Mechanism of N-terminal Autoinhibition in the Arabidopsis Ca\textsuperscript{2+}/H\textsuperscript{+} Antiporter CAX1*

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Regulation of Ca\textsuperscript{2+}/H\textsuperscript{+} antiporters may be an important function in determining the duration and amplitude of cytosolic Ca\textsuperscript{2+} oscillations. Previously the Arabidopsis Ca\textsuperscript{2+}/H\textsuperscript{+} transporter, CAX1 (cation exchanger 1), was identified by its ability to suppress yeast mutants defective in vacuolar Ca\textsuperscript{2+} transport. Recently, a 36-amino acid N-terminal regulatory region on CAX1 has been identified that inhibits CAX1-mediated Ca\textsuperscript{2+}/H\textsuperscript{+} antiporter. Here we show that a synthetic peptide designed against the CAX1 36 amino acids inhibited Ca\textsuperscript{2+}/H\textsuperscript{+} transport mediated by an N-terminal-truncated CAX1 but did not inhibit Ca\textsuperscript{2+} transport by other Ca\textsuperscript{2+}/H\textsuperscript{+} antiporters. Ca\textsuperscript{2+}/H\textsuperscript{+} antiporter activity measured from vacuolar-enriched membranes of Arabidopsis root was also inhibited by the CAX1 peptide. Through analyzing CAX chimeric constructs the region of interaction of the N-terminal regulatory region was mapped to include 7 amino acids (residues 56–62) within CAX1. The CAX1 N-terminal regulatory region was shown to physically interact with this 7-amino acid region by yeast two-hybrid analysis. Mutagenesis of amino acids within the N-terminal regulatory region implicated several residues as being essential for regulation. These findings describe a unique mode of antiporter autoinhibition and demonstrate the first detailed mechanisms for the regulation of a Ca\textsuperscript{2+}/H\textsuperscript{+} antiporter from any organism.

Transient elevations in cytosolic Ca\textsuperscript{2+} are elicited in response to a range of environmental and internal stimuli, and these Ca\textsuperscript{2+} elevations are then translated into a physiological response (1). The ability of a cell to generate specific Ca\textsuperscript{2+} oscillations requires tight regulation of the influx, via Ca\textsuperscript{2+} channels, and efflux, via Ca-ATPases (2). Ca\textsuperscript{2+}/Na\textsuperscript{+} antiporters in animal cells and Ca\textsuperscript{2+}/H\textsuperscript{+} antiporters in bacteria, yeast, and plants also mediate efflux of Ca\textsuperscript{2+} (3, 4). The antiporters are high capacity, low affinity Ca\textsuperscript{2+} transporters that are very efficient at removing the transient cytosolic Ca\textsuperscript{2+} spikes that occur during a signaling event (5). It is therefore important to determine the mechanisms of regulation of all of these Ca\textsuperscript{2+} transporters. Many animal and plant Ca-ATPases are regulated by calmodulin binding to a C-terminal or N-terminal autoinhibitor (2, 6, 7). Ca\textsuperscript{2+}/Na\textsuperscript{+} antiporters are regulated by intracellular concentrations of Ca\textsuperscript{2+} and Na\textsuperscript{+} and are activated by a variety of modulators such as protein kinase C (3). However, we still know little about the regulation of Ca\textsuperscript{2+}/H\textsuperscript{+} antiporters (4, 8).

Ca\textsuperscript{2+}/H\textsuperscript{+} antiporters have been characterized in plant, yeast, and microbial species (9–12) and also exist in some animal tissues (13, 14). These include ChaA from Escherichia coli and VCX1 from Saccharomyces cerevisiae (11, 12). Two Ca\textsuperscript{2+}/H\textsuperscript{+} antiporters of Arabidopsis thaliana, CAX1 and CAX2, were identified by their ability to suppress the Ca\textsuperscript{2+} sensitivity of a S. cerevisiae mutant lacking the vacuolar Ca-ATPase PMC1 and the Ca\textsuperscript{2+}/H\textsuperscript{+} antiporter VCX1 (9). In addition, Arabidopsis has other putative cation exchanger (CAX)\textsuperscript{3} proteins (15), including CAX3 and CAX4 (16, 17). As recent studies demonstrate with CAX2, these antiporters may also transport other metals (18). A central question is how these transporters are modulated to affect ion homeostasis.

