Identification of Interdependent Signals Required for Anterograde Traffic of the ATP-binding Cassette Transporter Protein Yor1p*

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The plasma membrane ATP-binding cassette (ABC) transporter Yor1p mediates oligomycin resistance in Saccharomyces cerevisiae. Its protein sequence places it in the multidrug resistance protein/cystic fibrosis transmembrane conductance regulator subfamily of ABC transporters. A key regulatory step in the biogenesis of this family of ABC transporter proteins is at the level of transport from the endoplasmic reticulum (ER) on through the secretory pathway. To explore the protein sequence requirements for Yor1p to move from the ER to its site of function at the plasma membrane, a series of truncation and alanine replacement mutations were constructed in Yor1p. This analysis detected two sequence motifs similar to the DXE element that has recently been found in other proteins that exit the ER. Loss of the N-terminal DXE element eliminated function of the protein, whereas loss of the C-terminal element only slightly reduced function of the resulting mutant Yor1p. Strikingly, although both of the single mutant proteins were stable, production of the double mutant caused dramatic destabilization of Yor1p. These data suggest that this large polytopic membrane protein requires multiple signals for normal forward trafficking, and elimination of this information may cause the mutant protein to be transferred to a degradative fate.

ATP-binding cassette (ABC)1 transporters proteins mediate energy-dependent transport of substrates across cell membranes (1). The cytoplasmic nucleotide-binding domain (NBD) is the defining feature of these proteins, with most ABC transporters containing two NBDs and two multispanning transmembrane domains (TMDs). ABC transporters have been classified into subfamilies based upon sequence similarity and spacing of NBD1 motifs (2, 3). One of the largest known subfamilies in humans contains the multidrug resistance proteins (MRPs), often overexpressed in tumors (4), and the cystic fibrosis transmembrane conductance regulator (CFTR) that, when mutated, results in cystic fibrosis (5, 6). Most members of this subfamily have a primary structure of two direct repeats of a unit composed of a TMD followed by an NBD.

Whereas MRP/CFTR ABC transporters show strong similarity in their NBDs, they have significant diversity at their N and C termini. MRP1 contains an additional TMD at its N terminus (TMD0) connected to the rest of the protein via a cytoplasmic linker region (L0). Deletion of TMD0 and L0 from MRP1 results in mislocalization of the protein and loss of substrate transport (7, 8). The CFTR N terminus interacts with its own regulatory domain for proper channel function (9), and both termini interact with components of the vesicular trafficking machinery (10–12). Mutation of the CFTR C terminus leads to instability of the protein and defects in its maturation (13, 14), and the extreme region of the CFTR C terminus interacts with PDZ domain proteins to regulate its activity and localization (15–17). C-terminal mutations in another member of this family, SUR1, result in hyperinsulinemic hypoglycemia (18, 19).

Saccharomyces cerevisiae expresses a variety of MRP/CFTR family members (20–22). The two first described yeast MRP/CFTR family members were Ycf1p and Yor1p. Ycf1p is a vacuolar glutathione conjugate transporter that sharesstrong sequence similarity with MRP1 (23–25), and Yor1p is a plasma membrane protein required for normal tolerance to oligomycin (26, 27). Intriguingly, both Ycf1p and Yor1p share a phenylalanine residue that is conserved with CFTR in the NBD1 region of these three proteins (27). Deletion of this phenylalanine residue from CFTR (Δ508) is the major disease-causing allele found at this locus (6) leading to ER retention and increased ER-associated degradation of the protein (28, 29). Analogous cell biological defects are seen when the corresponding phenylalanine residues are deleted from either Ycf1p (25) or Yor1p (30). Although these observations illustrate the similarities of NBD1 structure and function, the variability of N and C termini suggests these regions are important for functional specificity of ABC transporters. The structure of Yor1p, like that of CFTR (1), is predicted to have both termini cytoplasmically located (27).

