^{2+}\) antiporters in general have 10–14 membrane-spanning domains with about 400 amino acid residues (10). The H\(^{+}/Ca^{2+}\) antiporters contain a central hydrophilic motif rich in acidic amino acid residues (the acidic motif) that bisects the polypeptide into two groups of approximately equal length; however, little is known regarding the relationship between the primary sequence of any of these proteins and their biochemical properties. The *Escherichia coli* H\(^{+}/Ca^{2+}\) antiporter, chaA, contains an acidic motif possessing striking sequence similarity to the calcium-sequestering protein calsequestrin (7). However, this region has never been shown to mediate Ca\(^{2+}\) transport. In the yeast vacuolar H\(^{+}/Ca^{2+}\) antiporter, VCX1, amino acid residues within membrane-spanning domains appear to help mediate ion transport. A glycine to alanine change in the C-terminal membrane-spanning domain may modify H\(^+/Ca^{2+}\) transport (8). In another study, a point mutation in a central membrane-spanning domain has been implicated in altering the Mn\(^{2+}\) transport of VCX1 (11). In sum, these findings only hint at structural motifs involved in H\(^{+}/\)cation specificity.

Two *Arabidopsis thaliana* H\(^{+}/Ca^{2+}\) transporters, CAX1 (cation exchanger 1), and CAX2, were identified (9, 12) by their ability to sequester Ca\(^{2+}\) into yeast vacuoles in *Saccharomyces cerevisiae* mutants deleted for the vacuolar Ca\(^{2+}\)-ATPase (PM1C1) and H\(^{+}/Ca^{2+}\) antiporter. Experiments utilizing vacuolar membranes from yeast cells expressing CAX1 or CAX2 demonstrate that CAX1 is a H\(^{+}/Ca^{2+}\) exchanger and CAX2 is a H\(^{+}/\)heavy metal cation antiporter (9). The structural basis for the different cation transport properties between these gene products is difficult to discern in yeast because both gene products suppress yeast mutants defective in vacuolar Ca\(^{2+}\) transport.

A CAX homolog has recently been cloned from *Arabidopsis*. The putative protein is 77% identical (93% similar) to CAX1 and has been previously termed AtHCX1 for *A. thaliana* homolog of CAX1 (13). Recently the name of this gene was changed to CAX3 (10). CAX3 is more similar to CAX1 than CAX2 is to CAX1. Unlike CAX1 and CAX2, CAX3 fails to suppress yeast mutants defective in vacuolar Ca\(^{2+}\) transport despite high level expression of the protein (13). The *Arabidopsis* genome contains numerous open reading frames that are similar to these CAX genes and apparently do not function in yeast vacuolar Ca\(^{2+}\) transport (10, 12). The CAX homologs, particularly CAX3, in conjunction with yeast functional expression analysis, may serve as valuable tools with which to dissect the structural determinants of CAX1-mediated Ca\(^{2+}\) specificity.

The utility of yeast as an experimental tool to study amino acid residues involved in ion specificity is well documented (14). To identify Ca\(^{2+}\) domains within the CAX gene family, we localized CAX3 and CAX1 in yeast. We then interchanged regions of CAX1 and CAX3 and assayed for function. We mapped the minimal changes in CAX3 that confer yeast vacuolar H\(^{+}/Ca^{2+}\) exchange. Similar modifications in CAX2 increased the Ca\(^{2+}\) transport properties of this protein. These

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findings represent the first clues as to the structural basis of a new method described in detail elsewhere (18). All polymerase chain reaction-derived DNA clones were sequenced. The primers used for site-directed mutagenesis using a QuikChange kit (Stratagene). Bases to be altered are shown in bold.

a Mutagenesis using primers with a class 2S restriction enzyme site (18). Double underlines indicate the coding sequences of CAX1 and CAX3 were divided into four regions defined by unique internal restriction sites and the restriction sites on the vector. The chimeric clones were mobilized into the yeast expression vector and transformed into yeast.

