Topology mapping of the vacuolar Vcx1p Ca\textsuperscript{2+}/H\textsuperscript{+} exchanger from *Saccharomyces cerevisiae*

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*Saccharomyces cerevisiae* uses vacuolar storage to dynamically control the cytoplasmic calcium concentration. Vcx1p, a Ca\textsuperscript{2+}/H\textsuperscript{+} antiporter and a member of the CAX (Ca\textsuperscript{2+}/anion exchanger) family of exchangers, is one of the proteins that sequesters calcium into the vacuole. Although the biological importance of Vcx1p is clear, the molecular mechanism by which Vcx1p and its family members mediate Ca\textsuperscript{2+}/H\textsuperscript{+} exchange activity remains poorly understood. To provide a basic structural framework for understanding functional studies of the CAX proteins, we have mapped Vcx1p’s topology using three biochemical assays: C-terminal reporter localization, glycosylation mapping and proteolysis. We have found that the protein has an odd number of TM (transmembrane) domains and that its termini are located on opposite sides of the membrane, with the N-terminus in the cytoplasm. Our results indicate that loops 1, 3, 7 and 9 are luminal, while loops 6 and 8 are cytosolic. Our experimentally-based topology model for Vcx1p is in agreement with models derived from topology algorithms and with biochemical data reported by other groups. In addition, our studies suggest that the calcium domain, a nine-residue domain found to be critical for function in CAX proteins from plants, is not essential to Vcx1p activity.

Key words: Ca\textsuperscript{2+} transporter, dual topology reporter, glycosylation mapping, proteolysis.

INTRODUCTION

Calcium homeostasis in *Saccharomyces cerevisiae* is involved in numerous cellular processes, including responses to pheromones and osmotic stress [1], and consequently the cytoplasmic concentration of free Ca\textsuperscript{2+} is tightly controlled. In contrast to higher eukaryotes, where Ca\textsuperscript{2+} regulation results largely from extrusion or sequestration within the endoplasmic reticulum, both yeast and plant cells use the vacuole as the principal compartment for Ca\textsuperscript{2+} storage. Vacuolar Ca\textsuperscript{2+} transport results from the combined activity of three proteins: Pmc1p, a Ca\textsuperscript{2+} pump responsible for the ATP-dependent transport of Ca\textsuperscript{2+} into the vacuole [2], Vcx1p, a Ca\textsuperscript{2+}/H\textsuperscript{+} exchanger that brings Ca\textsuperscript{2+} into the vacuole with energy provided by the H\textsuperscript{+} gradient [3,4], and Yvc1p, a Ca\textsuperscript{2+} channel that facilitates Ca\textsuperscript{2+} release into the cytoplasm [5,6].

The first eukaryotic Ca\textsuperscript{2+}/H\textsuperscript{+} exchanger discovered, Vcx1p was originally identified to be important in Ca\textsuperscript{2+} homeostasis from a screen isolating proteins that permit pmc1 mutants to grow in high levels of Ca\textsuperscript{2+} [3]. Its activity in *vivo* is negatively regulated by calcineurin, the Ca\textsuperscript{2+}-dependent serine/threonine phosphatase, probably through an indirect mechanism [3]. At the molecular level, VCX1 encodes a 45 kDa protein that belongs to the CAX (Ca\textsuperscript{2+}/anion exchanger) family of cation exchangers. Members of the CAX family share several distinguishing features [7,8]. First, they contain 10–11 hydrophobic stretches long enough to be TM (transmembrane) domains. Secondly, the proteins appear to contain an internal tandem repeat: the hydrophobic stretches are in two clusters that are separated by the longest loop which contains a high concentration of acidic residues. Third, each tandem repeat contains a specific conserved sequence known as c-1 and c-2. Both c-1 and c-2 are largely predicted to lie in loop regions, and previous studies in the OsCAX1a (*Oryza sativa* CAX1a) protein, where they are the third and eighth loops, suggest that these loops may form re-entrant structures [9]. Re-entrant loops, in which the segment between two canonical TM domains is not located in aqueous solution but instead folds down into the core of the transporter, are now seen in a variety of integral membrane transport protein structures where they are generally located near or along the ion translocation pathway. Mutagenesis studies of Vcx1p, in which substitutions within the c-1 and c-2 segments result in Ca\textsuperscript{2+}-sensitive growth phenotypes, are consistent with c-1 and c-2 playing an analogous critical role in ion transport by Vcx1p [9,10].

A number of studies, both on Vcx1p as well as the related CAX proteins, are beginning to provide insights into the structure and function of these proteins. Recently, in a large-scale effort to determine the orientation of the C-termini of 617 different yeast proteins, Kim et al. [11] found that the C-terminus of Vcx1p is located in the lumen. In contrast, there is only indirect evidence for the location of the Vcx1p N-terminus: a large scale mass spectrometry study showed that Ser\textsuperscript{11} of the Vcx1p N-terminus is phosphorylated [12], consistent with a cytoplasmic location where it would be accessible to kinases and phosphatases. The precise locations of the loop domains are less well understood; the exceptions are loops four and eight, which cysteine accessibility studies of the OsCAX1a protein clearly indicate have a cytoplasmic location [9]. Several individual residues have been identified through site-directed mutagenesis as important for the function of Vcx1p (Leu\textsuperscript{228} and Met\textsuperscript{283}), however a mechanistic understanding of how these mutations exert their effects will require a more detailed structural model [3,13,14].