Two domains that control CAX1 activity have been identified (8, 17, 19). The first domain that regulates Ca\textsuperscript{2+}/H\textsuperscript{+} antiporter function has been termed the regulatory or autoinhibitory domain (8). We recently identified a CAX1 open reading frame (previously termed long-CAX1) that contains an additional 36 amino acids at the N terminus (8). These amino acids were not found in the original clone identified by suppression of the yeast vacuolar Ca\textsuperscript{2+} transport mutant (9). This longer version of CAX1 localizes to the yeast vacuole, but does not transport Ca\textsuperscript{2+} even in the presence of calmodulin (8). Sequence analysis suggests that an N-terminal regulatory region (NRR) may be present in all Arabidopsis CAX transporters. Using a series of N-terminal-truncated CAX (sCAX) chimeric constructs, a second domain, the Ca\textsuperscript{2+} domain (Cd), has been identified that appears to modulate Ca\textsuperscript{2+} transport (19). CAX3 and CAX4 can strongly suppress yeast vacuolar Ca\textsuperscript{2+} transport mutants if the 9-amino acid CaC of CAX1 is inserted into N-terminal-truncated versions of these transporters (sCAX3-9 and sCAX4-9; Refs. 17 and 19). Alternatively, both CAX3 and CAX4 can suppress yeast vacuolar Ca\textsuperscript{2+} transport mutants if N-terminal additions are made to these transporters (17). These findings imply that CAX-dependent Ca\textsuperscript{2+} transport depends on N-terminal modifications; however, the nature of the N-terminal regulation remains unclear.

Here we demonstrate that a peptide corresponding to the 36 amino acids of the CAX1 N terminus can specifically inhibit CAX1-mediated Ca\textsuperscript{2+}/H\textsuperscript{+} transport in yeast cells and Ca\textsuperscript{2+}-

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1 The abbreviations used are: CAX, cation exchanger; sCAX, N-terminal-truncated CAX; Cd, Ca\textsuperscript{2+} domain; NRR, N-terminal regulatory region; FCCP, carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone; SC, synthetic complete medium.
treated *Arabidopsis* roots. We identify individual amino acids within the N terminus that are important for this autoinhibition and map a region of CAX1 to which the NRR interacts. This novel autoinhibitory mechanism suggests that plants judiciously regulate Ca\(^{2+}\)/H\(^+\) transport and suggest a means to engineer plant Ca\(^{2+}\)/H\(^+\) transport.

**EXPERIMENTAL PROCEDURES**

**DNA Manipulation and Site-directed Mutagenesis**—CAX1 mutants were produced by PCR site-directed mutagenesis (20) using the following primers: T6A forward, 5'-GAAGATCTGGTCTCGAGCTAGCTCTTGGAGCGACCGCTCACAACTGTCTTCC-3' and reverse, 5'-GAATTCCTCGAGTTCCATGCAAAACTTTAAGAACATTCTCTG-3'; S10A forward, 5'-GAATTCCTGGTCTCGCTCGATCCTTTCGCCGT-3' and reverse (same as S10A reverse); S24A forward, 5'-GAAGATCTGGTCTCGGAAGAGCTGC-3' and reverse (same as S24A reverse); S25T forward, 5'-GAATTCCTGGTCTCAAGTGCCAGAAGAGGTTCCTG-3' and reverse (same as S25T reverse); T33A forward, 5'-GAATTCCTGGTCTCAAGTGCCAGAAGAGGTTCCTG-3' and reverse (same as T33A reverse); T33E forward, 5'-GAATTCCTGGTCTCAAGTGCCAGAAGAGGTTCCTG-3' and reverse (same as T33E reverse); T33S forward, 5'-GAATTCCTGGTCTCAAGTGCCAGAAGAGGTTCCTG-3' and reverse (same as T33S reverse); T33A forward, 5'-GAATTCCTGTGTGTTCCATGCAAAACTTTAAAGAACATTCTCTG-3' and reverse (same as T33A reverse); E7A/E13A forward, 5'-GAAGATCTGGTCTCGAGCTGAGAAGACGGATCAGT-3' and reverse (same as E7A/E13A reverse); B2A/B2A forward, 5'-GAATTCCTGGTCTCGAGCTGAGAAGACGGATCAGT-3' and reverse (same as B2A/B2A reverse); B1A/B1A forward, 5'-GAATTCCTGGTCTCGAGCTGAGAAGACGGATCAGT-3' and reverse (same as B1A/B1A reverse); T6B/T6B forward, 5'-GAAGATCTGGTCTCGAGCTGAGAAGACGGATCAGT-3' and reverse (same as T6B/T6B reverse); H11032.