Site-directed Mutagenesis—Site-directed mutagenesis was performed using a QuikChange site-directed mutagenesis kit (Stratagene) and a new method described in detail elsewhere (18). All polymerase chain reaction-derived DNA clones were sequenced. The primers used in the study are shown in Table I. For the names of the resulting clones, see Fig. 2.

Construction of Hemagglutinin (HA)1-tagged CAX1 and CAX3—The HA epitope (YPYDVPDYA) (8) was amplified by polymerase chain reaction with ligated in-frame oligonucleotides of CAX1 and CAX3 sequence.

Membrane Fractionation and Subcellular Immunolocalization—Preparation of vacuolar membrane-enriched vesicles for protein blot analyses was based on the method by Liang and Sze (19). The gradients and fraction density were prepared as previously described (20, 21, 23). Ten micrograms of protein from each fraction was separated on a polyacrylamide gel and blotted as previously described (13). Monoclonal mouse IgG against HA (Berkeley Antibody Co., Richmond, CA), yeast vacuolar alkaline phosphatase (Molecular Probes, Eugene, OR), and yeast dolichol phosphate mannose synthase (an endoplasmic reticulum indicator, Molecular Probes) were used at a 1:3000, 1:300 and 1:250 dilution, respectively. Affinity-purified polyclonal antibody against the CAX proteins in yeast, total proteins were isolated from pmc1::TRP1 vcx1::LEU2 (cxl1) was the yeast strain used to express chimeric and mutant genes (9). The plasmids were propagated in either pBluescript (Stratagene, La Jolla, CA) or pGEM-Zf+ (Promega, Madison, WI), and inserts were transferred to the shuttle vector pHiGpd (15) for their expression in the yeast. The plasmids were introduced into yeast by lithium acetate/polyethylene glycol transformation (16), and standard techniques were used to manipulate DNA (17).

Construction of Chimeric Genes—The double-underlined sequences are a BspMI, a class 2S restriction enzyme site.

a Mutagenesis using a QuikChange kit (Stratagene). Bases to be altered are shown in bold.

b Double underlines indicate the last 6 amino acids of the 9 amino acid region of CAX3 replaced with the equivalent from CAX1.

c First 6 amino acids of the 9 amino acid region of CAX3 replaced with the equivalent from CAX1.
Ca\textsuperscript{2+} transporters despite high level expression of CAX3 in these yeast cells (13). Potentially, CAX3 and CAX1 may differ in their biochemical properties in yeast because of localization on different membrane fractions. To test this hypothesis, we HA-tagged both CAX1 and CAX3 at similar regions of the N terminus. The HA-CAX1 suppressed yeast mutants defective in vacuolar Ca\textsuperscript{2+} sequestration, and HA-CAX3 expressed protein of the predicted molecular weight (data not shown). The colocalization of CAX1 and CAX3 is supported by the comigration of the two proteins in sucrose density gradients of membrane preparations obtained from yeast cells expressing the tagged proteins (Fig. 1). The sedimentation behavior of the membrane fraction containing both proteins is consistent with that of a vacuolar compartment. The two expressed plant transporters co-migrate with the yeast vacuolar marker alkaline phosphatase.