As a first step toward understanding the fundamental architecture of the CAX family, we have performed a basic topology mapping of the Vcx1p exchanger. In the short term, knowing which loops of the protein are readily accessible to the intracellular milieu will provide us with a framework for better understanding

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Abbreviations used: CA, C-terminus lacking the last six amino acids of the wild-type protein; CAX, Ca\textsuperscript{2+}/anion exchanger; CBd, C-terminus tagged with DTR at the very end of the full-length protein; Cl, C-terminal loop; d, DTR fusion; DTR, dual topology reporter; Endo H, endoglycosidase H; g, Suc2p, glycosylation site insertion; HA, haemagglutinin; L1 (etc.), loop number (etc.); NP-40, Nonidet P40; Nt, N-terminal loop; OsCAX, Oryza sativa CAX; Suc2p-frag, a small fragment of Suc2p; TM, transmembrane; x, Factor Xa consensus site insertion.

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of the function and regulation of Vcx1p as well as other family members. Eventually, a low-resolution map of membrane topology may also aid in obtaining and validating a high-resolution protein structure of these antiporters.

EXPERIMENTAL

Yeast strains

The STY50 (MATa his4-401 leu2-3,112 trp1-1 ura3-52 HOL1-1 suc2::LEU2) strain was obtained from Professor M. Hochstrasser (Department of Molecular Biophysics and Biochemistry, Yale University, U.S.A.) [15], and W303-1A (MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1) and K667 (MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 cnb1::LEU2 pmc1::TRP1 vcx1Δ) strains were from Professor K. W. Cunningham (Department of Biology, John Hopkins University, U.S.A.) [15], and W303-1A (Department of Molecular Biophysics and Biochemistry, Yale University, U.S.A.) [3]. K667 is congenic to W303-1A (also known as K601). The STY50 strain was used for gap repair cloning of the VCX1 truncations. The W303-1A strain was used for the expression and analysis of all the insertion mutants. The calcium sensitive K667 truncations. The W303-1A strain was used for complementation studies assaying growth in the presence of high calcium concentrations.

Yeast media

Rich and minimal yeast media were as previously described [16]; 300 mM CaCl2-containing minimal medium was prepared without phosphate salts and using ammonium chloride as the nitrogen source [4].

DNA methods

VCX1 construct

The VCX1 open reading frame was amplified with primers containing HindIII and XhoI restriction sites (5’ and 3’ ends respectively) and inserted into the pYES2/CT plasmid (Invitrogen).

DTR (dual topology reporter) truncations

DTR truncation constructs were generated in the pJK90 plasmid [15] by gap repair cloning. Fragments of VCX1 were amplified from the VCX1/pYES2/CT construct using ExTaq (Takara). The forward primer consisted of 30 bases of the OST4 promoter region of pJK90 and the first 21 nucleotides of VCX1. The reverse primers were complementary to bases 275 to 241 of pJK90 and a portion of the VCX1 gene starting at the sites indicated in Table 1. Primer sequences are given in the Supplementary online data, at http://www.BiochemJ.org/bj/414/bj4140133add.htm. pJK90 was linearized using a Smal restriction site 15 bp upstream of the start of the OST4-DTR fusion. Linearized pJK90 and PCR products were co-transformed into STY50 using the lithium acetate method, and uracil prototrophs were selected on solid agar plates (minimal medium lacking uracil). Liquid cultures were inoculated and grown to saturation in minimal medium lacking uracil and containing 2% galactose. Cells were diluted to a density of 600 units of cells harvested during mid-exponential growth as per the protocol, with the additional step of vortex mixing the cells (four 1 min treatments alternating with cooling on ice) with 150 μL acid-washed glass beads to ensure lysis. Half of each sample was treated with 0.005 units of Endo H (endoglycosidase H; Roche), whereas the second half served as a control; both aliquots were incubated at 37°C for at least 2 h. Proteins were separated using standard SDS/PAGE and visualized with a single round of QuikChange® mutagenesis (using oligonucleotides with 6 bases that looped out) at the locations shown in Table 1. Factor Xa or glycosylation site insertion mutants were then generated by inserting the appropriate cassette at the KasI site. All constructs were sequenced to verify the correct insertion. The Factor Xa cassette was generated from two oligonucleotides that encoded two consecutive protease consensus sites flanked by several spacer residues and finally the bases required to generate the KasI sticky ends. The amino acid sequence inserted was GAIEGRIEGRGTGA. The glycosylation cassette was generated by amplification of a fragment from the Suc2p protein containing the N-glycosylation sites from a plasmid template (pJK90) and using oligonucleotides encoding the KasI restriction site. The following sequence was inserted in these mutants: LTNWEDQPIAIAFKRNDSGAFSGSMVVDYNNTSGFFNDTIDPRQRVCVAVIWTY.

