Published
Journal of Biological Chemistry 279 (1) : 812-819 (2004)

~~~~~~

Residues in Internal Repeats of the Rice Cation/H^+ Exchanger are Involved in the Transport and Selection of Cations*

Takehiro Kamiya and Masayoshi Maeshima‡

From the Laboratory of Cell Dynamics, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan

To whom all correspondence should be sent:
Masayoshi Maeshima
Fax.: +81-52-789-4096; E-mail: maeshima@agr.nagoya-u.ac.jp

Running title: Functional Residues of the Rice Cation/H^+ Exchanger

(Summary)

In plants, the cation/H^+ exchanger translocates Ca^{2+} and other metal ions into vacuoles using the H^+ gradient formed by H^+-ATPase and H^+-pyrophosphatase. Such exchangers carrying 11 transmembrane domains have been isolated from plants, yeast and bacteria. In this study, multiple sequence alignment of several cation/H^+ exchangers revealed the presence of 36 residue highly conserved regions between the 3rd and 4th, and the 8th and 9th transmembrane domains. These two repetitive motifs are designated repeats c-1 and c-2. Using site-directed mutagenesis, we generated 31 mutations in the repeats of the *Oryza sativa* cation/H^+ exchanger, which translocates Ca^{2+} and Mn^{2+}. Mutant exchangers were expressed in a *Saccharomyces cerevisiae* strain that was sensitive to Ca^{2+} and Mn^{2+} due to the absence of vacuolar Ca^{2+}-ATPase and the Ca^{2+}/H^+ exchanger. Mutant exchangers were classified into 6 classes according to their tolerance to Ca^{2+} and Mn^{2+}. For example, the class III mutants had no tolerance to either ion and the class IV mutants had tolerance only to Ca^{2+}. The biochemical function of each residue was estimated. We investigated the membrane topology of the repeats using a method combining cysteine mutagenesis with sulfhydryl reagents. Our results suggest that the repeat c-1 re-enters the membrane from the vacuolar lumen side and forms a solution-accessible region. Furthermore, several residues in repeats c-1 and c-2 were found to be conserved in animal Na^+/Ca^{2+} exchangers. Finally, we suggest that these re-entrant repeats may form a vestibule or filter for cation selection.

Footnotes

‡ Corresponding author: E-mail, maeshima@agr.nagoya-u.ac.jp; Fax, +81-52-789-4096.

[1] The abbreviations used are: AMS, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid; BM, 3-(*N*-maleimidylpropionyl)biocytin; CAX, cation (Ca^{2+})/H^+ exchanger; OsCAX, *Oryza sativa* CAX; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; TM, trans-membrane domain; WT, wild-type.

The novel nucleotide sequence data published here have been submitted to the DDBJ, EMBL and GenBank sequence data banks and are available under accession number AB112656.
~~~~~~

(Introduction)

The regulation of the cytosolic concentrations of metal ions is essential for normal cell growth. Abnormal levels of essential or non-essential metal ions can cause alterations in cell growth rates. In plant cells, vacuoles serve as reservoirs for several ions and play a key role in maintaining ion homeostasis (1,2). In particular, Ca^{2+} is a common second messenger. A transient increase in the cytosolic free Ca^{2+} concentration plays a central role in signal transduction (2). The plant vacuolar membrane has 2 Ca^{2+} active transport systems: the Ca^{2+}-ATPase and the Ca^{2+}/H^+ exchanger (3,4). Recently the latter transporter was renamed the cation/H^+ exchanger (CAX) because it was found to translocate other metal ions, including Mn^{2+} and Cd^{2+} (1,5,6).

In plants, CAX is driven by the pH gradient generated by vacuolar H^+-ATPase and H^+-pyrophosphatase. cDNAs for CAXs have been cloned from plants (7,8) and *Saccharomyces cerevisiae* (9). Sequence information for CAX DNAs from *Neurospora crassa*, *Synecocystis* sp. PCC6803 and *Bacillus subtilis* is also available. The exchangers have 11 predicted transmembrane domains (TMs) and an acidic residue-rich region between TM6 and TM7. Recent studies on *Arabidopsis thaliana* CAX isoforms using heterologous expression in yeast have shown that the exchangers have 3 characteristic domains: the N-terminal regulatory region, the calcium domain and the C-domain. The N-terminal regulatory region has been shown to suppress Ca^{2+} transport activity by interacting with its neighboring N-terminal sequence. This regulatory region was found in *A. thaliana* CAX1 and CAX3 and in mung bean (*Vigna radiata*) VCAX1 (10), but not in other exchangers. In CAX1, the 9 amino acid calcium domain exists in the hydrophilic loop between TM1 and TM2. This domain is thought to be involved in the selection of Ca^{2+}; however, the sequence has not been found in other CAXs. The C-domain located in TM4 may be involved in the selection of Mn^{2+} by *Arabidopsis* CAX2, which is the only plant CAX known to be capable of Mn^{2+} transport (11).

Information on the domains that recognize and translocate Ca^{2+}, H^+ and other metal ions is needed to elucidate the functional mechanism of the CAX transporter. In the present study, we cloned a cDNA for the rice CAX (OsCAX1a) and determined the Ca^{2+}- and Mn^{2+}-transport activity. Multiple amino acid sequence alignment of CAXs of various organisms revealed 2 highly conserved regions whose sequences are similar to each other. The repeat motifs found in the present study may be the most likely candidates for the essential domain for ion transport activity. We investigated the conserved repeats of rice CAX using a combination of site-directed mutagenesis and heterologous expression in yeast. Furthermore, we performed cysteine-scanning mutagenesis against the repeats and their neighboring regions. The present study provides information on the function and structure of common sequences amongst CAXs. The similarity of the conserved motifs of CAXs and the animal Na^+/Ca^{2+} exchanger is discussed.

EXPERIMENTAL PROCEDURES

Yeast strain and Materials – *S. cerevisiae* strain K665 (*MAT*a, v*cx1*Δ*, pmc1::TRP1, ade2-1, can1-100, his3-11,15, leu2-3, 112, trp1-1, ura3-1*) was used (9). This strain lacks genes for the vacuolar Ca^{2+}/H^+ exchanger (*VCX1*) and Ca^{2+}-ATPase (*PMC1*). 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) and 3-(*N*-maleimidylpropionyl)biocytin (BM) were purchased from Molecular Probes. An immobilized streptavidin gel was purchased from Pierce Biotechnology.

cDNA cloning of Cation/H^+ Exchanger – Total RNA was isolated from rice (*Oryza sativa* japonica L. cv. Nipponbare) seedlings grown under constant light for 2 weeks at 30 °C. The

resulting RNA was used for the construction of cDNA. The RNAs were converted into cDNAs using Superscript™ II RNase H^- reverse transcriptase (Invitrogen Life Technologies) and an oligo-dT adapter primer [5'-CGGGATCCACTAGTTCTAGAGCGC$(T)_{17}$-3']. An *OsCAX1*-specific cDNA was then directly amplified using PCR with a pair of primers (forward, 5'-GAGGGATCCCTCTCGCGACTCGACTCTC-3'; reverse, 5'-AGGAATTCTATTGAGAGCTGCATAGATG-3'; *Bam* HI and *Eco* RI sites underlined). These were designed using information from the EST data base (accession number, D39665 and AU056400). The cDNA was then inserted into the *Bam* HI – *Eco* RI site of pBluescript KS(+) (Stratagene).

Heterologous Expression of Cation/H^+ Exchanger in Yeast Cells – An *OsCAX1aΔ27* cDNA encoding rice cation/H^+ exchanger 1a truncated in the N-terminal autoinhibitory domain (27 residues) was generated using PCR with specific primers (forward: 5’-GAGGAATTCATGTCGTCGTCGTCGCTGCG, *Eco*R I site underlined; reverse: 5’-GAGGTCGACTCATGCGGCCTGAACACTCA-3’, *Sal* I site underlined). The DNA fragment was inserted into the *Eco*R I-*Sal* I site of the yeast expression vector pKT10 (12). The construct was introduced into *S. cerevisiae* using the LiOAc/PEG method and selected in ACHW/Glc medium containing 50 mM potassium phosphate buffer (pH 5.5), 0.002% adenine sulfate, 0.002% tryptophan, 2% glucose, 1% casein hydrolysate and 0.67% yeast nitrogen base without amino acids.

Mutagenesis of the Exchanger – Site-directed mutagenesis of *OsCAX1aΔ27* was performed using a QuickChange site-directed mutagenesis kit (Stratagene) using the method of Kirsch and Joly (13). The identity of the mutated nucleotides was confirmed using DNA sequencing. A cysteine-less mutant of OsCAX1aΔ27 was generated by replacing 8 endogenous cysteine residues with serine. Single cysteine mutants were generated using the cysteine-less mutant as a template. The mutated nucleotides were confirmed using DNA sequencing.

Membrane Preparation from Yeast – The vacuolar membrane-enriched fraction was prepared as described previously (14,15). Yeast cells were grown to the exponential phase in ACHW/Glc medium and harvested. Cells were then treated with zymolyase and spheroplasts were obtained. The spheroplast suspension was homogenized using a Dounce glass homogenizer with a tight fitting pestle. Vacuolar membranes were isolated from the spheroplast homogenate using sucrose discontinuous gradient centrifugation. The membranes were suspended in 5 mM Tris-HCl (pH 7.6), 0.1 M sorbitol, 1 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM PMSF, 1 mg/l leupeptin and 2 mg/l pepstatin, and were stored at –80 °C until use.