**RESULTS**

A Synthetic Peptide Corresponding to the NRR of CAX1 Specifically Inhibits sCAX1 Ca\(^{2+}\)/H\(^+\) Antiport Activity—To determine whether the CAX1 NRR functions as an autoinhibitory domain, a synthetic peptide was generated that corresponds to all 36 amino acids (CAX1-NRR peptide, MAGIVTEPWSVAGGATCGGACAGAGAACTAAGACTTGGA-3') and reverse (same as S10A reverse); S24A forward, 5'-GAATTCCTGGTCATCAAGGAGACGAGAGAACTAAGACTTGGA-3' and reverse (same as S24A reverse); T33A forward, 5'-GAATTCCTGGTCTGAGAAGACGGATCAGT-3' and reverse (same as T33A reverse); T33E forward, 5'-GAATTCCTGGTCTGAGAAGACGGATCAGT-3' and reverse (same as T33E reverse); T33S forward, 5'-GAATTCCTGGTCTGAGAAGACGGATCAGT-3' and reverse (same as T33S reverse); T33A forward, 5'-GAATTCCTGTGTGTTCCATGCAAAACTTTAAAGAACATTCTCTG-3' and reverse (same as T33A reverse); E7A/E13A forward, 5'-GAAGATCTGGTCTCGAGCTGAGAAGACGGATCAGT-3' and reverse (same as E7A/E13A reverse); B2A/B2A forward, 5'-GAATTCCTGGTCTCGAGCTGAGAAGACGGATCAGT-3' and reverse (same as B2A/B2A reverse); B1A/B1A forward, 5'-GAATTCCTGGTCTCGAGCTGAGAAGACGGATCAGT-3' and reverse (same as B1A/B1A reverse); T6B/T6B forward, 5'-GAAGATCTGGTCTCGAGCTGAGAAGACGGATCAGT-3' and reverse (same as T6B/T6B reverse); H11032.

We wanted to verify that the inhibitory effect of the CAX1-NRR peptide on CAX1-dependent Ca\(^{2+}\)/H\(^+\) transport was not an artifact of the yeast expression system. MgATP-dependent H-ATPase-driven Ca\(^{2+}\)/H\(^+\) transport, in the presence of the Ca-ATPase inhibitor vanadate, was determined in vacuolar-enriched membrane vesicles from wild-type *Arabidopsis* roots (Fig. 2A). Given that CAX1 expression is highly induced by exogenous Ca\(^{2+}\) (24), we isolated vacuolar-enriched membrane vesicles from *Arabidopsis* plants treated with 100 μM Ca\(^{2+}\) for 18 h. Significant Ca\(^{2+}\)/H\(^+\) antiport activity could be measured only from Ca\(^{2+}\)-treated plants (Fig. 2A). Ca\(^{2+}\)/H\(^+\) antiport activity was inhibited in a concentration-dependent manner by the CAX1-NRR peptide (Fig. 2B). A peptide concentration of 9–10 μM was required for 50% inhibition of Ca\(^{2+}\)/H\(^+\) transport.

**The CAX1 NRR Interacts with the N Terminus of sCAX1**—To deduce which residues were important for interaction of the NRR with CAX1, the CAX1-NRR peptide was tested on sCAX1 chimeras that contain various domains of sCAX3 and sCAX2. These constructs were previously used to identify the Ca\(^{2+}\)/H\(^+\) antiport activity was inhibited in a concentration-dependent manner by the CAX1-NRR peptide (Fig. 2B). A peptide concentration of 9–10 μM was required for 50% inhibition of Ca\(^{2+}\)/H\(^+\) transport. The CAX1-NRR peptide interacts with the N terminus of sCAX1 (Fig. 2A). A chimera (sCAX3-9) was not inhibited by the NRR peptide (Fig. 3A). We then tested whether the NRR interacts with the CAX1 Ca\(^{2+}\)/H\(^+\) antiport transport by sCAX3-9 and by sCAX2 containing the CAX1 Ca\(^{2+}\)/H\(^+\) antiport transport (Fig. 3A; data download from www.jbc.org by guest on July 27, 2018