Complementation assays

Complementation of the Ca2+-sensitive growth phenotype of the K667 strain was examined for the DTR, Factor Xa and glycosylation insertion constructs. Factor Xa and glycosylation insertion constructs were transformed directly into K667 cells, while DTR constructs were freshly generated by transforming K667 with both linearized pJK90 and PCR products. Uracil prototrophs were selected on solid agar plates (minimal medium lacking uracil). Liquid cultures were inoculated and grown to saturation in minimal medium lacking uracil and containing 2% galactose. Cells were diluted to a density of 600 units of cells harvested during mid-exponential growth as per the protocol, with the additional step of vortex mixing the cells (four 1 min treatments alternating with cooling on ice) with 150 μL acid-washed glass beads to ensure lysis. Half of each sample was treated with 0.005 units of Endo H (endoglycosidase H; Roche), whereas the second half served as a control; both aliquots were incubated at 37°C for at least 2 h. Proteins were separated using standard SDS/PAGE and visualized

Table 1 Predicted topology of VCX1p and location of the modifications analysed in this paper

<table>
<thead>
<tr>
<th>Area of the Protein</th>
<th>Putative Loop Location</th>
<th>Modification Site</th>
<th>Associated Constructs</th>
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</thead>
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<td>N-terminus</td>
<td>1–32</td>
<td>13</td>
<td>Ntg, Ntx</td>
</tr>
<tr>
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<td>54–58</td>
<td>56</td>
<td>L1d, L1g, L1x</td>
</tr>
<tr>
<td>Loop 2</td>
<td>83–91</td>
<td>88</td>
<td>L2d, L2g, L2x</td>
</tr>
<tr>
<td>Loop 3</td>
<td>115–125</td>
<td>123</td>
<td>L3d, L3g, L3x</td>
</tr>
<tr>
<td>Loop 4</td>
<td>148–158</td>
<td>155</td>
<td>L4d, L4g, L4x</td>
</tr>
<tr>
<td>Loop 5</td>
<td>184–198</td>
<td>192</td>
<td>L5d, L5g, L5x</td>
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<td>Loop 6</td>
<td>227–251</td>
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<td>Loop 7</td>
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<td>L8d, L8g, L8x</td>
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<td>Loop 9</td>
<td>345–348</td>
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<td>Loop 10</td>
<td>373–378</td>
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<td>L10d, L10g, L10x</td>
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<td>C-terminus</td>
<td>400–411</td>
<td>404</td>
<td>Ctg, Ctx</td>
</tr>
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<td></td>
<td></td>
<td>405</td>
<td>Cad</td>
</tr>
<tr>
<td></td>
<td></td>
<td>411</td>
<td>Cb</td>
</tr>
</tbody>
</table>

Analysis of glycosylation

Glycosidase sensitivity of Vcx1p-DTR derivatives was examined largely following the protocol of Kim et al. [17]. Extracts were made from ten Dmax units of cells harvested during mid-exponential growth as per the protocol, with the additional step of vortex mixing the cells (four 1 min treatments alternating with cooling on ice) with 150 μL acid-washed glass beads to ensure lysis. Half of each sample was treated with 0.005 units of Endo H (endoglycosidase H; Roche), whereas the second half served as a control; both aliquots were incubated at 37°C for at least 2 h. Proteins were separated using standard SDS/PAGE and visualized...
by Western blots. For DTR experiments, constructs were grown in STY50 cells, proteins were analysed on a Tris/6% glycine gel, and the nitrocellulose was probed with an anti-HA (anti-haemagglutinin) monoclonal antibody (Covance). Glycosylation site insertion mutants were grown in the K601 strain, analysed on a Tris/12% glycine gel and probed with a mouse anti-V5 monoclonal antibody (Invitrogen).

**Factor Xa treatment**

Right-side out yeast membranes were prepared precisely as described in [18]. Membranes were resuspended in storage buffer (20 mM Tris/HC1, pH 7.5, 250 mM sorbitol, 50 mM potassium acetate and 1 mM 2-mercaptoethanol) using a Dounce homogenizer and stored at –80 °C. Proteolysis experiments were conducted on 100 μg of protein in Factor Xa buffer: 20 mM Tris/HC1, pH 7.5, 250 mM sorbitol, 100 mM NaCl and 1 mM EDTA. Reaction volumes were kept constant at 100 μl by adjusting the amount of buffer in each case: Factor Xa (New England Biolabs) was added at 1 μg per 20 μg of total protein, and when needed, NP-40 (Nonidet P40) was added to a final concentration of 0.2 % from a stock solution (1% in Factor Xa buffer). Factor Xa and NP-40 were both omitted from mock reactions. Reactions were incubated for 3 h on ice and stopped by the addition of 1 mM PMSF. Proteins were separated on a Tris/16.5% tricine gel and analysed by immunoblotting.

**Immunoblot analysis**

Proteins were transferred from polyacrylamide gels to PVDF membranes, blocked with 2% non-fat dried skimmed milk, probed with the appropriate primary and secondary antibodies, and visualized using Amersham ECL® (enhanced chemiluminescence) Plus (GE Healthcare). Primary antibodies: anti-HA mouse monoclonal antibody 16B12 (1:7000; Covance), anti-V5 mouse monoclonal antibody (1:7000; Invtrogen), and anti-Vcx1p rabbit polyclonal antibody generated against residues 8–25 in the N-terminus (Protein G-purified from rabbit sera generated by Open Biosystems, used at 470 ng/ml). HRP-conjugated secondary antibodies: goat anti-mouse IgG (1:7000; Invitrogen), and anti-V5 monoclonal antibody 16B12 (1:7000; Covance), anti-V5 monoclonal antibody (1:7000; Invtrogen), and anti-Vcx1p rabbit polyclonal antibody generated against residues 8–25 in the N-terminus (Protein G-purified from rabbit sera generated by Open Biosystems, used at 470 ng/ml). HRP-conjugated secondary antibodies: goat anti-mouse IgG (1:7000; Roche) for the anti-HA Western blots, goat anti-mouse IgG (1:7000; Sigma) for the anti-V5 Western blots, and donkey anti-rabbit IgG (1:5000; Amersham-GE Healthcare) for the anti-Vcx1p Western blots.