We have found that N- and C-terminal truncations of Yor1p result in ER localization and loss of oligomycin tolerance but generally no change in Yor1p stability. Alanine substitution mutagenesis identified residues important for normal expression and function of the protein, namely the ER localization defect was specifically observed for mutations in a diacidic DXE sequence (X is any amino acid) in the N terminus of Yor1p. DXE sequences have been shown to be required for efficient export of several proteins from the ER (31, 32), and this N-terminal DXE in Yor1p is one sequence that is required for its forward trafficking. Interestingly, this N-terminal DXE element is not sufficient for forward traffic of Yor1p because truncation mutations in the C terminus also fail to exit the ER.

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‡The abbreviations used are: ABC, ATP-binding cassette; CFTR, cystic fibrosis transmembrane conductance regulator; DAPI, 4′,6-diamidino-2-phenylindole; DIC, differential interference contrast, DXE, aspartate-any amino acid-glutamate ER export signal; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GFP, green fluorescent protein; MRP, multidrug resistance protein; NBD, nucleotide binding domain; TMD, transmembrane domain; YOR1, yeast oligomycin resistance; UPR, unfolded protein response.

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Mutation of a DXE sequence at the C terminus of Yor1p resulted in only a modest alteration in localization and oligomycin tolerance, whereas elimination of both DXE elements produced a form of Yor1p that failed to leave the ER and was strongly destabilized. These data indicate that multiple signals are required for forward traffic of Yor1p and suggest that elimination of the ability to exit the ER causes the mutant Yor1p to become a substrate for a degradative pathway.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Media**—The yeast strains used in this study were DK7 (ura3-52 leu2-3, -112 his3–Δ200 trp1–3901 lys2–801 suc2–Δ9 Mel 576–1:his5) and EY40 (ura3-52, leu2-3, -112 his3–Δ200 trp1–3901 lys2–801 suc2–Δ9 Mel–). EY40 replaces the wild-type PDR1 gene with the gain-of-function PDR1-3 allele. It was constructed by transforming DK7 with a SacI–Apal PDR1-3 fragment isolated from pRS315-PDR1 (33). Transformants were grown in YPD at 30 °C with shaking for 2 h prior to selection on YPD plates containing 0.4 μg/ml cycloheximide. Candidates containing the PDR1-3 allele were tested for increased cycloheximide and oligomycin resistance and for increased Yor1p expression by Western blotting of whole cell extracts after introduction of a low copy number plasmid carrying wild-type YOR1. DK7 was used for all experiments except fluorescence microscopy, where EY40 was used to enhance the GFP signal. All yeast transformations were carried out using the lithium acetate procedure (34). Cells were grown in cultures containing YPD (2% yeast extract, 1% peptone, 2% glucose) under nonselective conditions or appropriate SC media under selective conditions (35). Drug resistance was measured by the spot test assay on plates with either single concentrations of drug or gradient plates (27, 30). Oligomycin plates contained YPG media (2% yeast extract, 1% peptone, 3% glycerol, 3% ethanol).

**Plasmids**—Single-copy Yor1p-GFP-expressing plasmids were constructed using the method described by Longtine et al. (36). The forward primers contained 50 nucleotides of C-terminal YOR1 sequence followed by the F2 sequence described by Longtine et al. (36); the reverse primer contained 50 nucleotides of pRS316 sequence followed by the R1 primer sequence. With these primers the GFP(S65T)-TRP1 sequence was amplified from the corresponding pPA6a plasmid and co-transformed into DK7 with either the wild-type YOR1 plasmid pDK59 or the ΔPDR705 YOR1 plasmid pDK61 (30) linearized at the 3′ end of the YOR1 gene with Apal. Transformants were selected on Ura– media, then tested for TRP1 prototrophy, expression of Yor1p-GFP by Western blotting, plasma membrane localization, and complementation of yor1Δ oligomycin resistance. Plasmids were recovered using the method of Robzyk and Kassir (37) and sequenced to confirm the presence of the GFP fusion. The fusion to wild-type YOR1 was designated the ΔPDR705 fusion, and the ΔPDR705 fusion was named pEAE66.