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not shown), whereas transport by sCAX1 containing the equivalent CaD of CAX2 (sCAX1-9) was still inhibited. The effect of the peptide on Ca$^{2+}$/H$^{+}$ transport by sCAX3-A2 and sCAX1-A1 was then tested. In these constructs, the first quarter domain (residues 37–149) was further divided in half and comprised the immediate N terminus of sCAX3 (residues 37–73); only the second half of this domain was CAX1 (residues 74–149). No inhibition of Ca$^{2+}$/H$^{+}$ transport occurred for either of these constructs (Fig. 3A).

**FIG. 1. Inhibitory effects of the CAX1-NRR peptide.** A, the inhibitory effects of synthetic peptides on ∆pH-dependent 10 µM Ca$^{2+}$ transport by sCAX1, sCAX2, VCX1, and sCAX3-9 into yeast endomembrane vesicles measured at a 10-min time point. Concentrations of 8.5 µM CAX1-NRR peptide and 17 µM control peptide were used for sCAX1, 34 µM CAX1-NRR peptide was used for sCAX2 and sCAX-9, and 17 µM CAX1-NRR peptide was used for VCX1. B, the effect of CAX1-NRR peptide concentration on ∆pH-dependent 10 µM Ca$^{2+}$ transport by sCAX1 into yeast endomembrane vesicles measured at a 10-min time point. C, the kinetics of ∆pH-dependent Ca$^{2+}$ transport by sCAX1 into yeast endomembrane vesicles in the presence or absence of 6 µM CAX1-NRR peptide. Left, the initial rate of Ca$^{2+}$ transport was determined at 5 min after the addition of $^{45}$Ca$^{2+}$. Right, the data shown as a Hanes plot. All results in panels A–C are shown following subtraction of the gramicidin background values and are the means (±S.E.) of 2–4 independent experiments.

**FIG. 2. Ca$^{2+}$/H$^{+}$ antiport activity in Arabidopsis root endomembrane vesicles.** A, time course of 10 µM $^{45}$Ca$^{2+}$ transport into Ca$^{2+}$-treated Arabidopsis root endomembrane vesicles in the absence (closed circle) or presence (open circle) of 5 µM FCCP. Both treatments were performed in the presence of 200 µM vanadate. The Ca$^{2+}$ ionophore A23187 (5 µM) was added at 12 min. B, the effect of CAX1-NRR peptide concentration on ∆pH-dependent 10 µM Ca$^{2+}$ transport into Ca$^{2+}$-treated Arabidopsis root endomembrane vesicles measured at a 10-min time point. Results are shown following subtraction of the FCCP background values and shown as a percentage of Ca$^{2+}$ uptake of the control sample in the absence of peptide. All results are the mean (±S.E.) of three independent experiments.

The CAX1-NRR Interacts with a Region of 7 Amino Acids within the First Quarter of sCAX1—The amino acid sequences for CAX1 and CAX3 within the region of residues 37–73 were compared. Two regions of heterogeneity were identified within this sequence, a region of 2 amino acids and a region of 7 amino acids. Mutagenesis was performed on sCAX3-9 and sCAX1-A1 to change residues LV to VI (residues 50–51) or residues CK-TLKI to YKGLKDF (residues 56–62). Following mutagenesis, both constructs were able to transport Ca$^{2+}$. The CAX1-NRR peptide was unable to inhibit Ca$^{2+}$ transport by sCAX3-9 and sCAX1-A1 (Fig. 3A). This peptide was able to inhibit Ca$^{2+}$ transport by both constructs when they contained the 7-amino acid substitution (sCAX3-9-7aa and sCAX1-A1-7aa; Fig. 3A). However, no inhibition was found when they contained the 2-amino acid substitution. Furthermore, when mutagenesis was performed on CAX1 so that the 7-amino acid region was swapped with that of CAX3 (to give CAX1–7aa), CAX1–7aa was now able to suppress the Ca$^{2+}$-sensitive phenotype of K667, although not as strongly as sCAX1 (Fig. 3B). sCAX1 containing the 7-amino acid region of CAX3 (sCAX1–7aa) could suppress the Ca$^{2+}$-sensitive phenotype as strongly as sCAX1 (Fig. 3B).
However, Ca$^{2+}$ transport by sCAX1-7aa could not be inhibited by the CAX1-NRR peptide (Fig. 3C). A physical interaction between the NRR and the 7-amino acid region of CAX1 was tested using a yeast two-hybrid assay. When the CAX1 NRR in pACT2 was coexpressed with a hydrophilic N-terminal fragment of sCAX3 (residues 44–69 including the 7-amino acid region) in pAS2, the lacZ gene was activated, indicating that an interaction had occurred (Fig. 3D).