Identification of Domains in CAX1 That Confer Vacuolar Ca\textsuperscript{2+} Transport Properties on CAX3—CAX1 and CAX3 are highly similar throughout their deduced amino acid sequences. Thus, we made a series of chimeric constructs between the two open reading frames to determine which domains conferred the different properties of vacuolar Ca\textsuperscript{2+} transport. The CAX variants used in this study do not contain the first 36 amino acids of the CAX open reading frame. The CAX1 and CAX2 open reading frames initially isolated by their function in yeast and used in this study do not contain this 36-amino acid N-terminal region. Compared with this CAX1 variant, the N terminus of the CAX3 open reading frame contains additional 36 amino acids. If we delete this region, CAX3 still does not suppress the yeast mutants defective in vacuolar Ca\textsuperscript{2+} transport (Fig. 2A, 1). The chimeric constructs were created with the N-terminal 36 amino acids of CAX3 not present. Thus, the CAX1 and CAX3 constructs used here initiate at amino acid 37. The N terminus of CAX1 and CAX3 appeared to convey the different properties of Ca\textsuperscript{2+} transport (Fig. 2A, 2–5). The N terminus of CAX1 fused in-frame to CAX3 (L87I, A88C, N89T) conferred vacuolar Ca\textsuperscript{2+} transport properties onto K667 yeast cells (deleted in the yeast vacuolar Ca\textsuperscript{2+}-ATPase and vacuolar H\textsuperscript{+}/Ca\textsuperscript{2+} exchanger). Alternatively, if the CAX3 N terminus was fused in-frame to CAX1 (CAX3-I), K667 strains expressing this transporter did not suppress the Ca\textsuperscript{2+} sensitivity. If the central or C terminus of CAX3 was fused in-frame to CAX1 (CAX3-I/CAX1-9), the phenotype of K667 cells expressing these hybrid proteins was indistinguishable from CAX1-expressing cells. Conversely, if the central or C terminus of CAX1 was fused in-frame to CAX3 (CAX3-9/CAX1-9), these hybrid proteins did not suppress the K667 cells. In total, these findings showed that a region at the N terminus of these open reading frames determined the differences in vacuolar Ca\textsuperscript{2+} transport.

We then took the N terminus of CAX1 and CAX3 and divided this region in two. We demonstrated that the region between amino acids 74 and 149 defined the majority of the differences between CAX1 and CAX3. That is, if the region from 74–149 of CAX1 was fused in-frame to CAX3 (CAX3-9), this hybrid transporter suppressed the K667 yeast cells Ca\textsuperscript{2+} sensitivity (Fig. 2A, 4). Meanwhile, if the region from 37 to 73 of CAX1 was fused in-frame to CAX3 open reading frame (CAX3-9), the hybrid CAX3 protein did not strongly suppress the yeast mutant. Expression of this chimeric protein weakly suppressed the Ca\textsuperscript{2+} sensitivity of the yeast strains but only after multiple days of growth on the Ca\textsuperscript{2+}-containing media.

Site-directed Mutagenesis of CAX3—Once we had defined the region between 74 and 149 as important for vacuolar Ca\textsuperscript{2+} transport, we decided to specifically exchange the polymorphic 9-amino acid regions (amino acid residues from 87–95) in CAX1 and CAX3. The nine-amino acid region of CAX1 fused in-frame to CAX3 (termed CAX3-9) conferred Ca\textsuperscript{2+} suppression when expressed in yeast mutants defective in vacuolar Ca\textsuperscript{2+} transport (Fig. 2A, 5). The nine-amino acid region of CAX3 fused in-frame to CAX1 (termed CAX1-9) inhibited ability of CAX1 to suppress the K667 Ca\textsuperscript{2+}-sensitive phenotype. We have termed this nine-amino acid region of CAX1 the “Ca\textsuperscript{2+} domain.”

We proceeded to focus on CAX3. Expression of CAX3 chimeras containing the first six amino acids from the CAX1 Ca\textsuperscript{2+} domain of CAX1 suppressed the K667 Ca\textsuperscript{2+} sensitivity; however, CAX3 chimeras expressing the last six amino acids of the Ca\textsuperscript{2+} domain of CAX1 did not suppress the yeast mutant (data not shown). Expression of a CAX3 hybrid that expressed the first three amino acids from the CAX1 Ca\textsuperscript{2+} domain (termed CAX3-ICT) suppressed the yeast mutant (Fig. 2A, 6). This suppression was not as pronounced as that seen with the exchange of the entire nine-amino acid region; it usually took 2 days to observe significant colony growth on Ca\textsuperscript{2+}-containing media. Meanwhile, expression of hybrid CAX3 containing the second three amino acids or the last three amino acids from the Ca\textsuperscript{2+} domain of CAX1 did not suppress the K667 strain. In total, these observations specifically identified a three-amino acid region as an important determinant for CAX3 vacuolar Ca\textsuperscript{2+} transport in yeast.