A SacI site was engineered at YOR1 codons 58 and 59 in pEAE64 using a two-step PCR process (38) to generate plasmid pEAE93. In the first PCR step, two products were generated from pEAE64 using the oligonucleotides CGC TAT AGC CGT CAG TGG and GTG TTG GCG ATT ATT TCT GTG GCC AAC ATG ATA ATC ACC TGT AGC GAG and GCC GAT AAC TAT ACA GGA GCC for the first product, and GTG TTG GCG ATT ATT TCT GTG GCC AAC ATG ATA ATC ACC TGT AGC GAG and GCC GAT AAC TAT ACA GGA GCC for the second product. The underlined sequences are the SacI site, the c/g in lowercase is the base changed from G/C in wild-type YOR1 and various forward primers starting at the SacI site because of replacement of the triple alanine mutant sequence with the single or double mutant sequences and verified by sequencing.

The C-terminal deletions were constructed by the same method used to generate pEAE64 with the same reverse primer and different forward primers containing 50 nucleotides of YOR1 coding sequence up the end of each truncation followed by the F2 sequence for amplification of the GFP(S65T)-TRP1 template. The C-terminal alanine substitution mutant was constructed by the two-step PCR process described above using the same 5′ primer used to construct the GFP fusions, a 5′ primer starting at nucleotide 4117 in the YOR1 coding sequence, and complementary mutant primers containing the appropriate substitutions. The final products were cloned into pCR2.1 TOPO and then cut with SacI and Spal and cloned into the same sites of pEAE93.

**Plasmid Construction**—To generate the final plasmid, all truncations except the one from residues 2–57 were generated using the two-step PCR method described above with the same outside primers at –705 and 1122 for the final products, but with appropriate complementary mutagenic primers containing 20 nucleotides of 5′ YOR1 sequence up to and including the ATG followed by 20 nucleotides of coding sequence from truncations starting at amino acid residues 39, 78, 97, 114, and 201. The PCR products generated were cloned into pEAE93 as EcoRI/HinII/SphI fragments to replace the wild-

**Yor1p Traffic Signals**

34681
Yor1p Forward Traffic Signals

RESULTS

Localization and Expression of Yor1p-GFP—In previous work, the localization of Yor1p in intact cells was assessed by indirect immunofluorescence (27). Although this method localized the wild-type protein to the plasma membrane, it was unable to detect ER-retained Yor1p mutants. A fusion of the GFP to the C terminus of wild-type and ER-retained ΔF670 Yor1p was constructed to facilitate analysis of Yor1p mutants (Fig. 1A). The wild-type Yor1p-GFP fusion protein localized to the plasma membrane, consistent with our earlier experiments (30). Intracellular fluorescence was found to be within the vacuole lumen, as shown by co-localization with the vacuolar marker FM4-64. Because the wild-type protein is degraded by the vacuole, it is likely this signal is due to fluorescence of Yor1p-GFP molecules undergoing degradation. Fluorescence of the ΔF670 Yor1p was found only in perinuclear and subsurface compartments. This is consistent with endoplasmic reticulum localization, consistent with our previous findings (27). The GFP-tagged constructs were tested for their ability to confer oligomycin resistance and compared with the corresponding untagged versions (Fig. 1B). The addition of the GFP tag did not change the oligomycin resistance profile of either form of Yor1p. Expression levels of the GFP fusions were also measured (Fig. 1C). The ΔF670 Yor1p-GFP mutant was less abundant than the wild-type form, similar to results previously shown for the untagged version. These results show that fusion of a wild-type or mutant form of Yor1p to GFP produces a chimera that faithfully reproduces the behavior of the untagged protein.

