A Cation-regulated and Proton Gradient-dependent Cation Transporter from Chlamydomonas reinhardtii Has a Role in Calcium and Sodium Homeostasis

The CrCAX1 gene encoding a Ca\(^{2+}\)/H\(^+\) and Na\(^+\)/H\(^+\) exchanger was cloned and characterized from the unicellular green alga Chlamydomonas reinhardtii to begin to understand the mechanisms of cation homeostasis in this model organism. CrCAX1 was more closely related to fungal cation exchanger (CAX) genes than those from higher plants but has structural characteristics similar to plant Ca\(^{2+}\)/H\(^+\) exchangers including a long N-terminal tail. When CrCAX1-GFP was expressed in Saccharomyces cerevisiae, it localized at the vacuole. CrCAX1 could suppress the Ca\(^{2+}\)-hypersensitive phenotype of a yeast mutant and mediated proton gradient-dependent Ca\(^{2+}\)/H\(^+\) exchange activity in vacuolar membrane vesicles. Ca\(^{2+}\) transport activity was increased following N-terminal truncation of CrCAX1, suggesting the existence of an N-terminal auto-regulatory mechanism. CrCAX1 could also provide tolerance to Na\(^+\) stress when expressed in yeast or Arabidopsis thaliana because of Na\(^+\)/H\(^+\) exchange activity. This Na\(^+\)/H\(^+\) exchange activity was not regulated by the N terminus of the CrCAX1 protein. A subtle tolerance by CrCAX1 in yeast to Co\(^{2+}\) stress was also observed. CrCAX1 was transcriptionally regulated in Chlamydomonas cells grown in elevated Ca\(^{2+}\) or Na\(^+\). This study has thus uncovered a novel eukaryotic proton-coupled transporter, CrCAX1, that can transport both monovalent and divalent cations and that appears to play a role in cellular cation homeostasis by the transport of Ca\(^{2+}\) and Na\(^+\) into the vacuole.

Controlled ion homeostasis is critical to all organisms. This is certainly the case for alkali metals (Na\(^+\), K\(^+\)) and alkaline earth metals (Ca\(^{2+}\), Mg\(^{2+}\)) that play essential roles in many organisms but that must be carefully regulated with regard to their cellular concentrations to prevent osmotic and metal stress. For example, tightly controlled levels of Ca\(^{2+}\) play a critical role in cell signaling, but high concentrations of Ca\(^{2+}\) are very toxic to the cell (1). Likewise a careful balance between K\(^+\) and Na\(^+\) ions is required to prevent osmotic stress and the toxic effects of Na\(^+\) (2, 3). In unicellular organisms particularly, failure of Ca\(^{2+}\) or Na\(^+\) homeostasis will lead to death of that organism; therefore the tight control of these ions is paramount. This is certainly the case for many of these organisms that exist in potentially harsh ionic conditions. For example, many of the unicellular green algae of the Chlamydomonas genus can tolerate and adapt to multiple ion stresses. The acidophile Chlamydomonas acidophila and the halotolerant Chlamydomonas sp. W80 can tolerate very severe ion stresses (4, 5). The soil and freshwater living Chlamydomonas reinhardtii (herein referred to as Chlamydomonas), although not as tolerant to these stresses as the extremophile species, have adaptive mechanisms to many ion stress conditions (6), either by direct removal of an ion through efflux or by mediating Ca\(^{2+}\)-signaling processes.

Some of the mechanisms of Na\(^+\) and Ca\(^{2+}\) homeostasis and transport are well understood in model eukaryotes such as yeast (Saccharomyces cerevisiae) and the plant Arabidopsis thaliana. In both species, ΔpH-dependent cation/H\(^+\) exchangers are important in mediating the removal of excess Na\(^+\) and Ca\(^{2+}\) from the cytosol across the plasma membrane or the vacuolar membrane (7). Na\(^+\), K\(^+\), or Ca\(^{2+}\) transporting cation/H\(^+\) exchangers derive from at least four phylogenetic superfamilies. One of these is the CaCA (Ca\(^{2+}\)/cation antiporter) superfamily, which includes mammalian Na\(^+\)/Ca\(^{2+}\) exchanger genes and cation/H\(^+\) exchanger (CAX) genes (8, 9). CAX genes have been identified in bacteria, fungi, protozoa, plants, and lower vertebrates but are absent from higher animals (9). The majority of the CAX genes characterized to date encode H\(^+\)-coupled exchangers that transport Ca\(^{2+}\). Many are highly specific for Ca\(^{2+}\) such as the yeast and Arabidopsis vacuolar Ca\(^{2+}\)/H\(^+\) exchangers ScVCX1 and AtCAX1 (10, 11), whereas others have a broader specificity and can transport a range of divalent cations (12). An interesting exception is the yeast CAX family transporter ScVNX1, which can transport Na\(^+\) and K\(^+\) but appears not to transport Ca\(^{2+}\) (13). A disparate feature of these transporters is the mechanism of regulation. Ca\(^{2+}\) transport by ScVCX1 is negatively regulated by the protein phosphatase calcineurin (10), whereas a post-

Received for publication, September 16, 2008, and in revised form, November 10, 2008 Published, JBC Papers in Press, November 10, 2008, DOI 10.1074/jbc.M807173200

Jon K. Pittman\(^{1}\), Clare Edmond, Paul A. Sunderland, and Clifford M. Bray
From the Faculty of Life Sciences, University of Manchester, Manchester, M13 9PT, United Kingdom
A Chlamydomonas Cation/H⁺ Exchanger

translational mode of regulation involving an N-terminal domain has been demonstrated for plant CAX transporters. For example, when full-length AtCAX1 is heterologously expressed in yeast, it has no Ca²⁺/H⁺ exchange activity unless the N-terminus of the protein is truncated (14). Further analysis in yeast and plants confirmed that the N-terminal tail regulates AtCAX1 Ca²⁺ transport activity through an autoinhibitory mechanism (15–17). In addition, many of the plant CAX transporters are transcriptionally regulated by alterations in ion levels (18–20).

Chlamydomonas is a model organism for studying photosynthesis and other aspects of cell biology and should be an excellent model to study adaptive responses to stress and the evolutionary relationships of cation transporters among eukaryotes. The recently sequenced Chlamydomonas genome has indicated the wide range of transporters present including members of the CaCA superfamily (21, 22), the majority of which remain uncharacterized. Here we describe the first functional analysis of a cation/H⁺ exchanger from Chlamydomonas and demonstrate that it functions as a H⁺-coupled Ca²⁺ and Na⁺ transporter that provides a role in cation homeostasis.