We proceeded to individually substitute each of the three CAX3 amino acids for the corresponding amino acids found in the CAX1 Ca\textsuperscript{2+} domain (L87I, A88C, N89T). Only the CAX3 variant containing the leucine-to-isoleucine change (termed CAX3-1) conferred Ca\textsuperscript{2+} tolerance on the K667 yeast strain (Fig. 2A, 7). This suppression was not as pronounced as that seen with the exchange of the entire 9-amino acid Ca\textsuperscript{2+} domain, but substantial growth could be seen after 2 days on the Ca\textsuperscript{2+}-containing media.

To demonstrate that the changes in Ca\textsuperscript{2+} transport were not due to alteration in protein expression or stability, we assayed the yeast strains for expression of the CAX transporters. All yeast cells transformed with various CAX constructs appeared to express approximately equal amounts of CAX protein (Fig. 2B, data not shown).

ΔpH-dependent Ca\textsuperscript{2+} Transport—The assay of yeast growth on Ca\textsuperscript{2+}-containing media does not allow us to make precise measurements about changes in H\textsuperscript{+}/Ca\textsuperscript{2+} antiport activity. CAX1 has been shown to restore vacuolar H\textsuperscript{+}/Ca\textsuperscript{2+} antiport activity to yeast strains deficient in this transporter (9). Presumably, CAX3 fails to suppress yeast strains deficient in

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Fig. 1. Subcellular fractionation of HA-tagged CAX1 and CAX3. The pmc1vcx1cnb yeast strain was transformed with HA-CAX1 or HA-CAX3 and grown in standard yeast extract-peptone-dextrose medium and lysed, and membranes were fractionated on a sucrose gradient (15–48%, w/w). Protein extracts (10 μg) were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Proteins were immunodetected with an immune serum specific for hemagglutinin, yeast vacuolar alkaline phosphatase (ALP), the yeast endoplasmic reticulum protein dolichol phosphate mannosyl synthase (DPMS), and the yeast plasma membrane H\textsuperscript{+}-ATPase, PMA1. The distribution of membrane marker proteins was identical in both transformed yeast strains (data not shown).

| % Sucrose | HA-CAX1 | HA-CAX3 | ALP | PMA1 | DPMS |
|----------|---------|---------|-----|------|------|
| 18       | 23      | 27      | 30  | 33   | 37   |
| 30       | 40      | 30      | 45  | 48   |      |
vacuolar Ca\(^{2+}\) transport due to the inability of this transporter to drive Ca\(^{2+}\) transport into the yeast vacuole. It stands to reason that variants of CAX3 that suppress the vacuolar Ca\(^{2+}\) transport-deficient strains do so by H\(^+/\)Ca\(^{2+}\) antiport activity. To directly test H\(^+/\)Ca\(^{2+}\) antiport activity, we isolated yeast endomembrane vesicles purified from vector control, CAX1-, CAX3-, CAX3-9- and CAX3-I-transformed K667 cells, and examined their capacity for \(\Delta pH\)-dependent Ca\(^{2+}\) uptake.