**Membrane topology predictions**

MEMSAT [19], TMHMM 2.0 [20], HMMTOP 2.0 [21,22], and TopPred II [23] were used to predict Vcx1p topology.

**RESULTS**

**Predicted topology of Vcx1p**

We started by applying four widely-used membrane topology prediction algorithms to the Vcx1p sequence; TopPred II [23], MEMSAT [19], TMHMM 2.0 [20] and HMMTOP 2.0 [21,22]. Ultimately all prediction algorithms are limited by the quantity and diversity of known structures available for training. This poses particular limitations on membrane protein structure prediction, since especially by comparison with soluble proteins, only a relatively modest number of membrane protein structures have been solved. The four independent methods we applied here span a breadth of approaches including several which rely primarily on Hidden Markov Modeling algorithms while others focus more heavily on local protein structure. Among these, the Hidden Markov Model-based TMHMM has been found to perform the best at both identifying membrane-spanning regions and predicting sitedness [24].

All four approaches provided similar results overall, with three of the four programs (MEMSAT, TMHMM and HMMTOP) predicting 11 TM-spanning sections. The fourth, TopPred II, predicted only 10 TMs. That TopPred II predicted one fewer TM domain than other programs is not particularly surprising; a comparative study of prediction algorithms found that TopPred displays the highest rate of false negatives (missing nearly 50% of TM domains) among the algorithms tested (for instance, TMHMM missed less than 10% of TM segments) [24]. In application to Vcx1p, the difference in number of predicted TMs stems from the disposition of ~60 residues at the extreme N-terminus; according to TopPred, the entire segment is located in the lumen, while the other algorithms predict the region contains a cytoplasmic N-terminus and the first TM.

The four programs all agree on a luminal location for the C-terminus and the number and general position of the remaining TMs, although the precise length and boundaries of the domains differ slightly by algorithm. Table 1 shows the positions of non-TM segments all programs concur are located at the termini or in loops between TMs. We used the 11-TM domain model as a preliminary map for designing the subsequent topology studies to increase the likelihood of detecting the complete complement of TMs in Vcx1p.

**The Suc2-His4C DTR system**

We used the Suc2-His4C DTR to provide a preliminary map of Vcx1p topology [25]. In this approach, recombination is used to generate a series of fusion proteins in which the DTR segment is inserted at specified locations into Vcx1p. The DTR region itself encodes a segment of 126 kDa containing three consecutive HA epitopes and two independent protein domains: a segment of Suc2p (Suc2p) which undergoes N-linked glycosylation if located in the lumen, and a segment of His4p (His4C) which catalyses the conversion of histidinol to histidine in the cytoplasm. The DTR segment contains a stop codon and therefore the resultant fusion proteins contain only the N-terminal portion of Vcx1p adjoining with the DTR. The protein encoded by the DTR segment is soluble, and its membrane orientation in the context of the chimera is used to locate the segment of host protein immediately upstream of the fusion site.

We generated a panel of twelve fusion proteins. In ten cases, the DTR junction was located within a putative loop (see Table 1 for precise fusion location); the nomenclature we use for these constructs includes the loop location followed by ‘d’ to designate a DTR fusion, i.e. ‘L1d’. The final two fusions are both located for precise fusion location); the nomenclature we use for these constructs includes the loop location followed by ‘d’ to designate a DTR fusion, i.e. ‘L1d’. The final two fusions are both located within the C-terminus: the ‘CAd’ construct is lacking the last 6 residues at the extreme N-terminus; according to the DTR junction was located within a putative loop (see Table 1 for precise fusion location); the nomenclature we use for these constructs includes the loop location followed by ‘d’ to designate a DTR fusion, i.e. ‘L1d’. The final two fusions are both located within the C-terminus: the ‘CAd’ construct is lacking the last 6 residues at the extreme N-terminus; according to the DTR junction was located within a putative loop (see Table 1 for precise fusion location); the nomenclature we use for these constructs includes the loop location followed by ‘d’ to designate a DTR fusion, i.e. ‘L1d’. The final two fusions are both located within the C-terminus: the ‘CAd’ construct is lacking the last 6 residues at the extreme N-terminus; according to the DTR junction was located within a putative loop (see Table 1 for precise fusion location); the nomenclature we use for these constructs includes the loop location followed by ‘d’ to designate a DTR fusion, i.e. ‘L1d’. The final two fusions are both located within the C-terminus: the ‘CAd’ construct is lacking the last 6 residues at the extreme N-terminus; according to the DTR junction was located within a putative loop (see Table 1 for precise fusion location); the nomenclature we use for these constructs includes the loop location followed by ‘d’ to designate a DTR fusion, i.e. ‘L1d’. The final two fusions are both located within the C-terminus: the ‘CAd’ construct is lacking the last 6 residues at the extreme N-terminus; according to
prior to electrophoresis. Positions of molecular mass markers are shown on the left.