EXPERIMENTAL PROCEDURES

DNA Manipulations—CrCAX1 and N-terminally truncated CrCAX1 (sCrCAX1) cDNA was amplified by PCR from cDNA template derived from isolated Chlamydomonas RNA using primers CrCAX1F, CrCAX1R, and sCrCAX1F (see supplemental Table S1 for primer sequences). PCR products were cloned into a pGEM-T vector for propagation and sequencing. CrCAX1 and sCrCAX1 cDNAs were subcloned into the XbaI and SacI sites of the yeast expression vector pHiGpd and into a p35S-CAMBIA2300 plant kanamycin-resistant expression vector that had been modified to contain the CAMBIA2300 plant kanamycin-resistant expression vector site of pSP64. A mutant construct that encoded the first predicted 180 amino acids was amplified in vitro using primers CrCAX1TnTF and CrCAX1TnTR. A mutant variant was generated using the forward primer CrCAX1TnTF and N-terminally truncated and 3’ Ala so that translation would be predicted to initiate at Met91. The AtCAX1–67-GFP cDNA was subcloned where the predicted Met1 was mutated to Thr in a yeast expression vector piHGpd and into a p35S-CAMBIA2300 plant kanamycin-resistant expression vector using the standard pSP64 (Promega) for in vitro transcription-translation analysis. For comparison with AtCAX1, an AtCAX1 cDNA that encodes the first 67 amino acids was amplified using primers CAX1TnTF and CAX1a67R. This PCR product was fused to a previously amplified rsGFP sequence containing a 5’ BglIII site and a 3’ SacI site (23). The AtCAX1–67-GFP cDNA was subcloned into the PstI and SacI sites rsGFP (Promega) for in vitro transcription-translation analysis. For comparison with AtCAX1, an AtCAX1 cDNA that encodes the first 67 amino acids was amplified using primers CAX1a67R. This PCR product was fused to a previously amplified rsGFP sequence containing a 5’ BglIII site and a 3’ SacI site (23). The AtCAX1–67-GFP cDNA was subcloned into the PstI and SacI sites of pSP64. A mutant construct in which Met37 was substituted with Ala was generated using primers CAX1M37AF and CAX1M37AR. To generate C-terminal green fluorescent protein (GFP) fusions to CrCAX1 and sCrCAX1, a HindIII site was introduced into the 3’ end of the cDNA using the primer CrCAX1HR in combination with CrCAX1F or sCrCAX1F. A GFP cDNA was amplified from a synthetic GFP (S65G, S72A) plasmid (24) and was cloned into the HindIII and SacI sites of CrCAX1- and sCrCAX1-pGEM-T. The CrCAX1-GFP and sCrCAX1-GFP constructs were then subcloned into pHiGpd and p35S-CAMBIA2300.

Cell and Plant Growth—C. reinhardtii wild type strain 137C+ (CC-125 or CCAP 11/32C) was obtained from the UK Culture Collection of Algae and Protozoa (CCAP). The cells were grown in Tris-acetate-phosphate (TAP) liquid medium with agitation and on plates at 25 °C in 100 μmol of photons/m² x s⁻¹ light provided by fluorescent tubes. Cell growth was determined by measuring at A₅₇₀ nm. The yeast (S. cerevisiae) strains K667 (cnb1::LEU2 pmc1::TRP1 vvc1Δ) (10), ATX3 (nhx1::TRP1 ena1–4::HIS3 nha1::LEU2) (25), and nhx1Δ::kanMX4 (Euroscarf) were used for heterologous expression. Transformed yeast strains were grown in synthetic defined medium minus appropriate amino acids for selective growth for the expression plasmid and the mutations. For metal tolerance assays, serial dilutions of K667 yeast were grown at 30 °C on solid yeast extract-peptone-dextrose medium containing a range of metal salts including CaCl₂, NaCl, CoCl₂, and CdCl₂ for 4 days. For determination of ATX3 yeast growth rate in liquid NaCl solutions, yeast strains of the same starting cell density were inoculated in arginine-phosphate-dextrose medium containing 1 mM KC1 and a range of NaCl concentrations and grown at 30 °C, shaking for 48 h in 24-well flat bottomed plates, and cell growth was determined by measuring at A₆₀₀ nm. A. thaliana accession Col-0 was grown on soil or on solid 0.5× strength Murashige and Skoog medium at 22 °C in a 16 h/8 h light/dark cycle in 100 μmol of photons/m² x s⁻¹ light provided by fluorescent tubes. Total chlorophyll (chlorophyll a+b) measurements were determined as described (26).

DNA Transformations—Yeast strains were transformed with pHiGpd plasmid constructs using the lithium acetate/polyethylene glycol method as described previously (27). Arabidopsis was transformed with p35S-CAMBIA2300 plasmid constructs using the standard Agrobacterium tumefaciens-mediated floral dipping method. Following screening for kanamycin-resistant seeds, at least four homozygous lines were selected for each independent transformation with CrCAX1 and sCrCAX1.

RNA Extraction and RT-PCR—RNA was isolated from Arabidopsis using a RNA isolation kit (Qiagen). RNA was isolated from yeast by acid phenol extraction. RNA was isolated from Chlamydomonas cells grown in liquid TAP medium supplemented with various metal salts. The cells were collected by centrifugation, resuspended in water, and lysed in a lysis solution containing 50 mM Tris/ HCl, pH 7.5, 150 mM NaCl, 15 mM EDTA, 2% SDS, and 40 pg/ml proteinase K for 20 min at room temperature. RNA was purified from the lysed cells by four extractions with equal volumes of phenol/chloroform/isoamyl alcohol followed by two extractions of the aqueous phase with an equal volume of chloroform. RNA was precipitated in 2 volumes of 100% isopropanol, and the pellet was collected by centrifugation. First strand cDNA was produced from 1 μg of DNase-treated total RNA using Superscript II reverse transcriptase (Invitrogen) and an oligo(dT) primer. To determine CrCAX1 expression in transgenic Arabidopsis lines or yeast cells, RT-PCR was performed using sCrCAX1F and CrCAX1TnTR primers and actin or tubulin primers as a constitutive control. To determine relative CrCAX1 expression in

526 JOURNAL OF BIOLOGICAL CHEMISTRY
metal-treated Chlamydomonas cells, RT-PCR was performed using CrCAX1TN TF and CrCAX1TN TR primers and 18 S rRNA primers as a constitutive PCR. PCR products were amplified with a 60 °C annealing temperature for 25 cycles and detected with ethidium bromide.

