Structural basis for alternating access of a eukaryotic calcium/proton exchanger

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Eukaryotic Ca²⁺ regulation involves sequestration into intracellular organelles, and expeditious Ca²⁺ release into the cytosol is a hallmark of key signalling transduction pathways. Bulk removal of Ca²⁺ after such signalling events is accomplished by members of the Ca²⁺/cation (CaCA) superfamily. The CaCA superfamily includes the Na⁺/Ca²⁺ (NCX) and Ca²⁺/H⁺ (CAX) antiporers, and in mammals the NCX and related proteins constitute families SLC8 and SLC24, and are responsible for the re-establishment of Ca²⁺ resting potential in muscle cells, neuronal signalling and Ca²⁺ reabsorption in the kidney. The CAX family members maintain cytosolic Ca²⁺ homeostasis in plants and fungi during steep rises in intracellular Ca²⁺ due to environmental changes, or following signal transduction caused by events such as hyperosmotic shock, hormone response and response to mating pheromones. The cytosol-facing conformations within the CaCA superfamily are understood, and the transport mechanism remains speculative. Here we determine a crystal structure of the Saccharomyces cerevisiae vacuolar Ca²⁺/H⁺ exchanger Vcx1 at 2.3 Å resolution in a cytosol-facing, substrate-bound conformation. Vcx1 is the first structure, to our knowledge, within the CAX family, and it describes the key cytosol-facing conformation of the CaCA superfamily, providing the structural basis for a novel alternating access mechanism by which the CaCA superfamily performs high-throughput Ca²⁺ transport across membranes.

The CaCA superfamily is defined by the presence of two short, repeating homologous sequences, termed the α-repeats, found in predicted transmembrane regions. The α-repeats are opposite in topology and are believed to have arisen from a gene duplication event. Mutagenesis and recent structural data have identified this region as essential for ion binding and transport, and specifically two key acidic residues (Glu or Asp) are implicated in coordinating Ca²⁺ ions at the active site. Members of the CAX family are approximately 400 residues long with 11 predicted transmembrane helices. The first helix (MR), found in eukaryotic CAX members, has a regulatory role in plant members and is suggested to be involved in protein targeting and/or signalling in yeast. The 10 remaining transmembrane helices (M1–M10) perform the transport function, and are composed of two symmetrically related halves (M1–M5 and M6–M10) connected through a negatively charged loop termed the ‘acidic motif’. Saccharomyces cerevisiae Vcx1 catalyses low-affinity (Michaelis constant  Ka = ~25 μM), high-capacity (maximum rate Vmax = ~35 nmol Ca²⁺ min⁻¹ mg⁻¹) vacuolar Ca²⁺ exchange. To establish function of the purified protein, Vcx1 was reconstituted into liposomes and assayed for Ca²⁺ uptake activity. In this system, purified Vcx1 demonstrated Ca²⁺ uptake monotonically dependent on pH gradient (Supplementary Fig. 1). Vcx1 shares ~50% sequence identity with other members of the Ca²⁺/H⁺ exchanger family, including the canonical CAX proteins of Arabidopsis thaliana (Supplementary Fig. 2).

Vcx1 was solved experimentally to 2.3 Å resolution (Rfree of 22.5%) by molecular replacement, supported by iodine-based experimental phases (Fig. 1, Supplementary Table 1 and Supplementary Fig. 3). The structure encompasses residues 22–401 with the exception of a short loop (184–191) between M4 and M5. Two identical (root mean squared deviation (r.m.s.d.) 0.21 Å over 285 Cx atoms) monomers are found in the asymmetric unit. Six divalent cations are identified as Ca²⁺ or Mn²⁺ in the Vcx1 monomer, on the basis of their coordination geometry and anomalous scattering differences (Supplementary Fig. 4).

The shape of the Vcx1 monomer, viewed perpendicular to the membrane plane, resembles that of a wedge (Fig. 1). Viewed from the vacuolar side of the membrane, the tapered end of the wedge consists of two long antiparallel helices M1 and M6, which are intertwined and tilted ~30° with respect to the membrane normal. The central four-helix core contains the α-repeats, and is comprised of M2–M3 and M7–M8. M2 and M7 are kinked at their midpoints and change direction ~35° in the middle of the membrane plane to create M2a/M2b and M7a/M7b. These two oppositely related helix kinks meet in the mid-membrane plane, forming an hourglass shape, where the CAX family display the conserved GXXE(H) signature sequence necessary for calcium binding and transport. M3 and M8 are also tilted with respect to the membrane normal and line the interior of the hourglass. M4–M5 and M9–M10 form the outer components of a right-handed bundle which flank the central core and constitute the thicker side of the wedge shape. The 20-residue ‘acidic motif’ connecting the two duplicated halves of the protein between M5 and M6 is predicted to be disordered based on sequence. However, a clearly resolved α-helix (which we term the acidic helix) for this sequence is observed in the structure. This helix is oriented parallel to the membrane and lies directly underneath the α-repeat regions on the cytosolic side.