Figure 1(A) shows a blot of the entire panel of DTR constructs after treatment with Endo H to remove N-linked glycosylation; the decreasing mobility as the fusion junction is moved toward the C-terminus reflects the predicted increase in size of the corresponding fusion protein. To assess whether the DTR construct was glycosylated, the mobility of each Vcx1p-DTR construct was examined before and after treatment with Endo H (Figure 1B). Whereas some constructs showed a clear mobility shift upon Endo H treatment (L1d, L2d, L4d, L5d, L7d, L9d, CA and CBd), others were unaffected by the enzyme (L3d, L6d, L8d and L10d). The mobility change observed in the former constructs is consistent with the Suc2p portion of the fusion protein being glycosylated in those cases, while the Suc2p domains of the latter constructs are probably unglycosylated. Incomplete glycosylation is observed in several cases (L2d, L4d, L5d and CAD), where a small fraction of protein migrates more rapidly consistent with the fully unglycosylated form. Although in theory this might reflect a small population of protein with a different topological arrangement, it seems more likely to result from incomplete glycosylation of the over-expressed protein, perhaps due to position-specific limited accessibility to enzymes in the glycosylation pathway.

By probing Vcx1p topology with the Suc2-His4C DTR system, it was possible to examine before and after treatment with Endo H to remove N-linked glycosylation; the decreasing mobility as the fusion junction is moved toward the C-terminus reflects the predicted increase in size of the corresponding fusion protein. To assess whether the DTR construct was glycosylated, the mobility of each Vcx1p-DTR construct was examined before and after treatment with Endo H (Figure 1B). Whereas some constructs showed a clear mobility shift upon Endo H treatment (L1d, L2d, L4d, L5d, L7d, L9d, CA and CBd), others were unaffected by the enzyme (L3d, L6d, L8d and L10d). The mobility change observed in the former constructs is consistent with the Suc2p portion of the fusion protein being glycosylated in those cases, while the Suc2p domains of the latter constructs are probably unglycosylated. Incomplete glycosylation is observed in several cases (L2d, L4d, L5d and CAD), where a small fraction of protein migrates more rapidly consistent with the fully unglycosylated form. Although in theory this might reflect a small population of protein with a different topological arrangement, it seems more likely to result from incomplete glycosylation of the over-expressed protein, perhaps due to position-specific limited accessibility to enzymes in the glycosylation pathway.

The glycosylation of Suc2p at loops L1, L2, L4, L5, L7, L9 and and factor Xa constructs were also classified by their ability to complement the Ca\textsuperscript{2+}-dependent growth phenotype of the K667 strain (‘Functional?’: + +, complementation as wild-type; +, complementation, but less than wild-type; –, no complementation).

<table>
<thead>
<tr>
<th>Construct</th>
<th>DTR Location</th>
<th>Suc2p-frag Location</th>
<th>Factor Xa Functional?</th>
<th>Location</th>
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<td>Nt</td>
<td>lumen</td>
<td>–</td>
<td>–</td>
<td>cytoplasm</td>
</tr>
<tr>
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<td>–</td>
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<td>L10</td>
<td>lumen</td>
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**Glycosylation site insertions**

The truncated fusion proteins that form the basis of the DTR approach have a practical experimental limitation that is experimentally difficult to address: if the C-terminus affects protein folding, then its absence in the fusion proteins may alter the topology of the remaining protein. As a complementary and less perturbing approach, we used a ‘sandwich’ approach and inserted Suc2p-frag (a small fragment of Suc2p) containing two N-linked glycosylation sites at various positions within Vcx1p (Table 1). These constructs follow our standard naming convention: loop location followed by a ‘g’ indicating the Suc2p-frag glycosylation insert. We examined whether insertion of the Suc2p-frag affected transport by evaluating whether the insertion constructs still complemented the calcium-sensitive phenotype of the K667 strain. As seen in the first two rows of Figure 2, the K667 strain fails to grow in the presence of high [Ca\textsuperscript{2+}], but this growth defect can be rescued by the expression of Vcx1p. Four constructs, L1g, L2g, L9g and Ctg, complement the K667 Ca\textsuperscript{2+} sensitivity as well as wild-type Vcx1p, while Ntg (where Nt is the N-terminal loop), L3g, and L7g constructs also conferred K667 growth in high calcium albeit at a decreased rate. The slower growth might be due to a decrease in expression levels or exchange activity of the mutant proteins, but the observed complementation indicates that these proteins retain basic transport function, and probably therefore also the basic architecture, of the native Vcx1p. The remaining constructs (L4g, L5g, L8g and L10g) did not complement the calcium sensitivity of the K667 strain; these insertions may alter the levels of Vcx1p expression.

Figure 1 Probing Vcx1p topology with the Suc2-His4C DTR system

Table 2 Summary of results for the Vcx1p topology screens

DTR and Suc2p insertion constructs showing glycosylation are noted as having luminal locations; factor Xa insertion constructs undergoing cleavage in the absence of detergent are designated with a cytosolic location. + indicates that no positive data were obtained in a given test, * that no signal was seen on a Western blot and N.A. indicates ‘not attempted’. Suc2p and factor Xa constructs were also classified by their ability to complement the Ca\textsuperscript{2+}-dependent growth phenotype of the K667 strain (‘Functional?’: + +, complementation as wild-type; +, complementation, but less than wild-type; –, no complementation).
Membrane topology of Vcx1p

Figure 2 Complementation assay of the S2-insertion constructs

Each construct indicated in Table 1 was introduced into the K667 strain. Cells were grown to saturation, diluted to a $D_{600}$ of 0.5, and then serial dilutions (1:5) were plated on solid agar containing minimal medium plus the indicated carbon source and calcium chloride content.

in the K667 strain (see below), affect transport function or alter the conformation of the protein.