In Vitro Transcription and Translation—CrCAX1-pSP64, CrCAX1-M1A-pSP64, AtCAX1-pSP64, and AtCAX1-M37A-pSP64 constructs were transcribed and translated (TnT) in vitro, in the presence of 35S-labeled Met, using a TnT-coupled wheat germ cell-free system (Promega). TnT reactions were separated on a 6% denaturing polyacrylamide gel, dried, and analyzed using a BAS-1800 phosphorimaging device (Fuji Photo Film).

Vacuolar Membrane Vesicle Isolation and Transport Measurements—Vacuolar-enriched membrane vesicles were isolated from K667 or nhx1Δ yeast cells expressing sCrCAX1 and CrCAX1, as described previously (27). Ca2+/H+ exchange activity was determined by measuring pH gradient-dependent 45Ca2+ uptake into K667-derived vesicles, as described previously (28). Na+/H+ exchange activity was determined in nhx1Δ-derived vesicles by measuring Na+-dependent acidine orange fluorescence quench, essentially as described in Ref. 29 using a Jasco FP750 fluorescence spectrometer.

GFP Visualization—Cells expressing CrCAX1-GFP, sCrCAX1-GFP, or GFP alone were imaged by epifluorescence microscopy using a Leica DMR microscope and an enhanced GFP filter cube (Chroma Technology). The fluorescent yeast vacuole marker stain carboxy-5- and 6-carboxy-2′,7′-dichloro fluorescein diacetate (30) was used to visualize yeast vacuoles and was detected using a L4 fluorescence isothiocyanate filter cube (Leica Microsystems).

RESULTS

Identification of a CAX Gene from C. reinhardtii—Analysis of sequence from Chlamydomonas expressed sequence tag cDNA clones and the completed genome sequence has identified the presence of open reading frames homologous to Arabidopsis and S. cerevisiae CAX genes. One of these annotated open reading frames designated CrCAX1 (JGI I.D. 157233) was analyzed further by comparison with known CAX genes. Following DNA and amino acid sequence alignment, the accuracy of the sequence prediction was ascertained. PCR primers were designed to amplify the predicted CrCAX1 cDNA from C. reinhardtii 137C+ (wild type) RNA isolated from cells grown in liquid TAP medium. CrCAX1 (accession number FM253128) has a 1344-bp open reading frame and encodes a protein of 447 amino acids. Sequence comparisons of CrCAX1 found highest sequence identity with fungal and protozoan CAX genes (40% sequence identity with PbCAX1 from Plasmodium berghei, 35% identity with ScVCX1, and 30% identity with AtCAX1; supplemental Table S2). However, sequence alignment and transmembrane span prediction comparison of CrCAX1 with CAX genes from fungi and higher plants indicated that CrCAX1 encodes a protein with similar topology to higher plant CAX transporters including a long hydrophilic tail predicted to be cytosolic and 11 predicted transmembrane spans (supplemental Fig. S1). The CrCAX1 hydrophilic N-terminal tail sequence is longer (94 amino acids) than that of ScVCX1 (32 amino acids) and AtCAX1 (67 amino acids).

Detailed phylogenetic analysis was recently performed using 138 CAX gene sequences that were previously cloned or identified from genome sequences of bacteria, fungi, plants, protozoa, and lower vertebrates (9). This analysis indicated that CAX genes could be separated into three main phylogenetic groups (Type I, Type II, and Type III). Arabidopsis and S. cerevisiae CAX genes formed part of the Type I group, which was further divided into subtypes (Type I-A to Type I-H). Phylogenetic analysis performed with the CrCAX1 sequence and using the classification of Shigaki et al. (9) indicates that CrCAX1 falls within clade Type I-C, which includes CAX genes from protozoa, including Plasmodium falciparum and Cryptosporidium hominis, the red algae Thalassiosira pseudonana (Fig. 1), and the diatom Phaeodactylum tricornutum (data not shown).

In Vitro Translation Analysis of CrCAX1—To confirm that translation of CrCAX1 did initiate from the predicted first AUG start codon (Met1) rather than the downstream AUG (Met3), an in vitro cell-free transcription and translation experiment was performed. For comparison translation analysis was performed using the previously characterized AtCAX1. To improve translation efficiency, partial length constructs were used that encode the first 180 amino acids of CrCAX1 and the first 67 amino acids of AtCAX1. A single 35S-labeled protein band of the expected size was identified for CrCAX1, confirm-
A Chlamydomonas Cation/H⁺ Exchanger

ing that translation occurred solely from Met¹ (Fig. 2A), unlike for AtCAX1 where some translation (16% of total AtCAX1 protein) initiated from the second AUG (Met³⁷) (Fig. 2B). Analysis of an AtCAX1-M37A mutant confirmed that the lower protein band was due to initiation from Met³⁷. When CrCAX1 Met¹ was mutated to Ala, translation was able to initiate from Met⁹¹, and a smaller protein product was observed (Fig. 2A).

**Functional Analysis of CrCAX1 in S. cerevisiae**—To assess the transport function of CrCAX1, a yeast heterologous expression approach was used. This has been extremely successful previously to ascertain the function of higher plant cation/H⁺ exchangers (11, 14, 31). The K667 yeast mutant lacks vacuolar Ca²⁺/H⁺ exchange and Ca²⁺-ATPase activity and is therefore unable to sufficiently sequester Ca²⁺ into the vacuole in response to Ca²⁺ stress and cannot grow on high Ca²⁺-containing media (10). Many plant Ca⁺⁺ transporters including AtCAX1 possess an N-terminal regulatory domain that inhibits Ca²⁺ transport activity when expressed in a heterologous system (14); therefore for comparison an N-terminal truncation mutant of CrCAX1 (termed sCrCAX1) was generated in which translation was initiated from the first downstream AUG, which encodes Met⁹¹ immediately prior to the first predicted transmembrane span (supplemental Fig. S1). C-terminally tagged CrCAX1-GFP and sCrCAX1-GFP constructs were also generated and expressed in yeast. Both CrCAX1-GFP and sCrCAX1-GFP localized equivalently to the vacuole (Fig. 3). As predicted transmembrane proteins, CrCAX1 and sCrCAX1 are likely to be located at the vacuolar membrane, although imaging by epifluorescence microscopy was unable to unequivocally confirm this.