Ca^{2+} Uptake and Metal Ion Tolerance Assay – Ca^{2+} transport activity into membrane vesicles was measured using the filtration method with $^{45}CaCl_2$ (8). Yeast strains transformed with *OsCAX1aΔ27* and its derivatives were inoculated in AHCW/Glc medium and then grown overnight at 30 °C. Cell suspensions were diluted 100-fold with YPD (yeast peptone dextrose) medium supplemented with 50 mM Mes-Tris (pH 5.5), 0.5 mg/ml FK506 (Fujisawa Pharmaceuticals, Osaka, Japan) and an appropriate concentration (0 – 200 mM) of $CaCl_2$. Suspensions were then cultured in a microtiter plate at 30 °C. The reagent FK506 was added as a potent inhibitor of calcineurin (4). The optical density at 600 nm was measured using a Vient multi-spectrophotometer (model BT-MQX200, Bio-Tek Instruments, Inc., Winooski, VT). For plate assays, cell cultures were diluted 10-fold with distilled water and 5 μl aliquots

were spotted on YPD plates containing 0.5 mg/ml FK506 and $CaCl_2$ or $MnCl_2$. After incubation for 2 to 3 days at 30 °C, the plates were photographed.

Immunoblotting – We synthesized a peptide corresponding to the N-terminal region (22nd to 44th, NH_2-SRTAHNMSSSSLRKKSDAALVRK+[C]-COOH). For the preparation of antibodies, the peptide was linked with a carrier protein (keyhole limpet hemocyanin). The conjugate was homogenized with Freund's complete adjuvant for the initial injection, and Freund's incomplete adjuvant for the booster injections. The conjugate was then injected into rabbits. The obtained antibody was used as anti-OsCAX1a antibody. For immunoblot analysis, proteins were electroblotted from the SDS-polyacrylamide gel to a polyvinylidene difluoride membrane (Millipore) using a semidry blotting apparatus. Immunoblotting was performed using horseradish peroxidase-linked protein A and ECL Western blotting detection reagents (Amersham Biosciences).

Cysteine-Scanning Mutagenesis – Vacuolar membrane-enriched fractions with right-side-out orientation were prepared as described previously (14). Cysteine-scanning analysis with sulfhydryl reagents was conducted as described previously (16,17). Membranes (30 μg) were suspended in 5 mM Mes-Tris (pH 7.2), 0.3 M sorbitol, 25 mM KCl, 5 mM $MgCl_2$, 1 mM PMSF, 1 mg/l leupeptin and 2 mg/l pepstatin (labeling buffer) to a final volume of 100 μl. The sulfhydryl reagent AMS was added to a final concentration of 400 μM and then incubated on ice for 5 min. To stop the AMS reaction and to start the BM labeling reaction, 400 μl labeling buffer supplemented with 1 mM BM and 0.02% Triton X-100 was added. After incubation on ice for 30 min, the reaction of BM was stopped with 2 % β-mercaptoethanol. After centrifugation at 100,000 × *g* for 30 min, the pellet was resuspended in 1 ml labeling buffer and re-centrifuged. The resulting pellet was suspended in 200 μl 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1 % SDS, 1 mM PMSF, 1 mg/l leupeptin and 2 mg/l pepstatin (solubilization buffer). This mixture was then subjected to sonic oscillation for 10 min using a bath sonicator. The suspension was added to the solubilization buffer (800 μl), and then incubated on ice for 5 min. After centrifugation at 100,000 × *g* for 30 min, the supernatant (1 ml) was mixed with 15 μl streptavidin-agarose beads (Pierce) and then incubated with gentle agitation for 1 h at 4°C. The agarose beads were washed 5 times with 500 μl solubilization buffer. The resultant beads were suspended in 15 μl sample buffer for SDS-PAGE and then boiled for 10 min. SDS-PAGE was performed on 12.5 % polyacrylamide gel. The OsCAX1a protein was detected by immunoblotting with anti-OsCAX1a antibodies.

RESULTS

Expression of Rice Cation/H^+ Exchanger in Yeast as a Functional Enzyme – To characterize a constitutively active form of OsCAX1a, we truncated a nucleotide sequence corresponding to the N-terminal autoinhibitory domain (first 27 residues) from the original cDNA. Such domains in the Ca^{2+}/H^+ exchangers of *A. thaliana* (CAX1) and the mung bean (VCAX1) are known to inhibit ion transport activity (19,20). The 28th residue is the third methionine of OsCAX1a. The truncated form, OsCAX1aΔ27, was expressed in and accumulated in yeast strain K665, which lacks genes for the vacuolar Ca^{2+}-ATPase (*PMC1*) and Ca^{2+}/H^+ exchanger (*VCX1*). In immunoblot of the crude membrane fraction prepared from the transformant, anti-OsCAX1a antibody, which was prepared for the N-terminal region from 22nd to 44th, yielded a major band of 44 kDa and a minor band of 42 kDa (Fig. 1A). Here, OsCAX1aΔ27 was designated as a wild-type (WT) exchanger. The reaction of the

antibodies was completely suppressed with the authentic peptide, which was used for preparation of the antibodies (data not shown). Thus, we concluded that the lower band is likely a degradation product of the normal translation product (OsCAX1aΔ27); however, we cannot exclude other possibilities such as glycosylation.

Plate assay of the yeast transformant showed Ca^{2+} and Mn^{2+} transport activity (Fig. 1B). The strain K665, lacking *PMC1* and *VCX1*, was sensitive to a high concentration of Ca^{2+} (200 mM) and Mn^{2+} (4 mM; Fig. 1B, vector). Introduction of *OsCAX1aΔ27* into K665 rendered the strain tolerant to Ca^{2+} and Mn^{2+}. In the liquid medium assay, the transformant with *OsCAX1aΔ27* showed tolerance to Ca^{2+} at concentrations up to 200 mM. In contrast, the control yeast transformed with a vacant vector did not grow in medium containing Ca^{2+} even at concentrations of 50 mM (Fig. 1C). Thus, OsCAX1aΔ27 complements yeast vacuolar Ca^{2+}/H^{+} exchanger in the K665 strain. These results strongly suggest that in OsCAX1aΔ27 there is Ca^{2+} and Mn^{2+} transport activity in yeast vacuoles.

We confirmed the presence of $^{45}Ca^{2+}$ uptake activity into membrane vesicles (Fig. 1D). In this system, the pH gradient across vacuolar membranes was generated by V-ATPase. The membrane vesicles took up $^{45}Ca^{2+}$ from the medium in a time-dependent manner for up to 10 min. The accumulated $^{45}Ca^{2+}$ was released immediately after the addition of the Ca^{2+} ionophore, A23187. In the absence of ATP or in the presence of bafilomycin A_1, a potent inhibitor of V-ATPase, no such activity was detected (Fig. 1D). Membrane vesicles of yeast transformed with a vacant vector had no activity. These results indicate that OsCAX1aΔ27 expressed in yeast functions as a Ca^{2+}/H^{+} exchanger.

The apparent K_m of the WT exchanger for Ca^{2+} was 9.5 μM. Thus, OsCAX1a has a high affinity for Ca^{2+}, which K_m value is comparable to that of oat CAX (K_m, 10 μM) (21), *A. thaliana* CAX1 (K_m, 13.1 μM) (7) and *V. radiata* VCAX1 (K_m, 25 μM) (16). The V_{max} value of OsCAX1a in yeast membranes was 3.8 nmol/min/mg.

Structural Features of OsCAX – Based on results from the TMpred program, we predict that the 451 amino acid protein OsCAX1a may, like other CAX proteins, have 11 TM domains (Fig. 2A). The N-terminal region, which has a 59-residue hydrophilic sequence, is likely exposed to the cytosol. OsCAX1a has an acidic motif in the cytosolic loop between TM6 and TM7; similar findings have been described for *V. radiata*, *A. thaliana* and *S. cerevisiae* CAXs (7-9). In this study, multiple sequence alignment of 9 CAXs of plants and yeast revealed 2 novel, highly conserved regions. Both regions have 36 residues, 14 of which are identical. Furthermore, 7 residues are conserved in both motifs. The sequences of repeats c-1 and c-2 are $GNX_2EXIX_4AX_8VX_4LGSXLSNXLXV$ and $GNAAEHX_6AX_5DX_2LGX_3GSX_2QX_3FX$, respectively (Fig. 2B). The consensus sequence is $GNX_2EX_{21}GSX_8$ (Fig. 2B). Thus, we designated them as repeats c-1 and c-2, respectively. Repeat c-1 is located between TM3 and TM4 and faces the vacuolar lumen in this topological model (Fig. 2A). Repeat c-2 is located between TM8 and TM9 and faces the cytosol.