No interaction occurred with a similar N-terminal fragment of sCAX3 or a hydrophilic C-terminal fragment (residues 438–463) of CAX1. Similar results were obtained with other two-hybrid constructs; specifically, the NRR of CAX1 only interacted with constructs containing residues 44–69 of CAX1 (data not shown). No interaction was observed when CAX1 NRR was coexpressed with an N-terminal fragment of sCAX3 that included the 7-amino acid region of CAX1, although we did occasionally observe a weak interaction with some of the replicates (data not shown).

Modification of Amino Acids in the NRR of CAX1—We previously noted that the NRR of CAX1 contains putative phosphorylation sites (8). To investigate whether any of the five residues Thr$^{6}$, Ser$^{10}$, Ser$^{24}$, Ser$^{25}$, and Thr$^{33}$ may be phosphorylated, they were each mutated to Ala. This change was expected to block any phosphorylation event required to deactivate CAX1. All five CAX1 mutants were expressed at equal levels in yeast (data not shown), but only the T33A change strongly suppressed the K667 Ca$^{2+}$-sensitive phenotype (Fig. 4A). Thr$^{33}$ was further mutated to Asp to mimic continuous phosphorylation to see whether activation could be blocked. The T33D change and all other mutations of this residue (T33S or T33E) allowed yeast growth, suggesting that theThr$^{33}$ residue is critical for activation of CAX1.
caused very weak suppression of Ca\(^{2+}\) sensitivity, we further mutated this residue. A S25T change gave no suppression (Fig. 4B; data not shown) whereas a S25D change caused strong suppression of the K667 phenotype (Fig. 4A). The yeast growth was caused by Ca\(^{2+}\)/H\(^{+}\) transport activity conferred by CAX1S25D as demonstrated by the 45Ca\(^{2+}\) uptake into endo-membrane-enriched vesicles from CAX1S25D expressing yeast (Fig. 5). No Ca\(^{2+}\) uptake could be measured for CAX1 and CAX1S25A. When other Ser residues in the NRR were mutated to Asp (S10D or S24D) no suppression of the K667 phenotype was observed (Fig. 4B; data not shown).

The CAX1 NRR contains a number of charged residues, particularly Glu and Arg (Fig. 6). To investigate whether any of these residues have a role in the binding mechanism of the NRR, three sets of double substitutions were created to remove the charge of these residues: E7A/E13A, R26A/E27A, and R29A/R32A. Only the R29A/R32A mutation allowed CAX1 to strongly suppress the Ca\(^{2+}\)-sensitive phenotype of K667, comparable to the suppression by sCAX1 (Fig. 6).

**DISCUSSION**

The regulation of Ca\(^{2+}\) efflux mediated by high capacity Ca\(^{2+}\)/H\(^{+}\) exchange activity (and by Ca\(^{2+}\) pumping directly energized by ATP hydrolysis) is likely to be an important component in Ca\(^{2+}\) signaling (2). Vacuolar Ca\(^{2+}\)/H\(^{+}\) antiporters can sequester a sudden burst of cytosolic Ca\(^{2+}\) more efficiently than the higher affinity Ca-ATPase (5). However, the regulation of Ca\(^{2+}\)/H\(^{+}\) antiporters is poorly understood. For example, the yeast Ca\(^{2+}\)/H\(^{+}\) antiporter VCX1 appears to be negatively regulated at the post-translational level by the Ca\(^{2+}\)/calmodulin-dependent phosphatase calcineurin; however, the nature of this regulation remains unclear (12). Furthermore, nothing is known about the regulation of the *E. coli* Ca\(^{2+}\)/H\(^{+}\) antiporter chaA or the mung bean antiporter VCAX1 (10, 11). We recently described the presence of an NRR on *Arabidopsis* CAX1 (8). Here we demonstrate that the NRR of CAX1 regulates Ca\(^{2+}\)/H\(^{+}\) antiport activity by a unique autoinhibitory mechanism.