A centrally located Ca²⁺ ion occupies the active site of Vcx1, coordinated by Glu 302 on M7b and Ser 325 on M8 (Fig. 2). The Ser 325 residue is generally conserved throughout the CaCA superfamily, and in NCX and NCKX family members the analogous serine residue has been shown to have an important role in Ca²⁺ transport (Supplementary Fig. 5). Three ordered water molecules complete the octahedral coordination geometry of Ca²⁺ (Supplementary Fig. 4b). The presence of water molecules at the binding site suggest that the Ca²⁺ ion reaches the active site in a partially hydrated state, balancing the stronger binding of entropically ordered side chains with more loosely bound water to complete the coordination sphere. Glu 106, Asn 299 and the backbone carbonyl of Gly 102 coordinate the three water molecules. The remainder of the Ca²⁺ active site is stabilized by specific interactions from polar residues in the transmembrane regions of M2, M3, M7 and M8. The conserved Asn 299 and His 303 of M7b form a hydrogen bond to Ser 129 and Ser 132, respectively, of the adjacent M3 helix, and the conserved Asn 103 on M2b forms a hydrogen bond to Gln 328 on M8. M2a is bent away from the bundle of helices M2b, M3, M7 and M8, and in this configuration it is not packed tightly against the protein body (Fig. 3). M2a and the connected C-terminal half of M1 are bent away from the active site, exposing the central Ca²⁺ ion to the cytosol. The M2a/M1 arrangement creates a substantial

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vestibule that is accessible from the intracellular bulk solvent. This vestibule is conical in shape and has a negatively charged interior surface potential (Fig. 3c). The interior of the cavity vestibule is circumscribed by M2a, the C-terminal half of M1, M7b and the N-terminal half of M8, and allows access from the cytosol to the central Ca\textsuperscript{2+} binding site. Thus, the Vcx1 protein structure represents a substrate-bound, cytosol-facing conformation.

Lying across the cytosolic entrance to the vestibule, the acidic helix also coordinates two Ca\textsuperscript{2+} ions (Supplementary Fig. 4c). These two ions lie on the cytosolic side, 11 Å from the central Ca\textsuperscript{2+} site, coordinated by Asp 234 and Glu 230 of the acidic helix and by Glu 83 of M1 (Fig. 2c). The acidic motif has been suggested to have a role in Ca\textsuperscript{2+} binding\textsuperscript{23}. In mammalian NCX members, the analogous region connecting helices M5 and M6 contains a large intracellular calcium-binding domain (CBD1) responsible for stimulating activity in the transporter domain in the presence of Ca\textsuperscript{2+} (ref. 28) (Supplementary Fig. 5). The CBD1 Ca\textsuperscript{2+} binding sites are similarly formed from acidic motifs although they coordinate ions using \(\beta\)-sheets rather than \(\alpha\)-helical secondary structures\textsuperscript{29}. Molecular dynamics simulations performed with the Vcx1 structure suggest that the acidic helix maintains an \(\alpha\)-helical conformation in the presence of the two coordinated Ca\textsuperscript{2+} ions, and becomes more flexible in their absence (Supplementary Fig. 6). The increased rigidity of the Vcx1 acidic helix at higher Ca\textsuperscript{2+} concentrations indicates a possible Ca\textsuperscript{2+}-dependent regulatory function.

Figure 1 | Topology and fold of the Vcx1 protein. The symmetrically related halves of the Vcx1 monomer are coloured in a double colour spectrum from the N to C terminus. Helices of matching colour are related by symmetry. a–c, The Vcx1 monomer as viewed in the membrane along the axis of symmetry (a), rotated by 90° (b) and viewed from the vacuolar side of the membrane (c). d, Topology map of the Vcx1 monomer; CAX family conserved residues are coloured in red, \(\alpha\)-repeat sequences are denoted by dashed circles.

Figure 2 | Calcium binding sites in the Vcx1 crystal structure. a, Overview of site 1 and site 2 with helices MR, M1 and M6 removed for clarity. The cytosol is on the bottom of the image and Ca\textsuperscript{2+} ions are coloured in yellow. b, Active site Ca\textsuperscript{2+} substrate ion and interacting residues found in site 1. Hydrogen bonds are shown as dashed lines; numbers denote atomic distances (Å). 2m\(F_o\) – \(D_F\) map is shown contoured at 1σ (blue mesh). c, Ca\textsuperscript{2+} ions at the acidic helix in site 2 with interacting residues labelled. Hydrogen bonds are shown as dashed lines; numbers denote atomic distances (Å). 2m\(F_o\) – \(D_F\) map is shown contoured at 1σ (blue mesh).
function for this region, perhaps augmenting conductance in the presence of increased cytosolic Ca\(^{2+}\).

Comparison of the two structural repeats (M1–M5 and M6–M10) of Vcx1 reveals a structurally similar core region that is closely packed and rigid (M3–M5, M8–M10) (Supplementary Fig. 7b). In contrast, considerable differences are found in the M2a helix and C-terminal half of M1 when compared to M7a and M6. Superposition between helices M1–M2a and M6–M7a reveal a \(\sim 12^\circ\) and \(\sim 7^\circ\) asymmetric difference in the angle of M1 and M2a, respectively (Supplementary Fig. 7c, d). This structural divergence, in combination with loose packing and intracellular location, implicate this mobile region as the cytosolic gate. A dynamic straightening of the M1/M2a helices would collapse the cytosolic vestibule, and this motion could be coordinated by a structural rearrangement into a vacuole-facing conformation.