Comparison of CrCAX1 and sCrCAX1 in yeast grown on yeast extract-peptone-dextrose medium supplemented with a high concentration (200 mM) of CaCl₂ found that sCrCAX1 could efficiently suppress the Ca²⁺ hypersensitivity of K667 yeast in a manner equivalent to N-terminally truncated AtCAX1 (sAtCAX1) (Fig. 4A). Full-length CrCAX1 could only weakly suppress the Ca²⁺-sensitive phenotype. Transformed K667 yeast growth was evaluated on other metal conditions. CrCAX1 or sCrCAX1 could not suppress sensitivity to Mn²⁺ or Zn²⁺ but could provide some Cd²⁺ tolerance to yeast (data not shown). Growth of yeast on 0.75 mM NaCl was significantly enhanced following expression of either CrCAX1 or sCrCAX1, but there was no difference between full-length and truncated CrCAX1 in the ability to provide Na⁺ tolerance (Fig. 4A). Likewise, slight tolerance to 2.5 mM CoCl₂ was observed by expression of either CrCAX1 or sCrCAX1. sAtCAX1 was unable to provide tolerance to either Na⁺ or Co²⁺ stress, indicating that tolerance was not linked to altered Ca²⁺ homeostasis. The Ca²⁺ and Na⁺ tolerance by CrCAX1 and sCrCAX1 was clearly due to their expression in the yeast (Fig. 4B). To confirm the Na⁺ tolerance phenotype of CrCAX1, plasmids were transformed into AXT3 yeast, which lacks plasma membrane and endomembrane Na⁺ transporters and is hypersensitive to Na⁺ (25). Transformed AXT3 yeast strains were grown in liquid arginine-phosphate-dextrose medium supplemented with various concentrations of NaCl, and cell growth was measured. Both sCrCAX1 and CrCAX1 could provide AXT3 yeast with significant tolerance to high concentrations of NaCl, whereas AXT3 expressing empty vector or sAtCAX1 was unable to grow on this salt medium (Fig. 4C).

**Ca²⁺/H⁺ Exchange Activity of CrCAX1**—To confirm that growth of yeast on Ca²⁺-containing medium was due to enhanced vacuolar Ca²⁺/H⁺ exchange activity, ΔpH-dependent ⁴⁵Ca²⁺ uptake in the presence of the Ca²⁺-ATPase inhibitor vanadate was measured in tonoplast-enriched membrane vesicles isolated from K667 yeast expressing sCrCAX1. Ca²⁺/H⁺ exchange activity was determined for sCrCAX1, and this ⁴⁵Ca²⁺ uptake was significantly inhibited in the presence of 5 μM of protonophore FCCP (Fig. 5A). In contrast, Ca²⁺/H⁺ exchange activity mediated by full-length CrCAX1 was significantly reduced (by 78%) but was detectable over basal levels (Fig. 5B). A competition ⁴⁵Ca²⁺ uptake assay was performed to assess the substrate specificity of sCrCAX1. A 10-fold excess concentration of nonradioactive Ca²⁺ and Cd²⁺ could signifi-
cantly inhibit 10 μM 45Ca2+ uptake (Fig. 5C). A 10-fold excess of Na+ (as 0.1 mM NaCl), in addition to 0.1 mM NaN3, present in the uptake buffer, making the total Na+ concentration 0.2 mM, could inhibit ~60% of 45Ca2+ uptake, whereas Ca2+ and Cd2+ could inhibit ~85–95% of uptake. A 10-fold excess of Co2+ could only marginally inhibit sCrCAX1 45Ca2+ uptake.

Expression of CrCAX1 in Arabidopsis—CrCAX1 and sCrCAX1 were expressed highly under the control of the constitutive CaMV 35S promoter in wild type Arabidopsis Col-0 (Fig. 6B) to assess the impact that this cation transporter had to higher plant stress tolerance. CrCAX1- or sCrCAX1-expressing plants had no obvious alteration in morphology in nonstressed conditions. No phenotypic changes were observed between empty vector control and CAX-expressing lines under most ion stress conditions tested, except when grown under salt stress conditions. Multiple CrCAX1 and sCrCAX1 lines were able to provide significant tolerance when seedlings were germinated and grown on half-strength Murashige and Skoog medium containing 100 mM NaCl, as observed by enhanced growth and increased chlorophyll content compared with the vector control lines (Fig. 6, A and C). No significant difference was observed between the CrCAX1 and sCrCAX1 lines.

Na+/H+ Exchange Activity of CrCAX1—The salt tolerance phenotype of yeast and Arabidopsis seedlings expressing CrCAX1, coupled with the ability of Na+ to inhibit Ca2+/H+ exchange activity, suggested that Na+ may also be a substrate for transport. Na+/H+ exchange activity was assessed in vacuolar-enriched membrane vesicles isolated from nhx1Δ yeast lacking the endogenous endomembrane Na+/H+ exchanger NHX1 and expressing sCrCAX1 and CrCAX1. Na+-dependent H+ flux was determined by the fluorescence quenching of acridine orange. After H+ pumping into the vesicles was initiated by the addition of Mg2+-ATP, fluorescence quenching was observed until a steady-state pH gradient was obtained. The addition of 5 μM of the protonophore FCCP or 0.1% Triton X-100 completely abolished the pH gradient (data not shown). In the vector control vesicles from nhx1Δ yeast, basal levels of exchange were observed even until a steady-state pH gradient was obtained. The