A synthetic peptide composed of the 36 amino acids of the CAX1 NRR inhibited Ca\(^{2+}\)/H\(^{+}\) transport by the truncated sCAX1 at micromolar concentration, but did not inhibit transport by other Ca\(^{2+}\)/H\(^{+}\) antiporters (Fig. 1A). Furthermore, a control peptide with similar charge and size properties did not inhibit Ca\(^{2+}\) transport by sCAX1 (Fig. 1A). The CAX1-NRR peptide also substantially increased the \(K_m\) for Ca\(^{2+}\) of sCAX1 (Fig. 1C). The inhibition by the CAX1-NRR peptide was not caused by a reduction of the proton motive force across the membrane.
through a nonspecific interaction with lipids or proteins, because the peptide did not disrupt ΔpH-dependent Ca\(^{2+}\) transport by sCAX2, VCX1, or sCAX3-9 (Fig. 1A). It could also be argued that the peptide was directly binding Ca\(^{2+}\), as this would also raise the apparent K_m for Ca\(^{2+}\). However, we believe that this was not the case, because Ca\(^{2+}\) binding by the peptide would cause a reduction in free Ca\(^{2+}\) concentration that would reduce the level of Ca\(^{2+}\) transport by all antiporters, not just sCAX1. In addition, we observed a reduction in transport only for sCAX1. These results indicate that the 36-residue sequence does function like an inhibitory domain confirming that CAX1 is regulated by autoinhibition. We speculate that CAX1 only exists as a full-length version in tissue (25). We were able to measure Ca\(^{2+}/\)H\(^{+}\) antiport activity in vacuolar membranes from Arabidopsis roots (Fig. 2A). These plants were pretreated with 100 mM Ca\(^{2+}\), which has been shown to strongly induce expression of CAX1 (24) but not CAX2 (18). This antiport activity was strongly inhibited by the CAX1-NRR peptide in a manner similar to the inhibition of sCAX1-dependent Ca\(^{2+}\) transport in yeast (Fig. 2B). The ability of the peptide to inhibit the antiport activity suggests that under certain conditions (such as when high levels of exogenous Ca\(^{2+}\) are present) the majority of Ca\(^{2+}/\)H\(^{+}\) antiport activity at the root vacule is due to CAX1.

We speculate that CAX1 only exists as a full-length version in planta; therefore, the antiport activity measured was mediated by an activated CAX1. Therefore, this result indicates that the CAX1-NRR peptide can inhibit full-length CAX1. We may hypothesize that an activator protein interacts with the NRR and prevents autoinhibition, allowing the NRR peptide to bind to CAX1 instead and inhibit activity. Alternatively, the peptide may compete directly for the activator protein, preventing the activator from interacting with CAX1, which remains autoinhibited.

Identification of putative phosphorylation sites in the NRR led us to investigate whether CAX1 is regulated by a phosphorylation event. Our previous findings showed that minor deletions in the NRR can perturb inhibition (8); however, changing Thr\(^{33}\), Ser\(^{10}\), or Ser\(^{24}\) to Ala residues did not disrupt N-terminal inhibition (Fig. 4). Mutagenesis of Thr\(^{33}\) to Ala strongly suppressed the Ca\(^{2+}\) sensitivity of K667 expressing CAX1, initially indicating that this residue may be phosphorylated to block CAX1 activation. However, any mutation of this residue caused yeast growth to occur, suggesting that this Thr has an important structural role in the autoinhibition rather than in being phosphorylated. The Ser\(^{25}\) to Ala change caused minimal growth; however, when Ser\(^{25}\) was mutated to Asp to mimic continuous phosphorylation, CAX1 was as active as sCAX1 (Figs. 4 and 5). No suppression of the K667 phenotype was observed following mutation of Ser\(^{10}\) or Ser\(^{24}\) to Asp. This suggests that phosphorylation of CAX1 may activate Ca\(^{2+}\) transport. Some of the positively charged Arg residues also appear to be involved in the autoinhibition mechanism. Substitution of these residues to Ala, thereby removing their charge, activated CAX1 (Fig. 6). Binding of a regulatory protein to the CAX1 NRR may prevent autoinhibition and activate
CAX1. Some of the Ser, Thr, and Arg residues highlighted by this work may be candidates for part of a binding site for regulatory proteins. The identification of activators for CAX1 will enable us to understand how it is regulated and how it might relate to a particular Ca\(^{2+}\) signal transduction pathway.