Mutagenic Analysis of Amino Acid Residues in Conserved Repeats – We hypothesized that the two conserved repeats are essential to CAX function. To evaluate the functional significance of the 26 residues in the repeats, including the conserved repeats, we generated a series of OsCAX1aΔ27 mutants in which the residues were replaced with alanine (repeat c-1: G^{128}, N^{129}, T^{131}, K^{142}, K^{144}, K^{149}, S^{151}, G^{154}, S^{155}, S^{158} and N^{159}; c-2: G^{325}, N^{326}, H^{330}, K^{339}, N^{340}, K^{341}, T^{345}, G^{351}, S^{352}, T^{354} and Q^{355}) or conservative residues (c-1: E132Q, E132D, E146Q and E146D; c-2: E329Q, E329D, D343N and D343E), or His^{330} was replaced with arginine (H330R).

Each exchanger protein was clearly detected at 44 kDa on immunoblot (Fig. 3). In many

cases, a minor band of 42 kDa was detected, although no minor band was found in mutants K144A, K149A, S151A, G154A and K341A. As discussed above, this 42 kDa protein may be a degradation product. It is likely that a 2 kDa C-terminal region is removed by protease-mediated cleavage, as the anti-OsCAX1a antibodies recognize the N-terminal region (residues 22 to 44). Difference in the extent of proteolysis among mutants and an appearance of an addition band of 40 kDa in the N340A mutant may be due to the alteration of fine tertiary structure in the mutant exchangers.

The ion transport activity of the K665 strains with the WT (OsCAX1aΔ27) or 31 mutant exchangers was determined using a growth test on agar plates supplemented with $CaCl_2$ or $MnCl_2$ (Fig. 4). Tolerant cells grow normally and generate clear colonies on these plates. In this assay, mutants N129A, E132Q, G154A, E329Q, E329D, H330A and H330R were sensitive to 200 mM $CaCl_2$. Sensitivity to a high concentration of Mn^{2+} varied with the mutant. Sixteen mutants, including G128A and N129A, were not tolerant to Mn^{2+}. A mutant T354A was more tolerant to Mn^{2+} than the WT exchanger.

Classification of Mutants – Quantitative characterization of the Ca^{2+} tolerances of yeast strains with mutant exchangers was performed in liquid culture medium containing 50 or 200 mM $CaCl_2$ (Fig. 5). Strains with mutants that did not form colonies on agar plates (N129A, E132Q, G154A, E329Q, E329D, H330A and H330R) did not grow in the liquid medium even at a Ca^{2+} concentration of 50 mM. We classified the 31 mutants into 6 categories based on their tolerance of Ca^{2+} and Mn^{2+} (Table I).

Class I mutants (T131A, K142A, E146Q, E146D, N340A, D343N, D343E and T345A) had the same tolerance to both Mn^{2+} and Ca^{2+} as the WT. Class II mutants (G128A, E132D, K144A, K149A, N326A, K341A, G351A, S352A and Q355A) had a lower tolerance to both ions. Class III mutants (N129A, E132Q, G154A, E329Q, E329D, H330A and H330R) were sensitive to both ions, indicating that these residues are essential for ion transport. Class IV mutants (S155A, S158A, N159A, G325A and K339A) were tolerant to Ca^{2+} but sensitive to Mn^{2+}. Class V mutant (T354A) had the same tolerance to Ca^{2+} as the WT and a higher tolerance to Mn^{2+} than the WT. The class VI mutant (S151A) showed the same tolerance to Mn^{2+} as the WT, but and a lower tolerance to Ca^{2+}. The amino acid residues mutated in classes IV, V and VI may be involved in ion selectivity. In total, ion selectivity and transport activity was affected in 23 of the 31 mutant exchangers.

Mutagenesis of Cysteine Residues – To map the TM topology of the repeat motifs, we carried out cysteine replacement/biotinylation analysis. First we prepared a cysteine-less OsCAX1aΔ27 construct, in which all 8 endogenous cysteine residues were substituted with serine by site-directed mutagenesis. This construct was then expressed in a K665 strain. The cysteine-less mutant protein accumulated in yeast membranes as shown in an immunoblot (Fig. 6A). In membranes containing a cysteine-less mutant, Ca^{2+} uptake activity, which is sensitive to bafilomycin A_1, was 36% of that with the WT exchanger (Fig. 6B). We therefore concluded that the cysteine-less mutant retains its functional structure in yeast and can be used for cysteine-scanning mutagenesis.

We introduced 19 cysteine residues individually into repeat c-1 and its border regions in the cysteine-less mutant (Ser^{105}, Leu^{113}, Thr^{118}, Ala^{130}, Ala^{136}, Ala^{139}, Lys^{142}, Gly^{143}, Ile^{145}, Glu^{146}, Val^{147}, Val^{148}, Ser^{150}, Leu^{153}, Ser^{155}, Ser^{158}, Ser^{170}, Ala^{178} and Ser^{187}). All of the mutants expressed well, and 12 mutants (S105C, L113C, T118C, A130C, A136C, I145C, V148C, S150C, S158C, S170C, A178C and S187C) exhibited Ca^{2+} tolerance on an agar plate containing 200 mM $CaCl_2$, although the Ca^{2+} tolerance in liquid medium containing 50 mM varied with the mutant (Fig. 6, C and D). These 12 mutants were therefore used for cysteine-scanning mutagenesis. The remaining 7 mutants (A139C, K142C, G143C, E146C, V147C,

L153C and S155C) were not functional, suggesting that these 7 residues are essential for Ca^{2+} transport or for formation of the protein structure in yeast.

We also introduced 25 cysteine residues individually into repeat c-2 and its border regions. Eleven mutants of them showed Ca^{2+} tolerance (Figure 6D). The Ca^{2+} intolerance of 14 mutants (S317C, I335C, A337C, K339C, N340C, K341C, L342C, D343C, I344C, T345C, S352C, A353C, Q374C and D378C) suggests that these residues may be involved in the activity or the formation of the tertiary structure of OsCAX1a.

Membrane Topology of Repeats **–** In further experiments we treated right-side-out vacuolar membrane vesicles containing mutant exchangers with AMS and then with BM. AMS is a membrane-impermeable sulfhydryl reagent that reacts with cysteine residues that face the cytosol, but not with residues in vacuoles. BM is conditionally membrane permeable and reacts with residues in hydrophilic regions. After adding AMS, we added 0.02% Triton X-100 together with BM to render membranes permeable. In this system, BM is not able to react with cytosolic cysteine residues that have already reacted with AMS. Conversely, cysteine residues in the vacuolar lumen or the membrane are not labeled with AMS and therefore are able to react with BM. The BM-linked exchangers were then solubilized and selected using streptoavidin-agarose, which strongly binds to a biotinyl moiety of BM. The isolated exchangers were then subjected to immunoblotting with anti-OsCAX1a antibodies.

All of the mutant exchangers were labeled with BM in the absence of AMS, except for S158C, S170C, S314C, A331C, L338C, S357C and S364C (*e.g.*, see S158C, Fig. 7A). Four mutants of repeat c-1 (T118C, I145T, A178C and S187C) and 4 mutants of repeat c-2 (S308C, A333C, A349C and T354C) were not detected after treatment with AMS, suggesting that these residues are exposed to the cytosol (Fig. 7, B and C). Six mutants of repeat c-1 (S105C, L113C, A130C, A136C, V148C and S150C) and 2 mutants of repeat c-2 (G300C and G325C) were clearly labeled with BM even after treatment with AMS. We conclude that these residues are embedded in the membrane or are exposed to the vacuolar lumen.

DISCUSSION

The aim of this study was to identify the functional domains for the recognition and translocation of metal ions in the rice CAX. We focused our attention on the conserved repeats c-1 and c-2. In preliminary experiments using immunoblotting with anti-OsCAX1a antibodies, we found that OsCAX1a is a 46-kDa protein detectable in vacuolar membranes isolated from rice seedlings. Thus it appears that OsCAX1a is expressed and functions in the vacuolar membrane.

OsCAX1a and a Truncated Form **–** The CAXs of various organisms have a common motif of an acidic residue-rich region between TM6 and TM7 (7-9,22). OsCAX1a also contains such an acidic region (Fig. 2, residues 268–272). Although the detailed amino acid sequence is not conserved among CAXs, this acidic region is thought to be involved in the capture and selection of metal ions (7,18).

Recently, an N-terminal regulatory region has been found in *A. thaliana* CAX1 and CAX3 and mung bean VCAX1 (19,20). It should be noted that not all CAXs have an extensive N-terminal region. This region has been demonstrated to regulate the activity negatively by intramolecular interaction with a specific site in the N-terminal part (10,22). OsCAX1a also contains a long hydrophilic region at the N-terminus and has 5 residues in the tail (residues 21–26 in OsCAX1a, R*X*RTAH), which are identical to those of 3 other exchangers (CAX1, CAX3 and VCAX1). A full-length OsCAX1a protein showed reduced activity of Ca^{2+}/H^{+}

exchanger when expressed in yeast (data not shown). We suggest that the N-terminal sequence (27 residues) of OsCAX1a is a negative regulatory region. We therefore used the truncated form, OsCAX1aΔ27, as the WT exchanger in this study.

Presence of Common Repeats in CAXs – We found 2 conserved, repetitive sequences that are present in OsCAX1a and the CAXs of *A. thaliana*, *V. radiata*, *Zea mays*, and *S. cerevisiae* (Fig. 2). For OsCAX1a, 12 residues were identical between the two repeats. The repeat sequences were common to multiple CAX proteins and may have been generated by gene duplication at an early stage of molecular evolution.