Yor1p N-terminal Deletion Mutagenesis—Although Ycf1p and Yor1p are MRP/CFTR homologues in S. cerevisiae, these two yeast proteins differ dramatically at their N termini. The vacuolar Ycf1p, like MRP1, is predicted to contain five additional membrane-spanning segments that comprise TMD9, whereas Yor1p, like CFTR, does not appear to have this additional TMD region (27, 40). A hydropathy plot for the first 200 residues of Yor1p was determined, and the region was predicted to be primarily hydrophilic and intracellular (Fig. 2A) (41). To explore the possibility that the remarkably different N terminus in Yor1p might explain its differential trafficking in the cell, we constructed a series of N-terminal mutations for expression from the YOR1 promoter on low copy number plasmids (Fig. 2A). Each mutant is named by the final residue deleted in a given mutant. For example, Δ38 has residues 2–38 deleted, and the original residue 39 in Yor1p is now residue 2 in this mutant protein. The mutants were transformed into the yor1 strain DKY7 and tested for their ability to complement the oligomycin hypersensitivity of this strain. Deletion of residues 2–38 (Δ38) actually increased Yor1p-mediated resistance above wild-type levels. The Δ57 mutant provided some oligomycin resistance, although it was slightly less than the wild-type protein. Deletions beyond residue 57 resulted in the complete loss of oligomycin tolerance. Internal deletions starting from residue 60 and extending to residues 77, 96, or 113 were also unable to provide oligomycin resistance.

The expression levels of the mutant proteins were analyzed by Western blotting using antibodies against the C terminus of Yor1p (Fig. 2B). All mutants but the Δ77 deletion were expressed at least the same as wild-type Yor1p, indicating the loss of oligomycin resistance in the others was not due to reduced expression. Expression of the Δ38 mutant was slightly elevated above wild-type levels, consistent with its increased oligomycin tolerance. The Δ77 mutant most likely has a defect at the level of mRNA or protein synthesis as we were unable to detect any mRNA or protein for Δ77, shown previously (27) to increase transcription of YOFR1 on low copy number plasmids and transformed into the yor1-1 hisG PDR1-3 strain EEY40. Cells were labeled with FM4-64 and DAPI and visualized for GFP, FM4-64, DAPI, and DIC. B, equal numbers of cells from DKY7 (yor1-1 hisG) strains expressing no Yor1p (Δyor1) or wild-type or ΔF670 versions of Yor1p with or without a GFP tag were spotted in a row onto YPGE plates containing a gradient of oligomycin, increasing from left to right. C, whole cell extracts in the strain DKV7 expressing either no Yor1p (Δyor1), untagged Yor1p, wild-type Yor1p-GFP, and ΔF670 Yor1p-GFP were prepared. Equal amounts of protein (100 μg) were electrophoresed by SDS-PAGE, transferred to nitrocellulose, and Western-blotted using antibodies against the C terminus of Yor1p (top) and the vacuolar membrane protein Vph1p (bottom).

Fig. 1. Localization and expression of wild-type and ΔF Yor1p-GFP. A, the GFP sequence was fused to the C terminus of the YOR1-coding sequence for wild-type and ΔF670 YOR1 on low copy number plasmids and transformed into the yor1-1 hisG PDR1-3 strain EEY40.

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olar fluorescence was markedly reduced in the Δ38 mutant, suggesting that the increase in the steady-state level of this mutant protein might be explained by a reduction in the level of vacuolar degradation of the Δ38 mutant. Even though the Δ77 mutant was reduced in expression, a few cells were found with an endoplasmic reticulum fluorescence pattern, suggesting that this mutant was retained in this organelle. Similarly, other truncation mutants that failed to confer oligomycin tolerance were ER-localized as indicated by perinuclear fluorescence.