The mechanism of CAX1 autoinhibition appears to involve a physical interaction between two discreet domains, the NRR and residues C-terminal to this region (Fig. 7). In order to show this interaction we used two different approaches. Initially, a series of chimeric CAX constructs were used to discriminate the region of CAX1 that interacts with the CAX1 NRR. Peptide inhibition studies showed that the NRR peptide could inhibit any sCAX construct containing the N-terminal 7 amino acids from sCAX1. When N-terminal chimeras using other CAX transporters were analyzed, no peptide inhibition was seen. However, when 7 amino acids from CAX1 (residues 56–62) were inserted into sCAX3-9 at the N terminus, the CAX1 peptide could inhibit sCAX3-9 mediated Ca\(^{2+}/\)H\(^{+}\) transport. Furthermore, when the equivalent 7-amino acid region of CAX3 was swapped into sCAX1, peptide inhibition of Ca\(^{2+}/\)H\(^{+}\) transport was abolished. That this construct could still transport Ca\(^{2+}\) confirms that this region is not required for transport. CAX1 containing the CAX3 7-amino acid region (CAX1–7aa) was no longer autoinhibited, and this construct was able to suppress the Ca\(^{2+}\) sensitivity of the K667 yeast mutant. This chimeric CAX/CAX1-NRR approach was informative but rather unconventional. We also used yeast two-hybrid analysis to confirm an interaction between the CAX1 NRR and the CAX1 7-amino acid region by using a hydrophilic construct (residues 44–69) of sCAX1 (Fig. 3D). Similar results showed an interaction with the N terminus of sCAX1 (residues 37–222) using a fragment that contained some hydrophobic regions (data not shown). Surprisingly, no strong interaction was observed for CAX1 NRR with the construct containing the CAX1 7-amino acid region swapped into CAX3. This may be because of inappropriate folding of this protein. We term this 7-amino acid region the regulatory-dependent region. Our findings establish that these 7 amino acids are required for autoinhibition, although this analysis does not conclude that these are the only amino acids that interact with the N-terminal 36 amino acids. Given that CAX3 and CAX1 are very similar and many of the amino acids in their N termini are identical (Fig. 7A), other residues may be involved in this interaction. Further work is required to determine which residues in the NRR are required for interaction with its binding site. Residues Ser\(^{25}\), Arg\(^{29}\), Arg\(^{32}\), and Thr\(^{33}\) appear to be important, indicating that the C-terminal end of the NRR could contain the autoinhibitory binding site (Fig. 7). However, we have previously shown that removal of the first ten residues of the NRR prevents inhibition (8), indicating that the structure of the entire NRR may be important for inhibition.

Like CAX1, some plant Ca-ATPases with truncations at the N terminus are able to suppress yeast mutants that are defective in endomembrane Ca\(^{2+}\) transport; however, the N-terminal autoinhibitory mechanisms of plant Ca-ATPases appear to drastically differ from that proposed for CAX1. Arabidopsis Ca-ATPases ACA2, ACA4, and Brassica BCA1 are regulated by calmodulin binding or phosphorylation of an N-terminal autoinhibitory domain (26–29). A calmodulin-binding sequence is present within the N-terminal domain of ACA2, and the full-length pump demonstrates calmodulin-stimulated Ca\(^{2+}\) transport in yeast (29, 30). CAX1, however, is not activated by calmodulin (8). A Ca\(^{2+}\)-dependent protein kinase-binding site is present in the N terminus of ACA2 that phosphorylates a Ser near the calmodulin-binding site. This kinase activity inhibits ACA2 activity (27). However, our results suggest that CAX1 may be activated rather than repressed by phosphorylation (Fig. 4). Autoinhibitory domain peptides of Ca-ATPases have been shown to inhibit truncated and full-length calmodulin-activated pumps (30, 31). The degree of inhibition in these studies is similar to what we have observed for CAX1 (Fig. 1).