Role of Repeats in Ion Transport and Ion Selectivity – Amino acid substitution of residues in the repeats had a negative effect on CAX activity in many cases (Fig. 4 and Table I). Mutagenic analysis revealed that 13 residues, Gly^{128}, Asn^{129}, Glu^{132}, Lys^{144}, Lys^{149}, Gly^{154}, Asn^{326}, Glu^{329}, His^{330}, Lys^{341}, Gly^{351}, Ser^{352} and Gln^{355} (classes II and III), are essential for the transport of Ca^{2+} and Mn^{2+}. These residues are likely involved in translocation of metal ions. It should be noted that there are 3 pairs of conserved residues among the repeats c-1 and c-2 (Ans^{129} and Asn^{326}, Glu^{132} and Glu^{329} and Gly^{154} and Gly^{351}). These residues are essential for the translocation of both metal ions.

At present, we cannot exclude the possibility that these residues are involved in translocation of H^+. In the hard-soft acid-base theory, Ca^{2+}, Mn^{2+} and H^+ belong to the 'hard metal' group, and interact preferentially with oxygen atoms of carboxyl groups and a nitrogen atoms of amino groups in amino acid side chains. Thus, several essential residues, including Asn^{129}, Glu^{132}, Lys^{144}, Lys^{149}, Asn^{326}, Glu^{329}, Lys^{341} and Gln^{355}, may interact with Ca^{2+}, Mn^{2+} and/or H^+ during ion transport.

The class IV mutants (Ser^{155}, Ser^{158}, Asn^{159} and Gly^{325}) were tolerant to Ca^{2+} but sensitive to Mn^{2+}. The residues may recognize a difference in ion radius and select Mn^{2+}, since Mn^{2+} (ionic radius 80 pm) is smaller than Ca^{2+} (99 pm). The class V mutant T354A showed higher tolerance to Mn^{2+} than did the WT. The residue Thr^{354} in the WT exchanger may interfere with Mn^{2+} translocation. Most residues of classes IV and V may be located in the Mn^{2+} transport pathway. Recently, it has been reported that a 10 amino acid region (CAFFCGGLVF), especially the Cys-Ala-Phe motif in TM4, is responsible for the Mn^{2+} specificity of *Arabidopsis* CAX2 (11). This region may be specific for CAX2, since it is not conserved in OsCAX1a or other CAXs.

The mutant S151A (class VI), in which a side chain OH group was replaced with an H, was less tolerant to Ca^{2+} compared with WT (Fig. 5), although tolerance to Mn^{2+} was retained. Thus, this residue may be involved in the selection of and/or affinity for Ca^{2+}.

The class I mutations (Thr^{131}, Lys^{142}, Glu^{146}, Asn^{340}, Asp^{343} and Thr^{345}) did not affect tolerance to Ca^{2+} and Mn^{2+}, suggesting that these 6 residues make little contribution to ion transport function. The residues Lys^{142}, Asn^{340} and Thr^{345} are not conserved among CAXs. There was no effect in a T131A mutant. It is reasonable since the residue Thr^{131} in repeat c-1 corresponds to Ala^{328} in repeat c-2 and Glu^{146} corresponds to Asp^{343}. Mutants E146Q, E146D, D343N and D343E retained their tolerance to Ca^{2+} and Mn^{2+}. We conclude that the positions of Glu^{146} and Asp^{343} require an oxygen atom as a ketone.

We also constructed cysteine-replaced mutants. Several transformants did not grow in 200 mM Ca^{2+}, although the mutant exchangers were detected in yeast by immunoblotting. Therefore, we concluded that the 7 residues in repeat c-1 (Ala^{139}, Lys^{142}, Gly^{143}, Glu^{146}, Val^{147}, Leu^{153} and Ser^{155}) and 14 residues in repeat c-2 (Ser^{317}, Ile^{335}, Ala^{337}, Lys^{339}, Asn^{340}, Lys^{341}, Leu^{342}, Asp^{343}, Ile^{344}, Thr^{345}, Ser^{352}, Ala^{353}, Gln^{374} and Asp^{378}) are essential for Ca^{2+} transport function and/or the correct conformation of OsCAX1a.

Repeat Sequence as Re-entrant Loop – We determined the topological arrangement of repeats c-1 and c-2 using cysteine mutagenesis with sulfhydryl reagents. This method has been used to study several membrane transporters, including the Na^+/H^+ exchanger (23), Na^+/Ca^{2+} exchanger (24) and metal-tetracycline/H^+ antiporter (25). We found that Thr^{118}, Ala^{178} and Ser^{187} were located in cytosolic loops, as was predicted from hydropathy analysis. The residues Ser^{105}, Leu^{113}, Ala^{130}, Ala^{136}, Val^{148} and Ser^{150} were located in the membrane or vacuolar lumen. Interestingly, an Ile^{145} residue was found facing the cytosol or was accessible from the cytosol. Our topology model of repeat c-1 is shown in Figure 8A. We propose that repeat c-1, connecting TM3 and TM4, may re-enter the membrane from the lumenal side.

Common Motifs in Repeat Sequences between OsCAX and Na^+/Ca^{2+} Exchanger – We found that CAXs share common residues with the animal Na^+/Ca^{2+} exchanger (26-29), although the overall sequences are not conserved (Fig. 8B). The 4 conserved residues in repeats c-1 (Glu^{132}, Gly^{154}, Ser^{155} and Asn^{159}) and c-2 (Ala^{337}, Asp^{343}, Gly^{351} and Ser^{352}) of CAXs are also found in the animal Na^+/Ca^{2+} exchangers NCX1, NCX2, and CalX, which carry repeat sequences α-1 and α-2. The repeats α-1 and α-2 have been shown to form re-entrant loops and to enter the membrane with opposing orientations (26-28). We propose that the repeats c-1 and c-2 similarly form re-entrant loops in OsCAX1a and other CAXs. Our results suggest that the repeat c-1 loop may enter the membrane from the lumenal side and the c-2 loop from the cytosolic side.

The re-entrant loops were also found in other membrane proteins, such as the Na^+/H^+ exchanger (23), glutamate transporter (30), chloride ion channel (31) and water channel (32). In all cases, the re-entrant loops function as a filter. The repeat sequences of OsCAX1a contain several hydrophilic residues. Therefore, we hypothesize that the repeats form an ion filter in the solution-facing region. Both repeats share a GN*XX*E motif, and the Glu^{132} and Glu^{329} residues in the motif are essential for ion transport.

In conclusion, we propose that the solvent-accessible region of OsCAX1a is formed by the re-entrant loops c-1 and c-2, which carry highly conserved residues involved in cation translocation. This region may function as a vestibule for Ca^{2+}, Mn^{2+} and H^+. Our results suggest that CAXs are structurally similar to other cation exchangers, such as the Na^+/Ca^{2+} exchanger. Further studies including crystallography will shed light on the quaternary structure and the functional mechanisms of the cation/cation exchanger. Several residues (Ser^{155}, Ser^{158}, Asn^{159}, Gly^{325} and Ser^{352}) were found to define selectivity for Mn^{2+}. Manganese is one of the essential metals contained in enzymes such as manganese superoxide dismutase (33). In particular, the photosystem-II reaction center has a manganese cluster, in which 4 manganese atoms are ligated to amino acids and form an oxygen-evolving complex (34). By introducing these mutant exchangers into plants genetically, we hope to further evaluate the physiological roles of OsCAX1a and Mn^{2+}.

Acknowledgments – We are most grateful to Dr. Yoichi Nakanishi of Nagoya University for his stimulating discussions. Thanks are extended to Drs. Setsuko Komatsu and Yoshiyuki Tanaka of the National Institute of Agrobiological Sciences for the assistance and advice in the initial research of OsCAXs. This work was supported by Grants-in-Aid for Scientific Research 13142203, 13CE2005, and 10219203 (to M.M.) from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