To verify the fluorescence microscopy data, some of the mutants were also localized by Western blotting of sucrose density gradient fractions. Mutant derivatives of Yor1p that were predicted to be localized to the plasma membrane by fluorescence microscopy displayed a fractionation profile similar to that seen for wild-type Yor1p and the plasma membrane proton ATPase Pma1p (Fig. 4). Like wild-type Yor1p, the Δ57 mutant sedimented to the most dense sucrose fractions. This was also found for the Δ38 mutant (data not shown). ER-localized mutants, such as Yor1p lacking amino acids 60–77, co-fractionated with the ER resident protein Sec61p and not with the plasma membrane marker. These data confirm that N-terminal truncations beyond residue 57 and internal deletions from residue 60 are mislocalized to the ER.

Alanine Scanning Mutagenesis of Yor1p Residues 60–77—The smallest Yor1p deletion mutant that showed ER localization and loss of function corresponded to deletion of amino acids 60–77. These results suggest the possibility that the amino acid 60–77 region contains information important for proper trafficking of Yor1p. To analyze this segment further, a series of triple alanine scanning mutants were constructed beginning with residue 60 (Fig. 5A). Each mutant protein was then analyzed for its functional activity, steady-state expression, and subcellular localization. The RNK60AAA Yor1p mutant complemented the oligomycin tolerance of the yor1 null mutant nearly as well as wild-type Yor1p (Fig. 5B). The remainder of the triple alanine replacement mutant proteins conferred oligomycin tolerance to levels intermediate between the yor1 null mutant and YOR1 wild-type cells. These triple alanine substitution mutants were examined for steady-state expression by Western blotting (Fig. 5C). Strikingly, the RNK60AAA, PQT63AAA, and YLN66AAA mutants were expressed at lower levels relative to the wild-type protein. Expression of the other mutant proteins was similar to wild-type.

The localization of the triple alanine mutants showed a different pattern of defects than that found for drug resistance or expression. By fluorescence microscopy, the SDD69AAA and IEK72AAA were found primarily in the ER with no vacuolar fluorescence, whereas the rest appeared localized to the plasma membrane and displayed strong vacuolar fluorescence (Fig. 6). Sucrose gradient fractionation confirmed that the IEK72AAA mutant displayed the most severe mislocalization defect (Fig. 7). Very small amounts of the protein were found in the most dense fractions, consistent with its ability to confer a low level of oligomycin resistance. The similarity in resistance conferred upon expression of the SDD69AAA, IEK72AAA, and VYE75AAA proteins despite their different localization may
indicate that the VYE75AAA mutant has a greater functional defect than the ER-localized mutants. The other mutants analyzed by this method all showed significant amounts of protein in the most dense fractions. Even though the RNK60AAA and YLN66AAA mutants had slightly increased levels of Yor1p in the middle fractions, they are capable of reaching the vacuole at an amount significant enough to be visualized, unlike the SDD69AAA and IEK72AAA mutants, single D71A, E73A, and double D71A/E73A substitutions were constructed (Fig. 8B). These mutant proteins were expressed and analyzed as described for the triple alanine scanning mutants in Yor1p that were primarily ER-localized within the sequence SDDIEK. Spanning these two mutants is a di-acidic sequence similar to an element shown previously to be necessary for efficient export of other proteins from the ER (31, 32). This export signal was designated DXE for aspartate-any amino acid-glutamate. To determine whether mutation of the DIE sequence in this segment of Yor1p was responsible for the mislocalization found in the SDD69AAA and IEK72AAA mutants, single D71A, E73A, and double D71A/E73A substitutions were constructed (Fig. 8A). These mutant proteins were expressed and analyzed as described for the triple alanine scanning mutants above. Both the single and double alanine replacement alleles were defective in their ability to complement the oligomycin hypersensitivity of the yor1 strain (Fig. 8B). None of the alanine substitution mutants exhibited any expression defect (Fig. 8C) but each was now localized to the ER (Fig. 8D), indicating a defect in normal Yor1p subcellular trafficking. These data confirm the necessity of an intact DIE element for anterograde transport of Yor1p. Turnover of Yor1p Mutants—Mutant proteins retained in the ER are often mislocalized due to misfolding and are rapidly turned over by ERAD (42). A mechanism for turnover of mutants that reach the plasma membrane has also been identified (43). To assess the stability of the N-terminal mutants constructed, pulse-chase immunoprecipitations of [35S]methionine-labeled cells were performed using anti-C-terminal Yor1p antibodies. The turnover rates of the ER-localized deletion mutants were not different from that of wild-type Yor1p.
(Fig. 9, A and B). The ΔS8 mutant, however, was found to be more stable, consistent with its increased steady-state level. This could also explain the decrease in vacuolar fluorescence in the mutant if it is defective in normal turnover at the vacuole.