A peptide corresponding to the autoinhibitory domain of ACA2 inhibited 50% of activity (IC\(_{50}\)) of truncated ACA2 at 4 μM and increased the pump’s K\(_{m}\) for Ca\(^{2+}\) by 2-fold (30). Similarly, a peptide corresponding to the autoinhibitory domain of rabbit PMCA2b had an IC\(_{50}\) of 1 μM (31). The CAX1-NRR peptide only inhibited CAX1 (Fig. 1A). In contrast, peptides derived from some mammalian and plant Ca-ATPases were not as specific (30, 32). In these studies, a Ca-ATPase autoinhibitory domain peptide could inhibit different Ca\(^{2+}\) transporters, including the Ca\(^{2+} /Na\(^{+}\) exchanger (32). Similarly, a peptide corresponding to the autoinhibitory domain of ACA2 could inhibit activity of another Arabidopsis Ca-ATPase ECA1 (30).

Now that we have identified domains important for CAX1 autoinhibition, future work must assess possible conformational changes associated with autoinhibition. In animal Ca-ATPases, the autoinhibitory domain affects activity by causing self-association of the transporter (6). In some plant Ca-ATPases, the autoinhibitory domain binds to a stalk region and hydrophilic loops near the N terminus (7). Regardless of the specifics of inhibition, activation of the Ca-ATPases in plants and animals requires the sensing of Ca\(^{2+}\) fluxes that then activate calmodulin.

Our previous identification of a longer open reading frame of CAX1 suggested that the original N-terminal-truncated sCAX1 is most likely encoded by a partial-length cDNA. Thus, we concluded that only a truncated and deregulated version of CAX1 could suppress the Ca\(^{2+}\) sensitivity of the yeast mutant K667 (8). In this study we have shown how mutagenesis of single amino acids can be used to determine some of the mechanisms of autoinhibition. We have shown how to create a deregulated and activated Ca\(^{2+}/\)H\(^{+}\) antiporter. We have also shown how a peptide corresponding to the autoinhibitory domain can specifically inhibit the activity of deregulated CAX1 both in yeast and in Arabidopsis. This is the first report of peptide inhibition of Ca\(^{2+}/\)H\(^{+}\) antiporter activity. These findings suggest that it may be possible to temporally and spatially engineer expression of CAX1 NRR peptides in plants as a means to modulate CAX1-mediated Ca\(^{2+}\) transport.
Mechanism of CAX1 Regulation

18. Hirschi, K. D., Korenkov, V. D., Wilganowski, N. L., and Wagner, G. J. (2000) *Plant Physiol.* **124**, 125–134
19. Shigaki, T., Cheng, N.-H., Pittman, J. K., and Hirschi, K. (2001) *J. Biol. Chem.* **276**, 43152–43159
20. Shigaki, T., and Hirschi, K. D. (2001) *Anal. Biochem.* **296**, 118–120
21. Nathan, D. F., Vos, M. H., and Lindquist, S. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 1409–1414
22. Uemura, M., Joseph, R. A., and Steponkus, P. L. (1995) *Plant Physiol.* **109**, 15–30
23. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. (1993) *Cell* **75**, 805–818
24. Hirschi, K. D. (1999) *Plant Cell* **11**, 2113–2122
25. Gaxiola, R. A., Li, J., Undurraga, S., Dang, L. M., Allen, G. J., Alper, S. L., and Fink, G. R. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 11444–11449
26. Malmstrom, S., Akerlund, H.-E., and Akerlund, P. (2000) *Plant Physiol.* **122**, 517–526
27. Hwang, I., Sze, H., and Harper, J. F. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 6224–6229
28. Geisler, M., Frangne, N., Gomes, E., Martinoha, E., and Palmgren, M. G. (2000) *Plant Physiol.* **124**, 1814–1827
29. Harper, J. F., Hong, B., Hwang, I., Gao, H. Q., Stoddard, R., Huang, J. F., Palmgren, M. G., and Sze, H. (1998) *J. Biol. Chem.* **273**, 1099–1106
30. Hwang, I., Harper, J. F., Liang, F., and Sze, H. (2000) *Plant Physiol.* **122**, 157–168
31. Enyedi, A., Filoteo, A. G., Gardos, G., and Penniston, J. T. (1991) *J. Biol. Chem.* **266**, 8952–8956
32. Enyedi, A., and Penniston, J. T. (1993) *J. Biol. Chem.* **268**, 17120–17125
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