1. Maeshima, M. (2001) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 52, 469-497
2. Sanders, D., Brownlee, C., and Harper, J. F. (1999) *Plant Cell* 11, 691-706
3. Sze, H., Li, X., and Palmgren, M. G. (1999) *Plant Cell* 11, 677-690
4. Sze, H., Liang, F., Hwang, I., Curran, A. C., and Harper, J. F. (2000) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 51, 433-462
5. Hirschi, K. (2001) *Trends Plant Sci.* 6, 100-104
6. Hirschi, K. D., Korenkov, V. D., Wilganowski, N. L., and Wagner, G. J. (2000) *Plant Physiol.* 124, 125-133
7. Hirschi, K. D., Zhen, R. G., Cunningham, K. W., Rea, P. A., and Fink, G. R. (1996) *Proc. Natl. Acad. Sci. U S A* 93, 8782-8786
8. Ueoka-Nakanishi, H., Nakanishi, Y., Tanaka, Y., and Maeshima, M. (1999) *Eur. J. Biochem.* 262, 417-425
9. Cunningham, K. W., and Fink, G. R. (1996) *Mol. Cell. Biol.* 16, 2226-2237
10. Pittman, J. K., Shigaki, T., Cheng, N. H., and Hirschi, K. D. (2002) *J. Biol. Chem.* 277, 26452-26459
11. Shigaki, T., Pittman, J. K., and Hirschi, K. D. (2003) *J. Biol. Chem.* 278, 6610-6617
12. Tanaka, K., Nakafuku, M., Tamanoi, F., Kaziro, Y., Matsumoto, K., and Toh-e, A. (1990) *Mol. Cell. Biol.* 10, 4303-4313
13. Kirsch, R. D., and Joly, E. (1998) *Nuc. Acids Res.* 26, 1848-1850
14. Leng, X. H., Manolson, M. F., Liu, Q., and Forgac, M. (1996) *J. Biol. Chem.* 271, 22487-22493
15. Nakanishi, Y., Saijo, T., Wada, Y., and Maeshima, M. (2001) *J. Biol. Chem.* 276, 7654-7660
16. Leng, X. H., Nishi, T., and Forgac, M. (1999) *J. Biol. Chem.* 274, 14655-14661
17. Iwamoto, T., Nakamura, T. Y., Pan, Y., Uehara, A., Imanaga, I., and Shigekawa, M. (1999) *FEBS Lett.* 446, 264-268
18. Ueoka-Nakanishi, H., Tsuchiya, T., Sasaki, M., Nakanishi, Y., Cunningham, K. W., and Maeshima, M. (2000) *Eur. J. Biochem.* 267, 3090-3098
19. Pittman, J. K., and Hirschi, K. D. (2001) *Plant Physiol.* 127, 1020-1029
20. Pittman, J. K., Sreevidya, C. S., Shigaki, T., Ueoka-Nakanishi, H., and Hirschi, K. D. (2002) *Plant Physiol.* 130, 1054-1062
21. Schumaker, K. S., and Sze, H. (1990) *Plant Physiol.* 92, 340-345
22. Shigaki, T., Cheng, N. H., Pittman, J. K., and Hirschi, K. (2001) *J. Biol. Chem.* 276, 43152-43159
23. Wakabayashi, S., Pang, T., Su, X., and Shigekawa, M. (2000) *J. Biol. Chem.* 275, 7942-7949
24. Nicoll, D. A., Ottolia, M., Lu, L., Lu, Y., and Philipson, K. D. (1999) *J. Biol. Chem.* 274, 910-917
25. Tamura, N., Konishi, S., Iwaki, S., Kimura-Someya, T., Nada, S., and Yamaguchi, A. (2001) *J. Biol. Chem.* 276, 20330-20339
26. Nicoll, D. A., Hryshko, L. V., Matsuoka, S., Frank, J. S., and Philipson, K. D. (1996) *J. Biol. Chem.* 271, 13385-13391
27. Qiu, Z., Nicoll, D. A., and Philipson, K. D. (2001) *J. Biol. Chem.* 276, 194-199
28. Schwarz, E. M., and Benzer, S. (1997) *Proc. Natl. Acad. Sci. U S A* 94, 10249-10254
29. Iwamoto, T., Uehara, A., Imanaga, I., and Shigekawa, M. (2000) *J. Biol. Chem.* 275, 38571-38580
30. Slotboom, D. J., Sobczak, I., Konings, W. N., and Lolkema, J. S. (1999) *Proc. Natl. Acad. Sci. U S A* 96, 14282-14287

31. Dutzler, R., Campbell, E. B., Cadene, M., Chait, B. T., and MacKinnon, R. (2002) *Nature* 415, 287-294
32. Murata, K., Mitsuoka, K., Hirai, T., Walz, T., Agre, P., Heymann, J. B., Engel, A., and Fujiyoshi, Y. (2000) *Nature* 407, 599-605
33. Kwon, S. I., and An, C. S. (1999) *Mol. Cells* 9, 625-630
34. Rögner, M., Boekema, E. J., and Barber, J. (1996) *Trends Biochem. Sci.* 21, 44-49

Table I

Summary of Ca^{2+} and Mn^{2+} tolerance assays. Mutant proteins were classified into 6 classes according to their ion tolerance (Figs. 4 and 5). Ca^{2+} tolerance was assessed from Figure 4 by comparing with WT. Ca^{2+} tolerance is indicated by ++ (same tolerance as WT; growth rate, 80% - 120% of WT), + (reduced tolerance compared with WT; $p < 0.05$ versus WT, Student's *t*-test), and – (no tolerance, absence of colony on the plate in Fig. 4 and Fig. 5B). For the assessment of Mn^{2+} tolerance (Fig. 4), growth of yeast transformants in the presence of 4, 6, and 10 mM of $MnCl_2$ was individually quantified using the NIH image program. The values were summed up for each mutant and compared with WT. The tolerance is expressed by +++ (enhanced tolerance, more than 120% of WT), ++ (same tolerance as WT, 70% - 120%), + (reduced tolerance, 30% - 70%), and – (no tolerance, 0% - 30%).

Mutants	Ca^{2+} tolerance	Mn^{2+} tolerance	Class	Mutants	Ca^{2+} tolerance	Mn^{2+} tolerance	Class
WT	++	++	I	G325A	++	-	IV
G128A	+	-	II	N326A	+	-	II
N129A	-	-	III	E329Q	-	-	III
T131A	++	++	I	E329D	-	-	III
E132Q	-	-	III	H330A	-	-	III
E132D	+	-	II	H330R	-	-	III
K142A	++	++	I	K339A	++	+	IV
K144A	+	+	II	N340A	++	++	I
E146Q	++	++	I	K341A	+	+	II
E146D	++	++	I	D343N	++	++	I
K149A	+	+	II	D343E	++	++	I
S151A	+	++	VI	T345A	++	++	I
G154A	-	-	III	G351A	+	+	II
S155A	++	+	IV	S352A	+	-	II
S158A	++	-	IV	T354A	++	+++	V
N159A	++	-	IV	Q355A	+	-	II

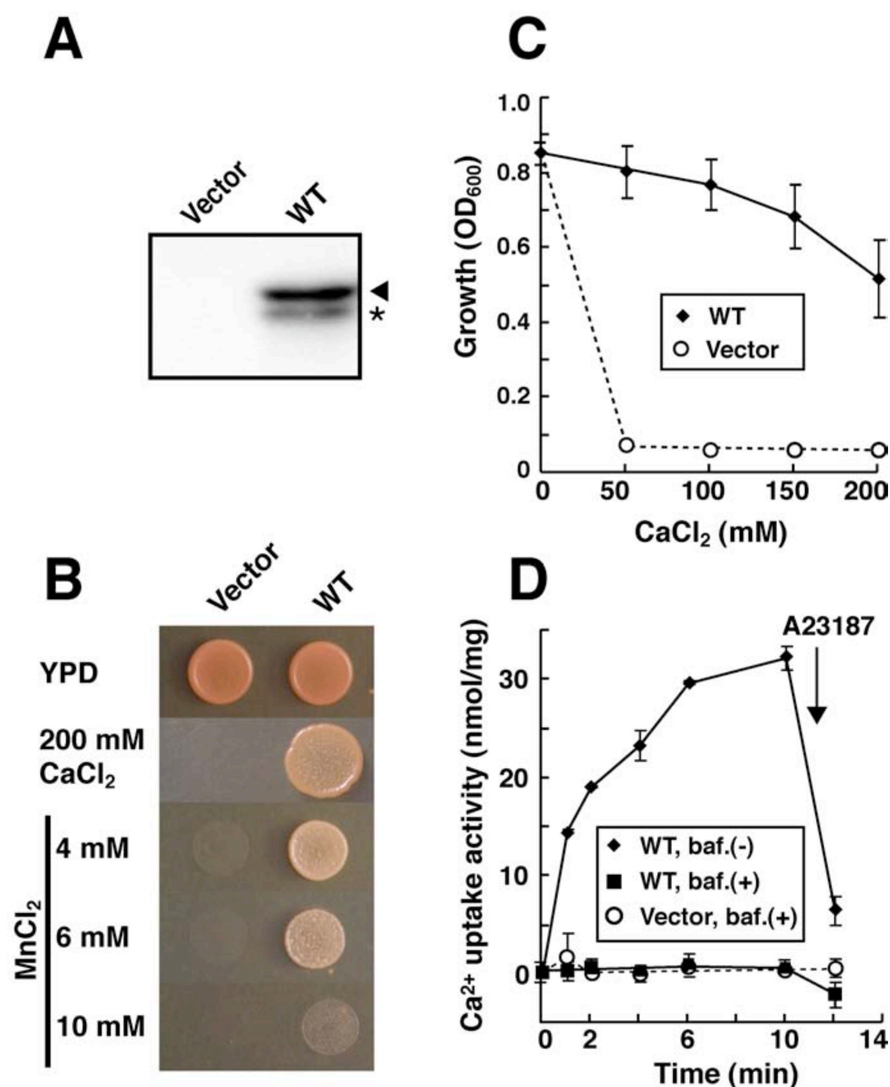


Fig. 1. Heterologous expression of OsCAX1a in yeast. *A*, a yeast strain (K665) was transformed with *OsCAX1aΔ27* cDNA. Vacuolar membrane fractions (5 μg) were subjected to immunoblot analysis using an anti-OsCAX1a antibody. An arrowhead and asterisk indicate the positions of OsCAX1aΔ27 (WT) and a lower band, respectively. *B*, cells cultured for 16 h were diluted to 10-fold and aliquots (5 μl) were spotted on a YPD plate containing $CaCl_2$ or $MnCl_2$. Growth was recorded after 2 days ($CaCl_2$) or 3 days ($MnCl_2$) of incubation at 30 °C. *C*, transformants (WT, ♦; vector, ○) pre-cultured for 16 h were diluted to 100-fold and then cultured in YPD liquid medium containing $CaCl_2$ for 16 h at 30 °C. Then absorbance at 600 nm (OD_{600}) was measured. *D*, membrane vesicles (10 μg) were assayed for Ca^{2+}-uptake activity. Aliquots (100 μg) of membrane suspension were pre-incubated in a reaction medium (1 ml) with (■) or without (♦, ○) 0.2 μM bafilomycin A_1 (baf.) for 5 min at 25 °C. The reaction was started by adding 100 μM $^{45}CaCl_2$ at 0 min. The calcium ionophore, A23187, was added at 11 min at a final concentration of 5 μM. Solid and broken lines in panels *B* and *D* indicate the strain transformed with *OsCAX1aΔ27* and vector, respectively. The data represents the means ± SD for 4 independent experiments.