The addition of MgCl\(_2\)/ATP and establishment of a steady-state \(\Delta pH\) by the V-ATPase associated with the vacuolar membrane before the addition of Ca\(^{2+}\) resulted in uptake by membrane vesicles from the CAX1-, CAX3-9-, and CAX3-I-transformed cells (Fig. 3A). The CAX1-, CAX3-9-, and CAX3-I-expressing vesicles had the uptake capabilities of \(-2.60, 0.95\) (36.5% of CAX1), and \(0.19\) (7.1% of CAX1) nmol/mg of protein, respectively. Although the rates varied between various membrane preparations, the CAX3-9 yeast vesicles always had less than 40% of the Ca\(^{2+}\) uptake capacity compared with CAX1 (data not shown). The CAX3-expressing vesicles showed no H\(^+/\)Ca\(^{2+}\) antiport activity. In CAX1-, CAX3-9-, and CAX3-I-expressing K667 vesicles, the inclusion of gramicidin in the uptake medium decreased \(^{45}\)Ca\(^{2+}\) uptake to a level similar to that seen in the absence of MgCl\(_2\)/ATP (data not shown). The low rate of uptake found in vesicles from K667 cells transformed with control vector or CAX3 was not inhibited by gramicidin or by the V-ATPase inhibitor, bafilomycin (data not shown). These results together with the release of \(^{45}\)Ca\(^{2+}\) seen with the addition of the Ca\(^{2+}\) ionophore, A23187, to CAX1, CAX3-9, and CAX3-I vesicles versus the small increase seen in \(^{45}\)Ca\(^{2+}\) uptake with vector control and CAX3 vesicles demonstrate that CAX1-, CAX3-9-, and CAX3-I-generated uptake is concentrative.

Inhibition of \(\Delta pH\)-dependent Ca\(^{2+}\) uptake by other metals was compared between CAX1- and CAX3-9-expressing vesicles. CAX1 was significantly inhibited by the presence of excess Ca\(^{2+}\) and Cd\(^{2+}\) but not Mn\(^{2+}\), whereas CAX3-9 was only inhibited by Ca\(^{2+}\) (Fig. 3B).

Regardless of whether antiport activity was assayed as V-ATPase-dependent (bafilomycin A\(_1\)-sensitive) \(^{45}\)Ca\(^{2+}\) uptake (data not shown) or \(\Delta pH\)-dependent (gramicidin-sensitive) uptake, the initial rate for vacuolar membrane-enriched vesicles purified for CAX3-9- and CAX3-I-transformed vesicles increased as a simple hyperbolic (Michaelian) function of Ca\(^{2+}\) concentration to yield an apparent \(K_m\) value of \(20\pm 10\) \(\mu M\) Ca\(^{2+}\) (Fig. 4).

Altering the Ca\(^{2+}\) Transport Properties of CAX2—CAX2 suppresses yeast mutants defective in vacuolar Ca\(^{2+}\) transport but has a lower capacity for Ca\(^{2+}\) transport than does CAX1 (9). To ascertain whether this nine-amino acid Ca\(^{2+}\) domain confers different transport properties in vacuolar Ca\(^{2+}\) transport onto these transporters, we moved the nine-amino acid domain from CAX1 into CAX2 (termed CAX2-9). Yeast cells harboring the CAX2-9 gene grew better in Ca\(^{2+}\)-containing media than did CAX2-containing cells (Fig. 5). We then purified endomembrane vesicles from vector control, CAX2-, and CAX2-9-transformed K667 cells and examined their capacity for \(\Delta pH\)-dependent Ca\(^{2+}\) uptake. The yeast cells expressing CAX2-9 were capable of H\(^+/\)Ca\(^{2+}\) transport at a rate approximately twice...
that of yeast cells expressing CAX2 (Fig. 5B). We also verified that equal amounts of CAX protein were expressed in the CAX2-9- and CAX2-expressing cells (data not shown).

It has been previously demonstrated that CAX2 is able to transport Cd\(^{2+}\) and Mn\(^{2+}\) as well as Ca\(^{2+}\) (9, 22). Experiments were performed to determine whether the pH-dependent uptake of \(^{45}\)Ca\(^{2+}\) into CAX2-expressing endomembrane vesicles could be inhibited by excess concentrations of Ca\(^{2+}\), Cd\(^{2+}\), or Mn\(^{2+}\). CAX2-dependent Ca\(^{2+}\) uptake was significantly inhibited by all metals (Fig. 5C). To determine whether this inhibition was altered by swapping the nine-amino acid domain of CAX1 into CAX2, this experiment was repeated with endomembrane vesicles from CAX2-9-transformed cells.