Figure 3 shows the effect of Endo H on the electrophoretic mobility of each Suc2p-frag insertion construct, similar to the approach described above for the DTR mutants. All insertion mutants also contain a C-terminal V5 epitope to assay protein levels; of the 12 constructs originally targeted, nine yielded detectable protein (L4g and L5g yielded undetectable protein product and we were unable to generate L6g). The wild-type Vcx1p migrates at the same position before and after treatment, as expected if the protein is not endogenously glycosylated. In contrast, L1g, L3g, L7g, L9g and Ctg display clear mobility shifts after Endo H treatment, indicating the glycosylation of the Suc2p-frag insertion in these mutants. Whereas L1g, L3g and L7g mutants appear almost completely glycosylated, the L9g and Ctg proteins undergo only partial glycosylation. Incomplete glycosylation is frequently observed even in native glycosylated proteins, and may simply reflect local steric constraints to the glycosylation machinery depending on the position of the Suc2p-frag insertion [26–28]. Four chimaeras (Ntg, L2g, L8g and L10g) show no mobility shift after Endo H treatment, consistent with these constructs not being N-glycosylated. The results of this assay indicate a luminal orientation for those constructs that are glycosylated (L1g, L3g, L7g, L9g and Ctg) (Table 2).

Protease accessibility experiments

Although the glycosylation mapping described above provides direct evidence for loops located in the lumen, cytoplasmic loops can only be assigned by indirect inference. We probed directly for loops located on the cytoplasmic surface by introducing a tandem pair of Factor Xa protease consensus sites within the loops and then examining protease susceptibility in intact vesicles. These constructs were named following our standard convention of loop location followed by 'x' to indicate insertion of the Factor Xa consensus sequence. We first tested whether insertion of the protease consensus sites impacted on Vcx1p function by examining the ability of each construct to complement the calcium-sensitive phenotype of the K667 strain (Figure 4). With the exceptions of insertions L4x, L5x and L10x, all the constructs complemented growth, suggesting that the global architecture required for transport function is retained in these mutant proteins.

Right-side-out membrane vesicles were made from yeast cells expressing each mutant construct, the vesicles were subjected to Factor Xa cleavage in the absence or presence of the detergent NP-40, and Vcx1p was visualized by Western blot using a C-terminal V5 epitope (Figure 5). The isolated vesicles show two principal immunoreactive products: the higher band reflects full-length Vcx1p, with an expected molecular mass of 45 kDa, while the
Figure 3  Topology mapping using the Suc2p-frag-insertion constructs

Total cellular extract was isolated from cells expressing the Suc2p-frag-insertion constructs shown in Table 1. Extracts were treated with Endo H as indicated, proteins were separated by SDS/PAGE and transferred to PDVF membranes, and Vcx1p constructs were visualized using an anti-V5 antibody.

Figure 4  Complementation assay of the Factor Xa insertion constructs

The K667 strain was transformed with each Factor Xa insertion construct described in Table 1. Cells were grown to saturation, diluted to a $D_{600}$ of 0.5, and serial dilutions (1:5) were plated on solid agar containing minimal medium and the indicated carbon source and calcium chloride concentrations.

second smaller band probably represents a degradation product of Vcx1p that includes the C-terminal epitope. Our analysis focuses on the protease susceptibility of the full-length species. Importantly, Factor Xa does not cleave the V5-tagged Vcx1p that lacks a Factor Xa insertion sequence (Figure 5A). For most constructs, the cleaved C-terminal fragment is large enough to also appear on the blot. However, for L10x and Ctx, the small cleaved V5 epitope-containing fragment runs off the gel. For both L10x and Ctx, we verified that all three samples contained similar levels of protein by probing analogous blots with a second polyclonal antibody generated against the Vcx1p N-terminus (Figure 5B).

Proteolysis experiments, especially those on intact membranes, are sensitive to precise experimental conditions of enzyme and substrate concentration and incubation time. Four constructs, Ntx, L6x, L8x and L9x, gave particularly straightforward results under our test conditions (Figure 5C). L6x and L8x underwent essentially complete cleavage by Factor Xa in the absence of NP-40, while L9x was not susceptible to cleavage until after...
Membrane topology of Vcx1p

Figure 5  Factor Xa insertion site mapping of Vcx1p topology

Right-side out membrane vesicles were made from cells expressing the Vcx1p constructs and subjected to Factor Xa incubation. As indicated, samples were either treated with Factor Xa protease (fXa), with both protease and the detergent NP-40, or incubated in a mock reaction to serve as a control. Proteins were separated by SDS/PAGE (Tris/16.5% tricine, unless indicated), transferred to PVDF membranes, and Vcx1p was visualized with the indicated antibody. (A) Samples from cells transformed with either pYES2/CT empty plasmid or the VCX1/pYES2/CT plasmid, in which wild type Vcx1p is tagged with a C-terminal V5 epitope. The PVDF membrane was probed with an anti-V5 antibody. (B) Membranes from cells expressing the Factor Xa insertion constructs L10 and Ct were treated with Factor Xa as indicated and, after immunoblotting, protein was visualized with a polyclonal anti-Vcx1p antibody directed against the N-terminus. (C) Samples from cells expressing each of the Factor Xa insertion constructs described in Table 1 were treated with Factor Xa as indicated, immunoblotted and probed with the anti-V5 antibody. (D) Nt samples shown in (C) were re-examined using a Tris/12% glycine SDS/acrylamide protein gel to achieve greater separation.