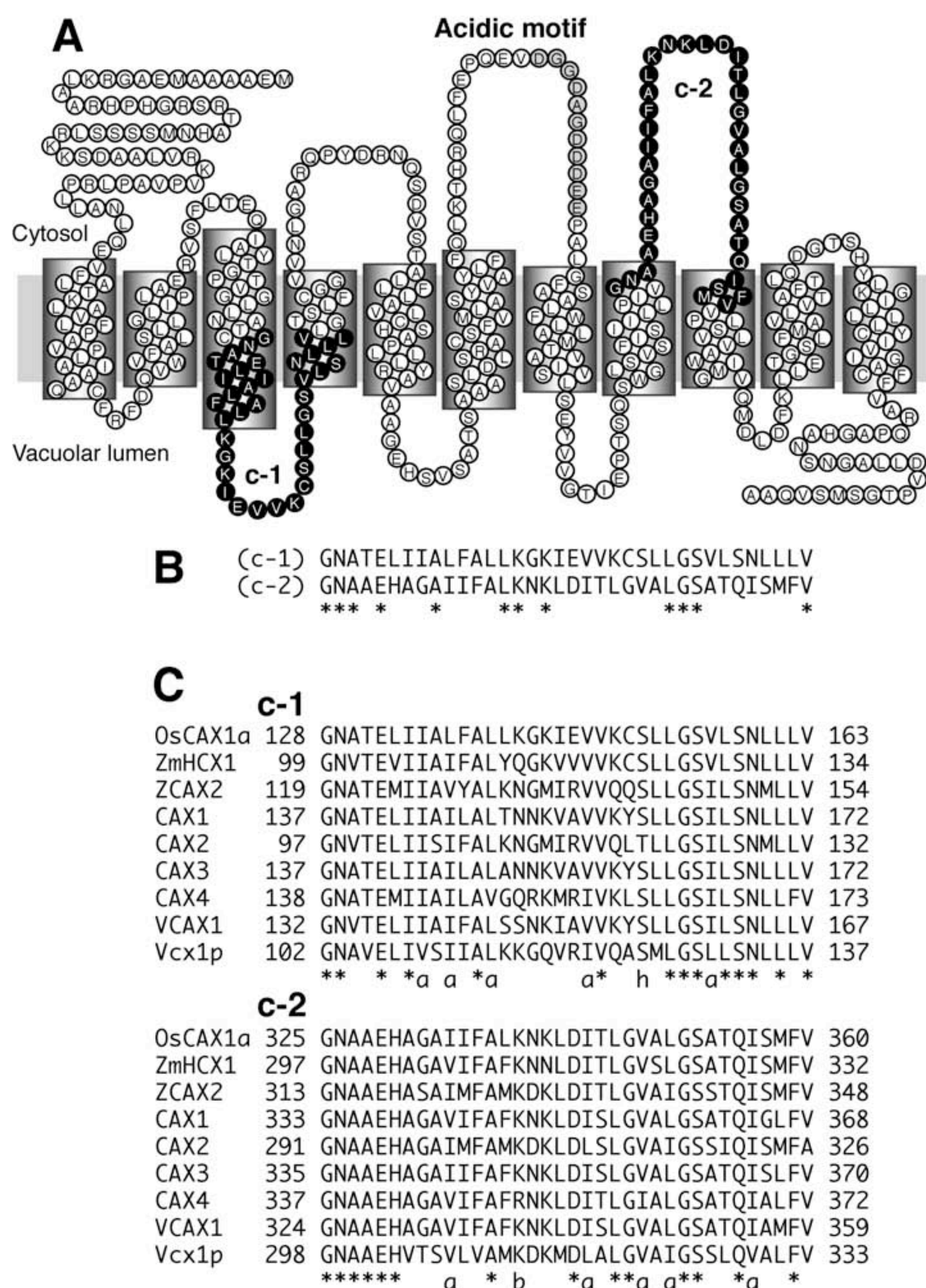


Fig. 2. A membrane topology model of CAX and repetitive conserved sequences amongst CAX members. *A*, a topology model of CAX predicted using the TMpred program (http://www/ch.embnet.org/software/TMPRED-form.html). Shaded boxes indicate putative TM domains. The repeats c-1 and c-2 are highlighted. Residues in the acidic motif are marked in gray. *B*, the consensus sequence amongst the repeats of OsCAX1a. *C*, sequence alignment of repeats c-1 (upper) and c-2 (lower). The accession numbers for the amino acid sequences aligned are as follows: OsCAX1a (*O. sativa*, AB112656), ZmHCX1 (*Zea mays*, AF256229), ZCAX2 (*Z. mays*, BAB61725), CAX1 (*A. thaliana*, AF461691), CAX2 (*A. thaliana*, AF424628), CAX3 (*A. thaliana*, AF256229), CAX4 (*A. thaliana*, AF409107), VCAX1 (*Vigna radiata*, AB012932) and Vcx1p (*S. cerevisiae*, U36603). Identical residues amongst all sequences are marked by *asterisks*. Aliphatic (Leu, Ile and Val; a), hydroxylic (Ser and Thr; h) and basic residues (His, Lys and Arg; b) are also marked. Shaded boxes indicate putative TMs. The *numbers* beside the amino acid sequences represent the sequence position of the amino acids.

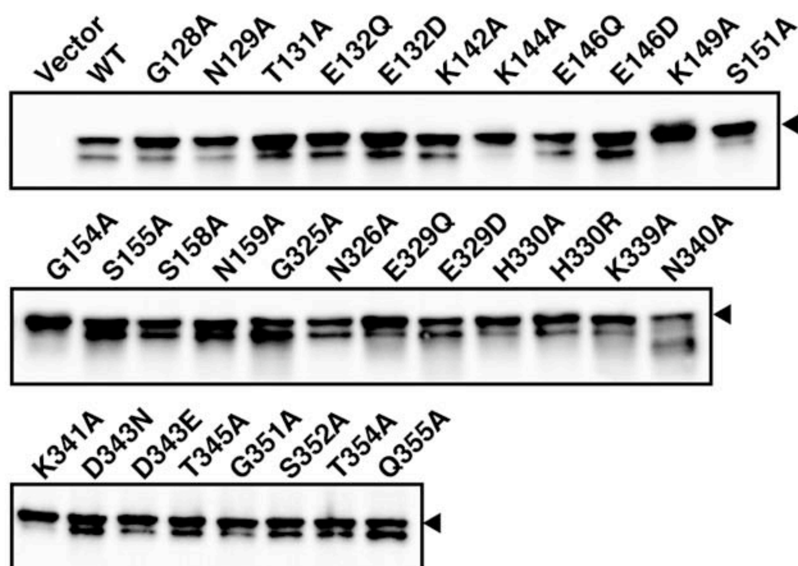


Fig. 3. Expression levels of mutant OsCAX1a proteins. Membrane fractions were prepared from yeast K665 expressing wild type OsCAX1aΔ27 (WT) and 31 mutants in which a certain residue of the exchanger was replaced with the indicated residue. OsCAX1aΔ27 protein in the membrane fraction (5 μg) of each mutant was quantified by immunoblotting with the anti-OsCAX1a antibody. *Arrowheads* indicate major immunostained bands of 44 kDa.