**FIGURE 4. Ca2+, Na+, and Co2+ tolerance of yeast mediated by CrCAX1.** A, saturated liquid cultures of K667 (cnb1 pmc1 vcr1) yeast expressing CrCAX1 in piHGpd, N-terminally truncated sCrCAX1 in piHGpd, sAtCAX1 in piHGpd, and empty vector alone (piHGpd) were serially diluted to the cell densities as indicated and then spotted onto selection medium lacking histidine (−his) and yeast extract-peptone-dextrose medium containing 200 mM CaCl2, 0.75 mM NaCl, and 2.5 mM CoCl2. Yeast growth at 30 °C is shown after 4 days. A representative experiment is shown. B, RT-PCR analysis of CrCAX1 and sCrCAX1 expression in K667 yeast compared with yeast expressing sAtCAX1 and empty vector alone (piHGpd). Transport measurements were determined in the presence of 0.1 mM NaN3, 10 mM KCl, 1 mM ATP, 1 mM MgSO4, and 0.2 mM orthovanadate (Ca2+-ATPase inhibitor). 45Ca2+ uptake in the presence or absence of 5 μM of the protonophore FCCP is shown. B, ΔpH-dependent uptake of 10 μM 45Ca2+ into vacuolar-enriched membrane vesicles isolated from K667 yeast expressing CrCAX1 and CrCAX1 measured over a 15-min time course. Ca2+/H+ exchange was measured as described in A. Ca2+ uptake values are shown after subtraction of the FCCP protonophore background values and empty vector control basal level. C, competition analysis of sCrCAX1 Ca2+ uptake. Ca2+/H+ exchange by sCrCAX1 as performed in A was measured after 10 min in the presence of 10-fold excess concentrations of indicated nonradioactive metals. All of the data represent the means of two to four replications from three independent membrane preparations, and the bars indicate S.E.

**FIGURE 5. Ca2+/H+ exchange activity of CrCAX1.** A, uptake of 10 μM 45Ca2+ measured after 10 min into vacuolar-enriched membrane vesicles isolated from K667 (cnb1 pmc1 vcr1) yeast expressing sCrCAX1 and empty vector (piHGpd). Transport measurements were determined in the presence of 0.1 mM NaN3, 10 mM KCl, 1 mM ATP, 1 mM MgSO4, and 0.2 mM orthovanadate (Ca2+-ATPase inhibitor). 45Ca2+ uptake in the presence or absence of 5 μM of the protonophore FCCP is shown. B, ΔpH-dependent uptake of 10 μM 45Ca2+ into vacuolar-enriched membrane vesicles isolated from K667 yeast expressing CrCAX1 and CrCAX1 measured over a 15-min time course. Ca2+/H+ exchange was measured as described in A. Ca2+ uptake values are shown after subtraction of the FCCP protonophore background values and empty vector control basal level. C, competition analysis of sCrCAX1 Ca2+ uptake. Ca2+/H+ exchange by sCrCAX1 as performed in A was measured after 10 min in the presence of 10-fold excess concentrations of indicated nonradioactive metals. All of the data represent the means of two to four replications from three independent membrane preparations, and the bars indicate S.E.
ing CrCAX1 and sCrCAX1 (Fig. 7). No significant K\(^+/H^+\) or Li\(^+/H^+\) exchange activity was observed. In the K667 yeast background lacking the Ca\(^2+/H^+\) exchanger ScVCX1 but with the endogenous Na\(^+/H^+\) exchanger ScNHX1 still present, significant background Na\(^+/H^+\) exchange activity was measured, although the expression of CrCAX1 did slightly enhance Na\(^+/H^+\) exchange activity (data not shown).

Transcriptional Regulation of CrCAX1 by Metal Stress Conditions—Chlamydomonas cells were grown in TAP medium supplemented with various metal salts, and CrCAX1 mRNA transcript expression was determined following 16 h of treatment by RT-PCR. CrCAX1 expression was not enhanced by any metal treatment tested (data not shown). Following treatment with a range of CaCl\(_2\) concentrations, there was no change in CrCAX1 expression until treatment with 50 mM CaCl\(_2\) and higher when a significant reduction of transcript was observed (Fig. 8A). Excess concentrations of NaCl above 100 mM similarly caused a significant reduction in CrCAX1 expression, but excess concentrations of other metal salts such as KCl did not. Despite a reduction in CrCAX1 transcript in 100 mM NaCl, growth of Chlamydomonas in this medium was not significantly impaired, although cell growth and chlorophyll content was reduced when cells were grown in 200 mM NaCl and in the 50 mM and 100 mM CaCl\(_2\) conditions (Fig. 8B).

DISCUSSION

Carefully regulated Ca\(^{2+}\) partitioning is an essential requirement in all cells to prevent toxic accumulation of Ca\(^{2+}\) in the cytosol but also to allow Ca\(^{2+}\) to fulfill a role as a cellular signal.
A complement of Ca$^{2+}$-binding proteins, Ca$^{2+}$ influx channels, and Ca$^{2+}$ efflux transporters comprising of Ca$^{2+}$-ATPases and ion-coupled Ca$^{2+}$ exchangers, allow cytosolic Ca$^{2+}$ levels to be dynamically shaped (1). In addition, various Ca$^{2+}$ sensing and effector proteins including Ca$^{2+}$-dependent kinases and phosphatases allow the generated Ca$^{2+}$ signals to be decoded and mediate downstream responses (32). Combinations of these components are likely to be present in all eukaryotic cells where Ca$^{2+}$-mediated signaling occurs, and indeed genomic sequence and proteomic analyses have begun to identify genes and proteins likely to be involved in Ca$^{2+}$ homeostasis and signaling in Chlamydomonas (21, 33). These include putative Ca$^{2+}$-ATPases, Ca$^{2+}$ exchangers, and Ca$^{2+}$ channels, some of which are similar to animal-type transporters not found in higher plants (21, 33). To date, all of these transporters are uncharacterized, one exception being a light-activated, Ca$^{2+}$-permeable cation channel COP4 that plays a role in photoreceptor mediated Ca$^{2+}$ signaling (34). In this report we have described the first characterization of a Chlamydomonas Ca$^{2+}$ efflux transporter and described the first analysis of a Type I-C CAX transporter (9).

An unexpected and intriguing feature of the CrCAX1 transporter is that in addition to having Ca$^{2+}$ transport activity, this exchanger could also transport Na$^{+}$ (Fig. 7), and this Na$^{+}$/H$^{+}$ exchange activity allowed CrCAX1 to provide significant tolerance to NaCl when expressed in yeast or plants (Figs. 4 and 6). The physiological significance of putative Na$^{+}$ transport activity by CrCAX1 in Chlamydomonas is likely to be due to the requirement of robust mechanisms of salt stress tolerance in a unicellular organism, particularly one such as Chlamydomonas that lives in aquatic environments with potentially harsh ionic conditions. By analogy with other unicellular organisms such as yeast, it is likely that Chlamydomonas will have multiple pathways for Na$^{+}$ extrusion from the cytosol. For example, genome sequence analysis indicates that Chlamydomonas also has NHX-type Na$^{+}$/H$^{+}$ exchangers (data not shown), although their function in Na$^{+}$ transport and tolerance has yet to be demonstrated. Further work will need to elucidate the relative roles of both NHX- and CAX-mediated Na$^{+}$ transport in Chlamydomonas.