Ca\(^{2+}\) Domains in the CAX-like Genes—The deduced amino acids that constitute this nine-amino acid Ca\(^{2+}\) domain populate one of the most polymorphic regions within the CAX group of putative transporters (Fig. 6) (13). None of the amino acids are identical in this region of CAX1, CAX2, and CAX3. The regions flanking this region are quite similar to each other within each region. In the first putative membrane-spanning domain, 35% (7/20) of the CAX1, CAX2, and CAX3 amino acids are identical. In the second putative membrane-spanning domain, 67% (10/15) are identical. However, this region is not the only domain that varies among the CAX open reading frames.
The region between the putative fifth and sixth membrane-spanning domain shares only 11% (2/18) identity among CAX1, CAX2, and CAX3. Furthermore, although the amino acids in the acidic motif are quite similar, they are less than 10% identical among these genes (13).

**DISCUSSION**

In plants, fungi, and bacteria control of cytosolic Ca\(^{2+}\) levels has been attributed in part to a low affinity, high capacity H\(^+\)/Ca\(^{2+}\) exchanger. These transporters have been biochemically characterized, and many of the open reading frames have been identified (7, 8, 10). Emerging genomic studies indicate common motifs among the characterized transporters and a burgeoning number of H\(^+\)/Ca\(^{2+}\) exchanger homologs (10). However, computer modeling studies, which attempt to ascribe transport function based on primary sequence information, are hindered by the lack of any biological studies focusing on the functional domains required for H\(^+\)/Ca\(^{2+}\) exchange. Utilizing the Arabidopsis CAX transporters and a robust yeast suppressor
sion screen, we have probed the structural basis of H+/Ca2+ exchange.

Recently, we have shown that CAX1 may contain an N-terminal autoinhibitory domain (23). CAX2 and CAX3 may also contain N-terminal autoinhibitory domains. Thus, there may be additional elements that modulate plant H+/Ca2+ exchange; however, in the experiments detailed here, we have uniformly used CAX variants (CAX1 37–463) that do not contain this regulatory region.

Utilizing a yeast screen, a region of 9 amino acids (residues 87–95 in the CAX1 construct utilized here) can account for a large portion of the vacuolar Ca2+ transport differences between CAX1 and CAX3 (Figs. 2–4). The fact that CAX1 and CAX3 both appear to be highly expressed in yeast and localize at the yeast vacuole (13) (Fig. 1) suggests that we have identified a domain involved in transport and not protein stability or membrane targeting. Furthermore, the chimeric constructs differed significantly in their transport properties (Figs. 2 and 3) but were all expressed at approximately equal levels (Fig. 2B). The acidic motif (residues 264–282 in CAX1), which has been implicated in Ca2+ transport (7), did not define the major difference between the Ca2+ transport properties of CAX1 and CAX3 (Fig. 2). In yeast assays, when the 9-amino acid region from CAX1 was mobilized into CAX3, the chimeric gene product had a low Km for Ca2+ and 36.5% of the Ca2+ transport capabilities of CAX1. The CAX3-9 construct differed from CAX1 in terms of ions that could compete with Ca2+ transport (Fig. 3B). Ca2+ uptake by CAX1 was inhibited by Cd2+ and Cd2+, indicating that it can be transported both of these metals, whereas it appears that CAX3-9 can transport Ca2+ but not Cd2+; this suggests that interchanging this domain does not significantly alter the transport of other ions. Although these experiments certainly imply that this region is important in Ca2+ specificity, the inability to fully confer CAX1-like phenotypes on CAX3 suggests that other regions in CAX1 affect some elements of Ca2+ transport. However, consistent with the premise that this region defines an important component of the Ca2+ recognition differences between CAX1 and CAX3, when the nine-amino acid region from CAX3 was placed into CAX1, it totally inhibited CAX1-mediated vacuolar Ca2+ transport (Fig. 2). Given these findings, we labeled this nine-amino acid region the Ca2+ domain.