The Vcx1 conformation also sheds light on the transport cycle of CaCA proteins by comparison with the recent structure of an archaeobacterial Na\(^+\)/Ca\(^{2+}\) exchanger from Methanococcus janaschii (mjNCX)\(^1\). Despite low sequence identity (14%) to Vcx1, the overall fold and topology of mjNCX is similar. However, unlike Vcx1, the mjNCX exchanger is closed to the cytosolic environment and instead represents a periplasm-facing conformation, as reflected in the overall displacement between similar atoms (r.m.s.d. 5.7 Å over 269 C\(_\alpha\) atoms). Structural alignment of the Vcx1 and mjNCX structures reveals a similar placement of the core region and of helices M7 and M1. Superposition of Vcx1 reveals a structurally similar core region that is closely packed and are shown from the axis of symmetry. b, View rotated by 90°. c, The cytoplasmic vestibule as oriented in panel a and depicted with a slab surface representation coloured by electrostatic potential (red to blue; \(-10\) to \(-10\) kT e\(^{-1}\)). Helices MR, M1 and M6 have been removed for clarity. Ca\(^{2+}\) ions (yellow spheres) pinpoint site 1 and site 2.

Figure 4 | Transport cycle of Vcx1 and structural comparison to mjNCX. a, Comparison of M2 and M7 and active site glutamate residues between Vcx1 (purple) and mjNCX (cyan). Ca\(^{2+}\) ions from each model are depicted as spheres. b, Comparison of M1 and M6 between Vcx1 (purple) and mjNCX (cyan). c, Schematic of Vcx1 turnover. Structures are coloured as in panel a. Proposed substrate movement is denoted by black arrows, and calcium by yellow circles. Red arrows show protein movement in the cytosol-facing state of Vcx1 (left) that results in the vacuole-facing conformation on the right. Return to the cytosol-facing state presumably requires reversal of the movements denoted by the red arrows.
Ca\(^{2+}\) is coordinated by the acidic helix, and Ca\(^{2+}\) is able to reach the active site. The Vcx1 side chains of Glu 302 and Ser 325 partially replace the Ca\(^{2+}\) hydration shell, and subsequent completion of coordination by Glu 106 displaces some of the remaining water molecules to bring helix M2b inward towards the active site. This movement of M2 towards the core can initiate M2a straightening and M1/M6 translation, closing the cytosolic vestibule. The translation of helices M1/M6 uncovers a vacular cleft and coordinates opening of M7a to expose the active site Ca\(^{2+}\) ion to the vacuole. The vacuole-facing conformation, in combination with the acidic pH in the vacuole, lowers the Ca\(^{2+}\) affinity of active site residues Glu 106 and Glu 302, leading to release of Ca\(^{2+}\) substrate into the vacuole. The cyclical pumping action of the M1/M6 ‘piston’, coupled to flexible helices surrounding the active site (M2a, M7a), provides an efficient framework for the rapid turnover necessary for high-throughput Ca\(^{2+}\) exchange.

In conclusion, Vcx1 is the first CAX family structure, and the first structure of the CaCA superfamily in a cytosol-facing conformation. It provides a structural basis for an alternating access mechanism for the Vcx1 protein and the CaCA superfamily in general. These findings lay the groundwork for future exploration of Ca\(^{2+}\) transport by CaCA superfamily members and lend insight to fundamental aspects of Ca\(^{2+}\) homeostasis and eukaryotic signal transduction processes.

METHODS SUMMARY

The Vcx1 protein from Saccharomyces cerevisiae (Uniprot ID Q99385) was expressed in S. cerevisiae and purified using a decahistidine affinity-tag. Solubilization and purification were performed using dodecyl-

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METHODS

Expression and purification. The Vcx1 protein from Saccharomyces cerevisiae (Uniprot ID Q99385) was incorporated into the 2µ expression plasmid p23-GAL1 modified with N-terminal and C-terminal purification tags, as described11. Transforme...
50. Jo, S., Lim, J. B., Klauda, J. B. & Im, W. CHARMM-GUI Membrane Builder for mixed bilayers and its application to yeast membranes. Biophys. J. 97, 50–58 (2009).
51. Berendsen, H. J. C., Postma, J. P. M., Van Gunsteren, W. F., Dinola, A. & Haak, J. R. Molecular dynamics with coupling to an external bath. J. Chem. Phys. 81, 3684–3690 (1984).
52. Bussi, G., Donadio, D. & Parrinello, M. Canonical sampling through velocity rescaling. J. Chem. Phys. 126, 014101–014101–7 (2007).
53. Parrinello, M. & Rahman, A. Polymorphic transitions in single crystals: A new molecular dynamics method. J. Appl. Phys. 52, 7182–7190 (1981).