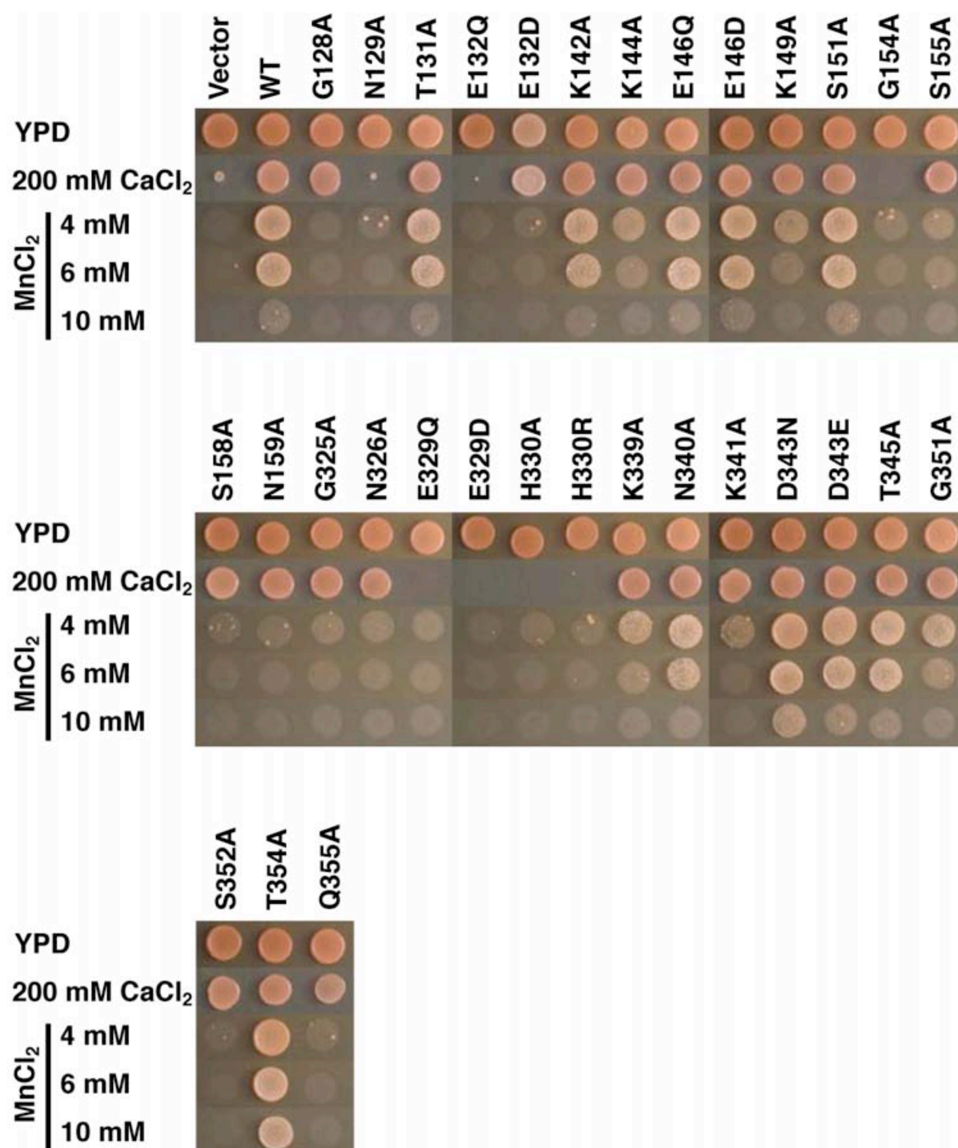


Fig. 4. Ion tolerance assay of mutant OsCAX1a on YPD plates containing Ca^{2+} or Mn^{2+}. Yeast cells expressing mutant exchangers cultured for 16 h were diluted and then spotted on a YPD plate containing $CaCl_2$ (200 mM) or $MnCl_2$ (4, 6, and 10 mM). Plates were incubated for 2 ($CaCl_2$) or 3 days ($MnCl_2$) at 30 °C under the same conditions as in Fig. 1C.

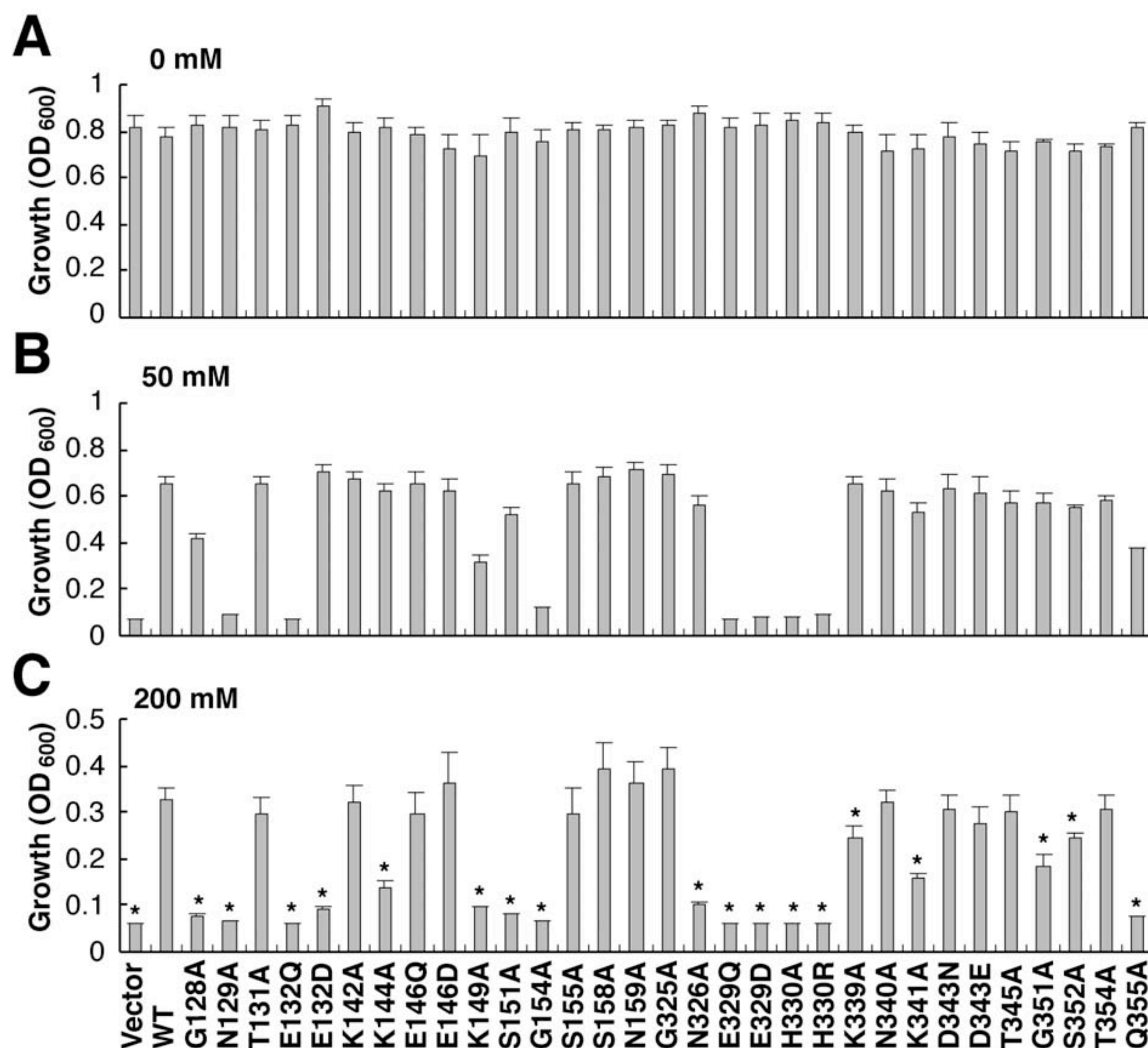


Fig. 5. Calcium tolerance assay in YPD liquid culture. Yeast transformants were cultured in YPD liquid medium that contained $CaCl_2$ at 0 mM (A), 50 mM (B) and 200 mM (C) at 30 °C. After incubation for 16 h absorbance at 600 nm was measured. The data represents means ± SD for 4 experiments. The mutants whose calcium tolerance was significantly decreased compared with WT are marked with asterisks ($p < 0.05$ versus WT, Student's *t* test).