Although some CAX transporters such as ScVCX1 are specific for Ca$^{2+}$ (10), others such as AtCAX2 have a broader substrate specificity and can transport a variety of divalent cations like Ca$^{2+}$, Mn$^{2+}$, Cd$^{2+}$, and possibly Zn$^{2+}$ (12). CrCAX1 is the first eukaryotic CAX transporter that has been shown to directly efflux both divalent and monovalent cations. There is some precedence for Na$^{+}$ transport by CAX-type exchangers like ScVNX1 (13), indicating that Na$^{+}$/H$^{+}$ exchange activity is not restricted to NHX-type exchangers (7). Similarly, a plant CAX transporter from soybean, GmCAX1, was previously shown to provide slight tolerance to Na$^{+}$ when expressed in Arabidopsis, but no direct Na$^{+}$ transport activity was demonstrated (20). In addition, some bacterial CAX- and NHX-type exchangers may transport both Na$^{+}$ and Ca$^{2+}$, as with chaA from Escherichia coli (35, 36), Aa-caxA from Alkalimonas amylolytica N10 (37), and ApNhaP from Aphanthece halophytica (38).

We might expect that the ability to bind and efflux Ca$^{2+}$ and Na$^{+}$ is determined by the amino acid sequence. Previous analyses of plant CAX transporters have identified two highly conserved sequence repeats named c-1 and c-2 (supplemental Fig. S1) that have been suggested to function as cation selectivity filters (39). These regions are similar and show sequence conservation with the α-1 and α-2 regions required for cation binding in mammalian Na$^{+}$/Ca$^{2+}$ exchangers (8, 39). Specific residues within the c-1 and c-2 regions have been shown to be conserved for Ca$^{2+}$ transport (39, 40), and these residues are required for Ca$^{2+}$ transport (39, 40), and these residues are conserved in the CrCAX1 c-1 and c-2 regions (Fig. 9). It is unknown whether these domains will also determine Na$^{+}$ binding. A comparison of the CrCAX1 c-1 and c-2 region with other CAX transporters, none of which have been shown to transport Na$^{+}$, highlights two residues in CrCAX1 that differ from the consensus sequence for these regions: Met$^{770}$ in c-1, which is Ile in the consensus sequence, and Cys$^{350}$ in c-2, which is Ala in the consensus sequence (Fig. 9). Future analysis will be able to determine whether these differences determine functional characteristics to CrCAX1. The fact that Na$^{+}$ transport is not regulated by the putative N-terminal auto-regulatory domain may suggest differences in the mechanisms of Na$^{+}$ and Ca$^{2+}$ transport. Thus, other domains of CrCAX1 may determine Na$^{+}$ selectivity and transport. A cytosolic region called the acidic domain between transmembrane spans 6 and 7 has been suggested to be involved in Ca$^{2+}$ binding (35), possibly analogous to the role of the cytosolic Ca$^{2+}$-binding β repeat domains of Na$^{+}$/Ca$^{2+}$ exchanger proteins (41). The CrCAX1 acidic domain is shorter and possesses fewer negatively charged residues than in AtCAX1 or ScVCX1 (supplemental Fig. S1).
Whether this region plays a role in cation selectivity also remains to be determined.

The identification and analysis of CrCAX1 allows further functional and regulatory comparison between the CAX transporters, particularly with those that have been studied in detail: *S. cerevisiae* Vcx1 and *Arabidopsis* AtCAX1. Despite having greater sequence similarity to ScVcx1 than AtCAX1 (Fig. 1 and supplemental Table S2), CrCAX1 has a broader substrate specificity than ScVcx1, showing both Ca\(^{2+}\)/H\(^+\) and Na\(^+\)/H\(^+\) exchange activity, and possibly the ability to transport Co\(^{2+}\) and Cd\(^{2+}\) (Figs. 5 and 7). A particularly intriguing feature of CrCAX1 is the structural similarities with the higher plant CAX transporters with regard to the extended hydrophilic N-terminal tail (supplemental Fig. S1). Comparison of full-length and an N-terminally truncated CrCAX1 mutant indicates that the autoinhibitory regulation mechanism is shared between *Chlamydomonas* and *Arabidopsis*, suggesting that this mechanism might be conserved in CAX transporters in all algae and higher plant lineages. Analysis of CAX transporters from mung bean and rice also show N-terminal regulation (31, 42), thus supporting this view. However, the N-terminal domain of CrCAX1 only regulated Ca\(^{2+}\)/H\(^+\) exchange activity but not Na\(^+\)/H\(^+\) exchange, suggesting that the autoinhibitory mechanism is more complex than just blocking cation transport.

Like many plant CAX transporters, CrCAX1 was transcriptionally regulated by excess ions (Ca\(^{2+}\) and Na\(^+\)) in the growth medium (Fig. 8A). AtCAX1 mRNA transcript level is significantly increased when seedlings are grown in high Ca\(^{2+}\) medium (18), presumably to allow sequestration of Ca\(^{2+}\) into the plant vacuole and provide Ca\(^{2+}\) tolerance. However, CrCAX1 transcript decreased as cells were grown in excess Ca\(^{2+}\). This is analogous to the observed inhibition of ScVcx1 by calcineurin in response to elevated cytosolic Ca\(^{2+}\) (10). Although CrCAX1 can clearly play a role in providing tolerance to excess concentrations of Ca\(^{2+}\) and Na\(^+\), as shown by the yeast expression experiments, CrCAX1 levels may be moderated *in vivo* to prevent excessive accumulation of ions into the cell. This may be important for unicellular organisms that have a finite capacity for intracellular ion storage. This may also suggest that CrCAX1 plays an important role in the generation of Ca\(^{2+}\) signals rather than solely for providing Ca\(^{2+}\) tolerance.