The mechanism by which the Ca2+ domain regulates ion transport is unknown. This region appears to be a hydrophilic loop that separates membrane-spanning domains 1 and 2 (Fig. 6). The CAX1 hydrophobicity plot predicts that the N-terminal regulatory region and the Ca2+ domain are located on opposite sides of the membrane (orientation unknown). Furthermore, our preliminary results suggest that the N-terminal autoinhibitory domain does not directly interact with the Ca2+ domain. Thus, these two domains may act independently to regulate ion transport. Given the regulatory role of the N terminus, we favor a model in which the N terminus is cytoplasmic. This orientation would be consistent with the regulatory C terminus for animal Na+/H+ antiporters (24). With this membrane topology, the Ca2+ domain would be found inside the vacuole. Given that Ca2+ binding or transport sites are usually acidic amino acids and no acidic amino acids are within this domain, it is unlikely the Ca2+ domain directly binds Ca2+ ions. This region may restrict the movement of the transmembrane-spanning domains so that a Ca2+ ion can either enter or be blocked from the “pore.”

The modifications of the CAX2 Ca2+ domain further support the role of this region in Ca2+ selectivity. Expression of CAX2 in yeast demonstrates that this transporter has a lower capacity for Ca2+ transport than does CAX1 (9). In our assay conditions in yeast, we found the apparent Km for Ca2+ of both CAX1 and CAX2 to be $20 \pm 10 \mu M$ (data not shown); thus, the difference between these transporters appears to lie in their capacity to transport Ca2+. In plants, CAX2 localizes to the plant vacuolar membrane and transports metals and Ca2+ into the vacuoles (22). When the Ca2+ domain of CAX1 was fused to CAX2, the result was an increase in vacuolar Ca2+ transport with no noticeable change in transport of other ions (Fig. 5).

A single leucine-to-isoleucine change in the Ca2+ domain allowed the CAX3 gene product to weakly suppress the yeast vacuolar transport mutant (Figs. 2 and 3). Furthermore, this single amino acid change conferred a relatively low apparent Km for Ca2+ to the chimeric CAX3 (Fig. 4). This isoenceule may interact with side chains of specific amino acids from various transmembrane segments of CAX3 to aid in co-ordination of Ca2+ or H+. Thus, the CAX transporters may contain several Ca2+ domains that collectively coordinate ion transport. Given that our experimental procedure compared the CAX transporters to each other, we may have simply identified the Ca2+ domain that is the most polymorphic between these transporters.

Although structure/function studies of H+/Ca2+ exchangers are limited, other cotransporters such as Na+/H+ antiporters have been extensively studied (25–27). Histidine residues are candidates for H+-sensing amino acids in Na+/H+ antiporters, and these face the cytoplasmic side. CAX1 contains only six histidine residues, but their role in transport has not been evaluated. Structural studies demonstrate a novel membrane-protein motif for the E. coli Na+/H+ antiporter, NhaA. Other studies identify the mechanisms involved in pH sensitivity and cation binding and transport by Na+/H+ antiporters (24). The fundamental mechanism of cotransporters may be conserved, so insights from those studies may help direct future work related to H+/Ca2+ exchangers.

Only in relatively few structure/function studies of Ca2+ and heavy metal transporters have some of the amino acid residues that determine ion specificity, binding, or transport been identified (5, 6, 14, 28). This study identified residues in one particular domain of a H+/Ca2+ exchanger that may be important for Ca2+ ion specificity. This type of approach may allow researchers to alter the duration of cytosolic Ca2+ pulses in different organisms. Some microbial antiporters appear to be able to mediate both Na+ and Ca2+ transport (7, 29). Using the appropriate yeast suppression screens, it may be feasible to map the substrate-determining regions for these transporters. In plant systems, the ability to modulate CAX-mediated transport and ectopically express these modified genes in plants offers the potential to manipulate both nutrient content and signal transduction events.

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