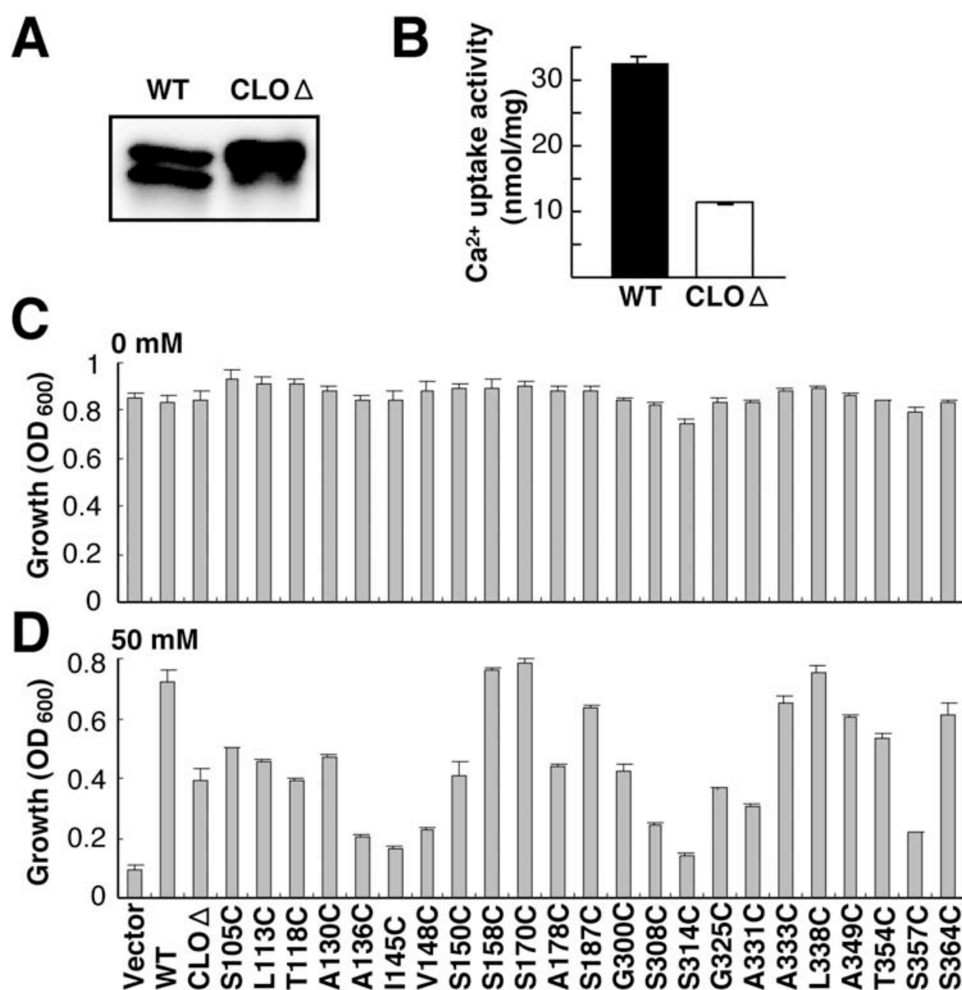


Fig. 6. Cysteine mutants of repeats c-1 and c-2. *A*, an immunoblot of vacuolar membranes prepared from yeast expressing wild-type (WT) and cysteine-less OsCAX1a (CLOΔ). *B*, Ca^{2+} uptake activity of membrane vesicles (30 μg) of WT and cysteine-less mutant. The data represents means ± SD for 4 experiments. *C* and *D*, Ca^{2+} tolerance assay in YPD liquid culture that contained $CaCl_2$ at 0 mM (*C*) or 50 mM (*D*). Yeast transformants were cultured at 30 °C for 16 h, and then absorbance at 600 nm was measured. The data represents means ± SD for 4 experiments.

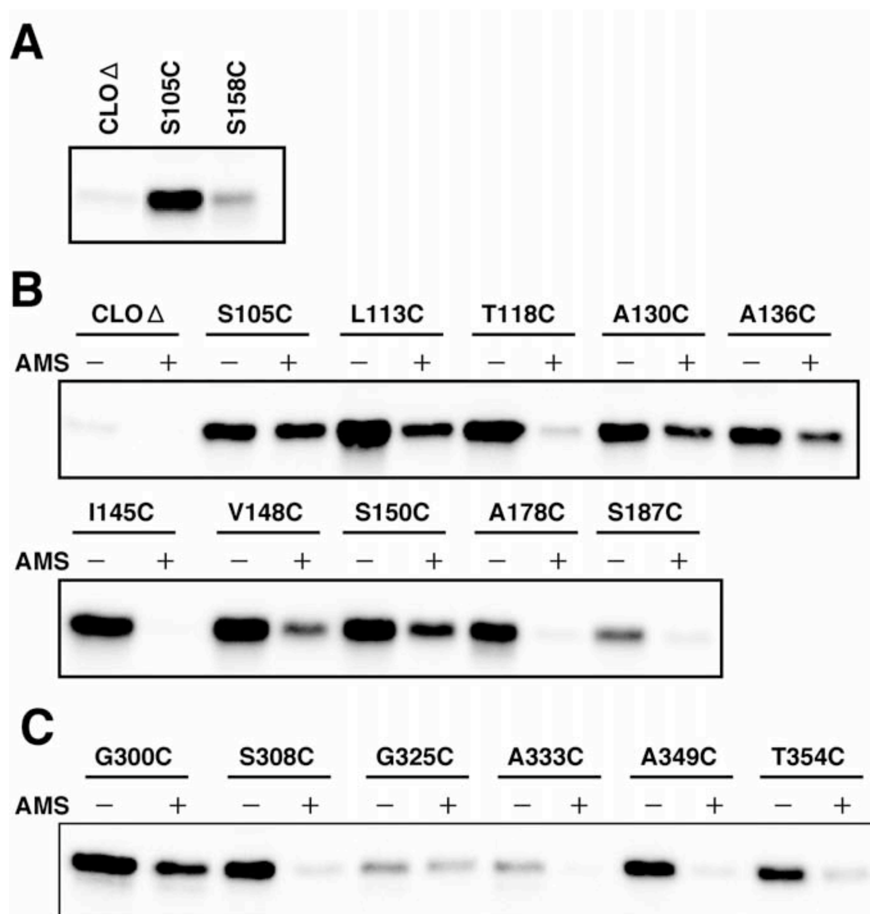
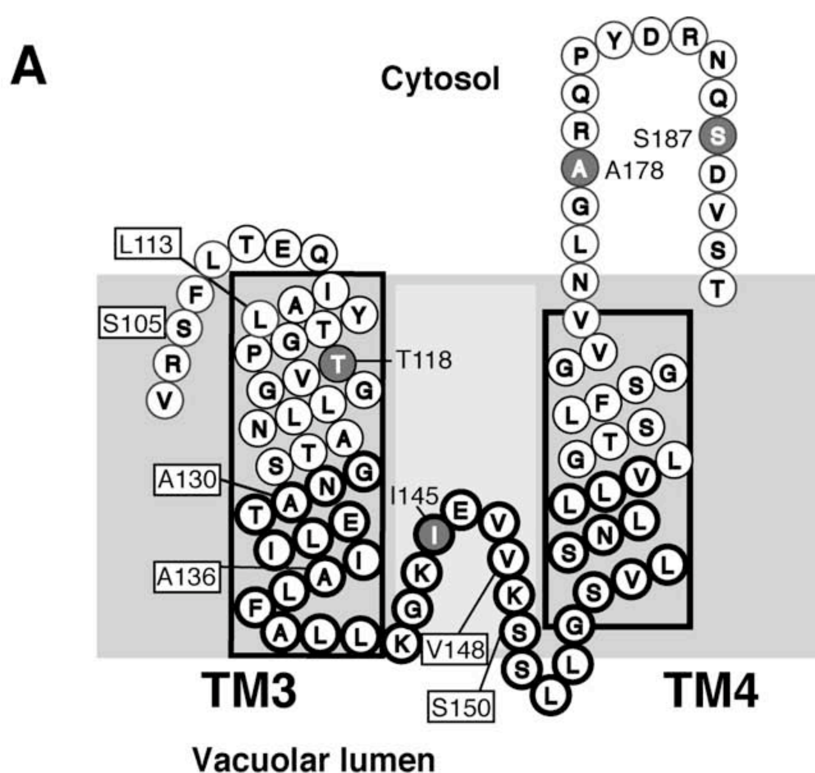


Fig. 7. Cysteine scanning mutagenesis and membrane topology test. *A*, reactivity of cysteine-less (CLOΔ), S105C and S158C mutant exchangers with BM in the presence of 0.1% SDS and 1% Triton X-100. *B* and *C*, membranes prepared from yeast expressing the cysteine-less mutant and single cysteine mutants of the repeat c-1 (*B*) and c-2 (*C*) were incubated in the absence (–) or presence (+) of 400 μM AMS, followed by 5-fold dilution and labeling with 1 mM BM in the presence of 0.02% Triton X-100. Membranes were treated with 0.1% SDS and 1% Triton X-100 and then OsCAX1a labeled with BM was isolated using streptavidin-agarose. The proteins obtained were subjected to immunoblotting with the anti-OsCAX1a antibody.



B

```
α-1
NCX1    141 SSAPEILLSVIEVCGHNFTAGDLGPSTIVGSAAFNMFII
NCX2    138 SSAPEILLSVIEVCGHNFQAGELGPGTIVGSAAFNMFVV
CalX    190 SSAPEILLSVIEIYAKDFESGDLGPGTIVGSAAYNLFMI
OsCAX1a 128 GNATELIIALFALL---KGKIEVVKCSLLGSVLSNLLLV
CAX co      GN..E.Ia...Aa.---.....aa..h..GSaa.N.L.V
              * aa   a          a  h  **   *  a
α-2
NCX1    843 TSVPDTFASKVAA--TQDQYADASIGNVTGSNAVNVFLG
NCX2    793 TSIPDTFASKVAA--LQDQCADASIGNVTGSNAVNVFLG
CalX    822 TSIPDTFASMIAA--KHDEGADNCIGNVTGSNAVNVFLG
OsCAX1a 128 GNAAEHAGAIIFAL---KNKLDITLGVALGSATQISMFV
CAX co      GNAAEH......A.---....Da......GS..Qa....
                -       *        *      **
```

Fig. 8. A topological model of the repeat c-1 and its borders. *A*, the residues that were accessible from the cytosol are highlighted (Leu[113], Thr[118], Ile[145], Ala[178] and Ser[187]) and those not accessible from the cytosol are boxed (Ser[105], Ala[130], Ala[136], Val[148] and Ser[150]). Residues in the repeat c-1 are circled with a thick line. *B*, sequence alignment with Na^+/Ca^{2+} exchangers. NCX1 (M57523), NCX2 (P48768) and CalX (AF009897), OsCAX1a and consensus sequences of CAXs (CAX co) are aligned. Large boxes indicate putative TMs. *Asterisks* indicate identical residues. Aliphatic (Leu , Ile and Val; a), and acidic residues (Asp and Glu; -) are also marked.