These studies also suggest that there may be divergent mechanisms of regulation between CrCAX1 and AtCAX1 at the level of translation. The *in vitro* transcription and translation experiment confirmed that translation of CrCAX1 did initiate from the predicted AUG start codon at position 1 with no translation initiation from the downstream AUG codon (Fig. 2). As a comparison, we performed the same experiment with an AtCAX1 construct, which has two AUG codons in close proximity at the 5′ end, with a second AUG 109 bp from the first, encoding a Met residue at position 37 (supplemental Fig. S1). Although the majority of translation initiated from the first AUG (M1), a small proportion (16%) of translation initiated from the second AUG (M37). Such alternate translation initiation from a single mRNA transcript has previously been observed. Alternate initiation of *Arabidopsis* DNA ligase 1 alters the intracellular targeting of the AtLIG1 protein (23). The experiment in this report suggests that alternate translation initiation could also be a means to regulate activity of some proteins, such as AtCAX1.

We have previously demonstrated that removal of the first 36 residues of AtCAX1 up to Met\(^{37}\) significantly activates Ca\(^{2+}\) transport activity (14); thus this apparent alteration in translation of AtCAX1 could signify an additional level of regulation. Further experiments will be required to determine whether AtCAX1 is alternatively translated in planta.

Expression of CrCAX1-GFP in yeast showed localization at the vacuole, consistent with the ability of CrCAX1 to suppress the Ca\(^{2+}\)-hypersensitive phenotype of a yeast mutant lacking two vacuolar Ca\(^{2+}\) transport pathways. Unfortunately we were unsuccessful in attempts to observe expression of CrCAX1-GFP in *Chlamydomonas* despite using a codon-optimized GFP tag. However, localization of CrCAX1-GFP to the tonoplast in yeast and *Arabidopsis* (data not shown) indicates that CrCAX1 is similarly localized to a vacular membrane *in vivo*. In addition, CrCAX1 is predicted to be vacuolar localized by computer-based prediction (22). Antibodies raised against plant tonoplast proteins (vacuolar H\(^+\)-pyrophosphatase) detect proteins at the tonoplast in *Chlamydomonas* (43), suggesting some conservation of protein vacuolar targeting between *Chlamydomonas* and higher plants. *Chlamydomonas* are not as vacuolated as typical plant or yeast cells. They possess two contractile vacuoles that are involved in osmoregulation and numerous small acidic vacuoles that appear similar to acidocalcisomes (43, 44). Acidocalcisomes are acidic organelles that contain a matrix of pyrophosphate and polyphosphates bound to Ca\(^{2+}\) (45). They have been identified and characterized predominantly in protozoan parasites and are proposed to play a role in cation storage and adaptation to environmental stress. The *Chlamydomonas* acidocalcisome membrane, like that of the plant vacuolar membrane, contains both a H\(^+\)-pyrophosphatase and a V-type H\(^+\)-ATPase that drive H\(^+\) transport into the organelle and establish a pH gradient (43). This gradient would therefore be sufficient to energize cation/H\(^+\) exchange by CrCAX1 of Ca\(^{2+}\) and Na\(^+\) into the acidocalcisome. It is also interesting to note that the genomes of many of the protozoan species that store Ca\(^{2+}\) in acidocalcisomes including *P. falciparum* possess single CAX genes that cluster in the *Type I-C clade* alongside *Arabidopsis* and *higher plants*. *Chlamydomonas* acidocalcisome, like that of the plant vacuolar membrane, shows the conservation of protein vacuolar targeting between *Chlamydomonas* and higher plants. *Chlamydomonas* acidocalcisomes are acidic organelles that contain a matrix of pyrophosphate and polyphosphates bound to Ca\(^{2+}\) (45). They have been identified and characterized predominantly in protozoan parasites and are proposed to play a role in cation storage and adaptation to environmental stress. The *Chlamydomonas* acidocalcisome membrane, like that of the plant vacuolar membrane, contains both a H\(^+\)-pyrophosphatase and a V-type H\(^+\)-ATPase that drive H\(^+\) transport into the organelle and establish a pH gradient (43). This gradient would therefore be sufficient to energize cation/H\(^+\) exchange by CrCAX1 of Ca\(^{2+}\) and Na\(^+\) into the acidocalcisome. It is also interesting to note that the genomes of many of the protozoan species that store Ca\(^{2+}\) in acidocalcisomes including *P. falciparum* possess single CAX genes that cluster in the *Type I-C clade* alongside *Arabidopsis* (1). We can rule out the localization of CrCAX1 at another membrane such as the contractile vacuole.

We have provided evidence that *Chlamydomonas* possesses a CrCAX1 protein that can transport both Ca\(^{2+}\) and Na\(^+\) by a cation/H\(^+\) exchange mechanism and have suggested a role for this transporter in providing tolerance to cation stress based on heterologous expression experiments in yeast and *Arabidopsis*. The identification of CrCAX1 as a Na\(^+\) transporter may help understand osmotic stress regulation by this unicellular organism. In addition, the identification of a *Chlamydomonas* Ca\(^{2+}\) transporter gene provides the potential to advance our understanding of Ca\(^{2+}\) signaling in this model eukaryote. *Chlamydomonas* is already established as an excellent model for Ca\(^{2+}\) signaling studies. Ca\(^{2+}\) signaling is required in phototaxis and aspects of flagellar function including excision require specific Ca\(^{2+}\) signals (34, 46–48). It was recently demonstrated that the efficient delivery of Ca\(^{2+}\) indicator dyes into *Chlamydomonas* cells provides the ability to monitor cytosolic Ca\(^{2+}\) oscillations, such as in response to deflagellation (48, 49). Further charac-
terization of CrCAX1 and homologous Ca\(^{2+}\) transporters coupled with the ability to monitor Ca\(^{2+}\) changes should further our knowledge in the generation and specificity of these Ca\(^{2+}\) signals in this species.

Acknowledgments—We thank Dr. Toshiro Shigaki for advice on phylogenetic analysis of CAX genes and Dr. Toshiro Shigaki and Dr. Kendall Hirschi for critical comments to the manuscript. We are grateful to Dr. Anil Day for providing the GFP plasmid.