The turnover rates of several alanine substitution mutants were also analyzed (Fig. 9, A and C). Only the RNK60AAA mutant was degraded at a significantly different rate than wild-type Yor1p. The increased turnover of this mutant was consistent with its decreased steady-state expression, although it is not known if this increased degradation occurs at the ER or vacuole. Because the protein reaches the vacuole, it is likely that the vacuole contributes to its degradation, but the finding of increased RNK60AAA Yor1p in the less dense fractions indicates the protein may accumulate in the ER with subsequent turnover by ERAD. The ER-retained alanine substitution mutants within the DIE sequence were not turned over more rapidly than the wild-type protein, likely indicating they are not substrates for ERAD.

Azido-ATP Labeling of Yor1p Mutants—To assess more directly the folding of the N-terminal mutants, we measured their ability to bind 8-azido-ATP. Although ATP binding occurs at the NBDs downstream of the N-terminal mutants, measurement of this binding can serve as an indication of the protein to achieve a properly folded confirmation. Membrane-enriched protein extracts were incubated with α-32P-labeled 8-azido-ATP and photo-cross-linked, followed by immunoprecipitation with anti-C-terminal Yor1p antibodies and analysis by SDS-PAGE and fluoroanalysis. A polypeptide at the predicted molecular weight of Yor1p was immunoprecipitated only in cells expressing Yor1p and a smaller nonspecific band was also found in all cells independent of Yor1p (Fig. 10A). Both a plasma membrane-localized mutant (ΔS8) and an ER-localized mutant (ΔS6) were able to bind ATP. Appearance of both the specific Yor1p band and nonspecific band was dependent on the Yor1p antibody, and labeling of both was reduced when cold ATP was added to compete with the labeled 8-azido-ATP (Fig. 10B). Analysis of the alanine substitution mutants showed that all are capable of binding ATP (Fig. 10C), consistent with the idea that none of these lesions caused an extensive unfolding of Yor1p.

C-terminal Deletion Mutants—By having provided evidence that the N terminus of Yor1p is important in specifying plasma membrane localization of this protein, we constructed a set of C-terminal truncations to determine whether this segment of the 1477-residue Yor1p contributed targeting information. Each C-terminal truncation mutant was named based on the position of the last remaining Yor1p residue. These mutant proteins were expressed as GFP fusions and were analyzed for their ability to complement the yor1 mutant strain, steady-state expression level, and localization by fluorescence microscopy (Fig. 11).

The Δ1453 mutant protein was able to confer significant oligomycin tolerance compared with wild-type Yor1p. Two more extensive truncations (Δ1427 and Δ1402) were both
highly defective in oligomycin tolerance. All of these mutants were expressed at normal levels, but clear differences were found in their subcellular location. H9004Yor1p was partially associated with the ER but also produced some plasma membrane fluorescence. Consistent with the defect in Yor1p forward trafficking in the H9004Yor1p mutant, no vacuolar fluorescence was observed. Both the H9004Yor1p and H9004Yor1p mutants appeared to localize primarily to the ER. These data indicate that the presence of the DIE motif in the Yor1p N terminus is not sufficient to allow this protein to traffic normally and suggest that informational contributions from both termini are required to deliver properly this protein to its normal subcellular residence.