The addition of detergent. Nt also undergoes cleavage in the absence of detergent; the gel shift elicited by proteolysis is small but reproducible. This change in electrophoretic mobility is more evident when the mutants are analysed under different electrophoresis conditions (Figure 5D). These results suggest a cytosolic localization for Ntx, L6x and L8x, and are consistent with the luminal location for L9x previously indicated by the glycosylation studies.

Although many of the remaining constructs provided data, the results were more ambiguous than those above for Ntx, L6x, L8x and L9x. Because we did not work to optimize the conditions for each loop position, we are reluctant to draw firm
conclusions from them and include the results here only for completeness. One set of constructs (L1x, L9x and L10x) shows incomplete cleavage even in the presence of NP-40, as though the Factor Xa site is shielded from proteolysis by surrounding protein. The remaining constructs (L2x, L3x, L4x, L7x and Ctx) show complete cleavage in the presence of NP-40, but only partial cleavage in its absence. This is unlikely to result simply from leaky vesicles; L9x was protected from cleavage until detergent addition, arguing for the integrity of the vesicle preparation. Instead, the incomplete cleavage probably reflects a limited susceptibility to protease, either as a result of shielding imposed by the phospholipid bilayer or of conformational changes during the transport cycle. Of these latter mutants, a substantial fraction of L2x and L4x constructs was cleaved in the absence of NP-40; this is consistent with L2 and L4 being located in the cytoplasm, with slightly reduced enzyme accessibility by comparison with those at L6 and L8. In addition, L7x displays almost complete protection of Factor Xa proteolysis, again consistent with the luminal location indicated by glycosylation studies.

DISCUSSION

We analysed the membrane topology of Vcx1p using several distinct biochemical approaches. Each of the methods required engineering a set of constructs containing novel ‘tags’ into the protein.

Our initial studies used the DTR approach. Since its development, DTR mapping has proven useful with a variety of yeast membrane proteins [11,15,17,29]. The DTR approach benefits from the relative ease with which constructs are made using homologous recombination. Our localization of the luminal loops on the basis of glycosylation of the Suc2p DTR studies could in theory be complemented by the identification of cytoplasmic loops by testing for complementation of the his+ phenotype. However, the DTR approach has several distinct disadvantages. First, protein folding, and thus membrane topology, could easily be affected by the insertion itself, which at 126 kDa is quite large by comparison with Vcx1p. Secondly, the use of these constructs assumes that the truncation does not significantly alter the topology or folding of the protein, since the truncated protein lacks any folding information provided by the sequences C-terminal to the DTR insertion point. Folding effects probably have the least impact on fusions made near the C-terminus, where relatively little Vcx1p is removed in the final fusion protein. Ultimately the acid test for a correctly-folded transporter lies in confirming its functional properties but, since even the most distal DTR insertions failed to complement the Ca2+ sensitivity of the K667 strains, it is impossible to gauge with certainty the extent of the structural impact of the DTR fusion and accompanying C-terminal truncation. Consequently we focused on independent tests of Vcx1p topology using ‘sandwich’ biochemical approaches that might have a smaller overall impact on Vcx1p folding, using the DTR results as a foundation.

Similar to the DTR approach, ‘sandwich’ topology scanning methods, in which a tag is introduced in-frame within the coding sequence for a gene, also rely on the protein folding correctly in the presence of the tag. Many of the Vcx1p loops are predicted to be quite small (in the range of only 3–5 amino acids, see Table 1), and the two tags we used were by comparison relatively large (14 residues for the Factor Xa site, 52 for the Suc2p-frag insertion). Strikingly, of the ten loops at which we attempted insertions, we obtained functional constructs for all but three (L4, L5 and L10). The relative ease with which Vcx1p accommodated the insertions at the remaining seven positions implies substantial structural tolerance at those sites, consistent with their original designation as loop regions. The preservation of activity in the sandwich constructs strongly suggests that these mutant proteins have a molecular architecture similar to the wild-type and are suitable for topological analysis.

The two tags provided complementary topological data: the presence of glycosylation indicated a luminal orientation, while Factor Xa cleavage in the absence of detergent indicated a cytoplasmic location. Recent membrane protein crystal structures show numerous unexpected non-canonical membrane segments that do not completely span the bilayer, such as a traditional TM helix, bending within the membrane, or even forming re-entrant loops. The emerging high resolution structures highlight the importance of relying on positive results when evaluating topology data; i.e. although the observation of glycosylation is a strong indicator that a segment is located in the lumen, the failure to observe glycosylation does not indicate a cytosolic location, since it could also result from a vacuolar location from which the glycosylation machinery is physically occluded, or a location embedded within the membrane in a non-canonical hairpin.

Together, glycosylation and Factor Xa place a broad set of constraints on Vcx1p topology that are shown in the topological model in Figure 6. First, the Factor Xa susceptibility indicates that the N-terminus is cytoplasmic, while glycosylation results (both in the Suc2p-frag insertion as well as the DTR experiments) point to a luminal position for the C-terminus. These results require that Vcx1p has an odd number of TM domains, and rules out the model generated by the TopPred II prediction algorithm.