REFERENCES

1. McAinsh, M. R., and Pittman, J. K. (2008) *New Phytol.*, in press
2. Maser, P., Gierth, M., and Schroeder, J. I. (2002) *Plant Soil* **247**, 43–54
3. Lang, F. (2007) *J. Am. Coll. Nutr.* **26**, 6135–6235
4. Spijkerman, E., Barua, D., Gerloff-Elias, A., Kern, J., Gaedke, U., and Heckathorn, S. A. (2007) *Extremophiles* **11**, 551–562
5. Tanaka, S., Suda, T., Ikeda, K., Ono, M., Miyasaka, H., Watanabe, M., Sasaki, K., and Hirata, K. (2007) *FEBS Microbiol. Lett.* **271**, 48–52
6. Mendez-Alvarez, S., Leisinger, U., and Eggen, R. I. (1999) *Int. Microbiol.* **2**, 15–22
7. Luo, G. Z., Wang, H. W., Huang, J., Tian, A. G., Wang, Y. J., Zhang, J. S., and Chen, S. Y. (2005) *Plant Physiol.* **137**, 428–446
8. Hanikenne, M., Kramer, U., Demoulin, V., and Baurain, D. (2005) *Plant Physiol.* **137**, 428–446
9. Sunderland, P. A., West, C. E., Waterworth, W. M., and Bray, C. M. (2006) *Plant J.* **47**, 356–367
10. Primavesi, L. F., Wu, H. X., Mudd, E. A., Day, A., and Jones, H. D. (2008) *Transgenic Res.* **17**, 529–543
11. Shigaki, T., Barkla, B. J., Miranda-Vergara, M. C., Zhao, J., Pantoja, O., and Takabe, T. (2001) *J. Biol. Chem.* **276**, 356–367
12. Quarmby, L. M., and Hartzell, H. C. (1994)
13. Apse, M. P., Sottosanto, J. B., and Blumwald, E. (2003) *Plant J.* **36**, 229–239
14. Roberts, C. J., Raymond, C. K., Yamashiro, C. T., and Stevens, T. H. (1991) *Methods Enzymol.* **194**, 644–661
15. Kamiya, T., Akahori, T., and Maeshima, M. (2005) *Plant Cell Physiol.* **46**, 1735–1740
16. Apse, M. P., Sottosanto, J. B., and Blumwald, E. (2003) *Plant J.* **36**, 229–239
17. Berridge, M. J., Bootman, M. D., and Roderick, H. L. (2003) *Nat. Rev. Mol. Cell. Biol.* **4**, 517–529
18. Wheeler, G. L., and McAinsh, M. R. (2006)
19. Wei, Y., Liu, J., Ma, Y. H., and Krulwich, T. A. (2007) *J. Bacteriol.* **189**, 2975–2980
20. Hanikenne, M., Kramer, U., Demoulin, V., and Baurain, D. (2005) *Plant Physiol.* **137**, 428–446
21. Sunderland, P. A., West, C. E., Waterworth, W. M., and Bray, C. M. (2006) *Plant J.* **47**, 356–367
22. Primavesi, L. F., Wu, H. X., Mudd, E. A., Day, A., and Jones, H. D. (2008) *Transgenic Res.* **17**, 529–543
23. Quintero, F. J., Blatt, M. R., and Pardo, J. M. (2000) *FEBS Lett.* **471**, 224–228
24. Porra, R. J., Thompson, W. A., and Kriedemann, P. E. (1989) *Biochim. Biophys. Acta* **975**, 384–394
25. Pittman, J. K., Cheng, N. H., Shigaki, T., Kunta, M., and Hirschi, K. D. (2004) *Mol. Microbiol.* **54**, 1104–1116
26. Pittman, J. K., Shigaki, T., and Hirschi, K. D. (2005) *FEBS Lett.* **579**, 2648–2656
27. Hanikenne, M., Kramer, U., Demoulin, V., and Baurain, D. (2005) *Plant Physiol.* **137**, 428–446
28. Hanikenne, M., Kramer, U., Demoulin, V., and Baurain, D. (2005) *Plant Physiol.* **137**, 428–446
29. Apse, M. P., Sottosanto, J. B., and Blumwald, E. (2003) *Plant J.* **36**, 229–239
30. Roberts, C. J., Raymond, C. K., Yamashiro, C. T., and Stevens, T. H. (1991) *Methods Enzymol.* **194**, 644–661
31. Kamiya, T., Akahori, T., and Maeshima, M. (2005) *Plant Cell Physiol.* **46**, 1735–1740
32. Apse, M. P., Sottosanto, J. B., and Blumwald, E. (2003) *Plant J.* **36**, 229–239
33. Kamiya, T., Akahori, T., and Maeshima, M. (2005) *Plant Cell Physiol.* **46**, 1735–1740
34. Ivey, D. M., Guffanti, A. A., Zemsky, J., Pinner, E., Karpel, R., Padan, E., Schuldiner, S., and Krulwich, T. A. (1993) *J. Biol. Chem.* **268**, 11296–11303
35. Ohyama, T., Igarashi, K., and Kobayashi, H. (1994) *J. Bacteriol.* **176**, 4311–4315
36. Wei, Y., Liu, J., Ma, Y. H., and Krulwich, T. A. (2007) *Microbiology* **153**, 2168–2179
37. Waditee, R., Hinbino, T., Tanaka, Y., Nakamura, T., Incharoensakdi, A., and Takabe, T. (2001) *J. Biol. Chem.* **276**, 36931–36938
38. Kamiya, T., and Maeshima, M. (2004) *J. Biol. Chem.* **279**, 812–819
39. Shigaki, T., Barkla, B. J., Miranda-Vergara, M. C., Zhao, J., Pantoja, O., and Hirschi, K. D. (2005) *J. Biol. Chem.* **280**, 30136–30142
40. Lytton, J. (2007) *Biochem. J.* **406**, 365–382
41. Pittman, J. K., Sreevidya, C. S., Shigaki, T., Ueoka-Nakanishi, H., and Hirschi, K. D. (2002) *Plant Physiol.* **130**, 1054–1062
42. Ruiz, F. A., Marchesini, N., Seufferheld, M., Govindjee, and Docampo, R. (2001) *J. Biol. Chem.* **276**, 46196–46203
43. Komine, Y., Eggink, L. L., Park, H. S., and Hoober, J. K. (2000) *Plant Cell Physiol.* **41**, 987–990
44. Bothwell, J. H. F., Brownlee, C., Hetherington, A. M., Ng, C. K. Y., Wheeler, G. L., and McAinsh, M. R. (2006) *Plant J.* **46**, 327–335