Analysis of a C-terminal DXE Sequence—Within the interval of Yor1p deleted in the H9004Yor1p mutant, sequence inspection showed the presence of a second diacidic sequence, 1472DFE. This sequence is located three residues before the end of Yor1p-coding sequence. To test the possible role of this DXE motif in Yor1p forward trafficking in the H9004Yor1p mutant, no vacuolar fluorescence was observed. Both the H1402 and H1427 mutants appeared to localize primarily to the ER. These data indicate that the presence of the DIE motif in the Yor1p N terminus is not sufficient to allow this protein to traffic normally and suggest that informational contributions from both termini are required to deliver properly this protein to its normal subcellular residence.

DISCUSSION

In contrast to the apparent dispensability of TMD0 of MRP1 (7), most of the Yor1p N terminus is required for proper function and localization. The functional results resemble those found for the MRP-like ABC transporter Ycf1p, the yeast cadmium resistance factor, in which drug resistance is lost when the N-terminal TMD0 is deleted (25). However, the rationale explaining loss of function in the case of N-terminally truncated Ycf1p or Yor1p is different. Removal of TMD0 from Ycf1p still allows this protein to leave the ER, although function is lost. Loss of the N-terminal domain of Yor1p greatly increases the level of protein found associated with the ER, which is likely to explain the absence of Yor1p function. Along with their dramatically different topologies, the N-terminal domains of Yor1p and Ycf1p have different roles in regulating the anterograde movement of these membrane proteins.

The H9004Yor1p N-terminal truncation mutant had an unex-
labeled and either competed with 1 m M cold ATP or immunoprecipitated from DKY7 (yor1-1) cells expressing the forms of Yor1p indicated were photolabeled with [α-32P]8-azido-ATP, immunoprecipitated with anti-C-terminal Yor1p serum, and analyzed by SDS-PAGE and fluorography. A, strains expressing no Yor1p (Δyor1), wild-type (wt) Yor1p, and the Δ38 or Δ96 deletions were labeled and immunoprecipitated. A band corresponding to the molecular weight of Yor1p was present only in the Yor1p-expressing strain but not in the Δyor1 strain. A nonspecific band (NS) was recovered in all strains. B, the wild-type Yor1p strain was labeled and either competed with 1 m M cold ATP or immunoprecipitated without Yor1p antibody. C, labeling of alanine substitution mutants. The starting residue of each triple alanine substitution mutant is indicated in each lane. Expected effect. The plasma membrane-localized Δ38 Yor1p is hyper-resistant to oligomycin and does not appear to accumulate in the vacuole. An explanation consistent with these data is that Δ38 Yor1p can reach the plasma membrane in a similar fashion to the wild-type protein but fails to be normally endocytosed and delivered to the vacuole for degradation. This increased residence on the plasma membrane could allow Δ38 Yor1p to confer greater oligomycin resistance. Further experiments are underway to test this idea.

The multiple defects associated with different mutants in the N-terminal region from residues 60 to 79 demonstrate that this domain has influence on the functional status of Yor1p. Removal of this segment eliminated detectable function of the resulting mutant Yor1p, whereas triple alanine scanning mutagenesis identified subdomains critical for steady-state synthesis (Yor1p residues 60–68), anterograde traffic (69–74), and activity (63–79). We interpret our results to indicate that the N-terminal segment of Yor1p is multifunctional in nature and crucial for the biogenesis of this ABC transporter. Comparison of the behavior of the stable but ER-retained Δ60–77 Yor1p with the unstable but primarily plasma membrane localized triple alanine mutants (RNK60AAA, PQT63AAA, and YLN66AAA) suggests that blocking the forward traffic of the protein in the ER is epistatic to the stability defects seen in the three alanine scanning mutations. Perhaps these three alanine replacement forms of Yor1p have an accelerated rate of arrival in and degradation by the vacuole.