The results further confirm that the L1, L3, L6 L7, L8 and L9 insertions are located in loops, and provide positive evidence for their locations. On the basis of the presence of glycosylation in the S2 insertion constructs, L1, L3, L7 and L9 are all located in the vacuolar lumen. Since the N-terminus is cytoplasmic, a luminal orientation of L1 thus requires the presence of an initial TM within the first 60 residues, consistent with all of the computer predictions with the exception of TopPred II. Both L6 and L8 displayed complete cleavage in response to Factor Xa, as expected for a cytoplasmic location. The longest loop in Vcx1p, L6, is located at the junction of the two tandem internal repeats of the TM regions. L3 and L8 are analogous to the L3 and L8 of OsCAX1a that are proposed to form re-entrant loops on the lumenal and
cytoplasmic surfaces respectively [9]. The cytoplasmic position of L8 in Vcx1p is consistent with that observed in OsCAX1a, while our glycosylation mapping of L3 provides the first direct evidence of a lumenal location for that loop.

We note with particular interest the disparities between the results obtained using DTR and Suc2p-frag sandwich approaches (Table 2). Both approaches used glycosylation mapping, with precisely the same glycosylation sites in the Suc2p protein segments. Yet although the results are identical for many sites, particularly those located in the C-terminal half of Vcx1p, they conflict directly for the disposition of L2, L3 and L4. Why the discrepancy? The simplest explanation is that the C-terminal region of Vcx1p influences the fold of the N-terminal segment. Perhaps in the absence of the C-terminal region, the DTR constructs lack the full complement of structural constraints, and although the N-terminus still folds (as evidenced by the alternating pattern of accessibility for L2–L4), it is simply not properly inserted. In our hands, the results obtained with the less perturbing series, the Suc2p-frag insertion constructs, were consistent with those obtained from the Factor Xa insertion experiments. Regardless, the finding that proper folding depends on the presence of the C-terminal region urges caution in the application and interpretation of the DTR approach.

Finally, in some members of the CAX family, a nine residue stretch known as the ‘calcium domain’ is critical to calcium transport [30]. Located near the N-terminus, the precise effect of this segment remains unknown as it contains no acidic residues likely to interact directly with Ca$^{2+}$, and it is hypothesized to affect Ca$^{2+}$ accessibility to the conduction pathway through an indirect mechanism. At the molecular level, the calcium domain segment shows little conservation among different Ca$^{2+}$ mechanisms. At the molecular level, the calcium domain segment strongly suggests that the calcium domain is not critical to Ca$^{2+}$ phenotypic complementation from both L1g and L1x, constructs where single point mutations within the calcium domain can affect the first luminal loop L1. In contrast to the CAX family members, the equivalent segment in Vcx1p lies between residues 52 and 60, which our results position within the class of proteins. The equivalent segment in Vcx1p influences the fold of the N-terminal segment. Perhaps in the absence of the C-terminal region, the DTR constructs lack the full complement of structural constraints, and although the N-terminus still folds (as evidenced by the alternating pattern of accessibility for L2–L4), it is simply not properly inserted. In our hands, the results obtained with the less perturbing series, the Suc2p-frag insertion constructs, were consistent with those obtained from the Factor Xa insertion experiments. Regardless, the finding that proper folding depends on the presence of the C-terminal region urges caution in the application and interpretation of the DTR approach.

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SUPPLEMENTARY ONLINE DATA

Topology mapping of the vacuolar Vcx1p Ca²⁺/H⁺ exchanger from Saccharomyces cerevisiae

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PRIMER SEQUENCES FOR THE DUAL TOPOLOGY REPORTER TRUNCATIONS of Vcx1p

Below is a list of the primer sequences used to generate the different C-terminal Vcx1p truncation mutants.

Forward primer used to generate all the constructs

The stretch of the primer that is homologous to the promoter region upstream of OST4 in the pJK90 plasmid is capitalized. The remainder of the sequence is homologous to first 20 nucleotides of VCX1.

5′-GTTTGTTACGCATGCAAGCTTGA TATCGA Aatggatgca-actaccccaact-3′

Truncation primers (reverse orientation)

The stretch of the primer that is complementary to the top strand of pJK90 is capitalized. The remainder of the sequence is complementary to the top strand end of the area whose topology is being evaluated.

L1 5′-AGATGGTCTAGAGGTGTAACCACTTGAGTTgaagtgtcgcc-caaatgcaacc-3′

L2 5′-AGATGGTCTAGAGGTGTAACCACTTGAGTTctttagegca-acttcgg-3′

L3 5′-AGATGGTCTAGAGGTGTAACCACTTGAGTTctgcaacatc-tgacttgacc-3′

L4 5′-AGATGGTCTAGAGGTGTAACCACTTGAGTTgttgaatgtct-gttggactc-3′

L5 5′-AGATGGTCTAGAGGTGTAACCACTTGAGTTgatgaagtg-gtttcctgcc-3′

L6 5′-AGATGGTCTAGAGGTGTAACCACTTGAGTTcaaagagtga-tggtgattctgg-3′

L7 5′-AGATGGTCTAGAGGTGTAACCACTTGAGTTgttgctatcgt-acccag-3′

L8 5′-AGATGGTCTAGAGGTGTAACCACTTGAGTTttatctccatg-gccaccaag-3′

L9 5′-AGATGGTCTAGAGGTGTAACCACTTGAGTTcatggaactc-gatcactccag-3′

L10 5′-AGATGGTCTAGAGGTGTAACCACTTGAGTTctcaccatc-gaattaagtaatgg-3′

CA 5′-AGATGGTCTAGAGGTGTAACCACTTGAGTTgcaggttctt-ttctggcttg-3′

CB 5′-AGATGGTCTAGAGGTGTAACCACTTGAGTTacacttatcc-aatagagttcaag-3′

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