A diacidic sequence containing DXE has been shown to be necessary for normal ER export of several proteins, including the vesicular stomatitis virus glycoprotein, lysosomal acid phosphatase (31), and a yeast Golgi protein Sys1p (32). In the process of protein transport from the ER to the Golgi, proteins concentrate at specific ER export sites prior to concentration into COPII-coated vesicles (44). ER export signals containing acidic residues (including the sequences EXD or EXE) have also been found in the potassium channels Kir2.1 and -1.1 (45), and a diphenylalanine motif found in p24 proteins has also been shown to facilitate ER export (46). Our finding that mutation of the 71DIE sequence in the N terminus of Yor1p reduces the ability of the resulting mutant protein to exit the ER is consistent with the view that this represents an ER export signal for Yor1p. Surprisingly, mutation of the C-terminal 1472DFE sequence resulted in a much less severe localization defect than the 71DIE mutant, but loss of both sequences produced an unstable ER-retained mutant.

Mutant proteins are often retained in the ER because they are misfolded and are subsequently and rapidly turned over by ERAD (42, 47). This was found for the ΔF670 mutation in Yor1p that was degraded in a proteasome-dependent manner four times faster than the rate of wild-type Yor1p (30). The ER-retained mutants identified here do not appear to be rapidly degraded by ERAD, as their steady-state expression was not reduced compared with wild-type, and those analyzed by...
pulse-chase immunoprecipitation were degraded at a similar rate as the wild-type. To assess folding of the mutants, binding of Yor1p to 8-azido-ATP was measured. All the mutant proteins we analyzed, whether ER-retained or not, still possessed ATP-binding activity. Although this is an indirect method to assess folding, it does show that a function dependent on folding is preserved in the mutants. The unfolded protein response (UPR) is another method of ER quality control where accumulation of misfolded proteins in the ER up-regulates target genes to assist in folding and clearance of unfolded proteins (42, 48, 49). To assess activation of the UPR, we measured expression of one of its targets, Kar2p (BiP), in several Yor1p mutants and did not find increased Kar2p expression, even when the Yor1p mutants were overexpressed (data not shown). We believe the N terminus of Yor1p contains information required for ER export because of the following: 1) mutation of a diacidic sequence was found to block forward trafficking of Yor1p; 2) ER-retained mutants are not rapidly degraded by ERAD; 3) ER-retained mutants bind ATP and are not severely misfolded; and 4) ER-retained mutants do not activate the UPR.

The ER retention of short C-terminal truncations in the presence of a normal N terminus and the additive effect of mutating both N- and C-terminal DXEs implies that localization determinants in Yor1p may be interdependent. Furthermore, these findings suggest that exit of complex, polytopic membrane proteins like Yor1p might require more extensive sequence information than proteins with fewer transmembrane segments. Although it is formally possible that the C-terminal truncation mutants may be misfolded, the similarity of steady-state expression between the C-terminal mutants and wild-type Yor1p indicates they are likely not substrates for ERAD. The ability of the DFE1472AFA mutant to function at a level similar to wild-type also argues against misfolding as an explanation for its defect. The destabilization and complete loss of oligomycin tolerance with the double 71AIA, 1472AFA mutant is consistent with the idea that the loss of both forward trafficking signals completely abolishes forward trafficking of the protein leading to identification and degradation by ERAD mechanisms. However, it is possible that simultaneous removal of both DXE elements elicits misfolding, and further experiments are underway to distinguish between these two explanations. The variability of forward trafficking sequences previously identified and the variability in the contribution of the diacidic sequences in Yor1p indicate that more than primary sequence information may be required to constitute an ER export signal and, as noted previously (32), may contribute

**Fig. 12. Mutation of a C-terminal DXE in Yor1p.** A, the double DFE to AFA mutant was constructed in the C terminus of Yor1p on a low copy number plasmid. The period indicates the end of the Yor1p coding sequence. A double mutant of DlE71AIA, DFE1472AFA was also constructed. B, the N- and/or C-terminal DXE to AXA substitutions were transformed into DKY7 and grown on YPGE oligomycin gradient plates. C, expression of Yor1p and Vph1p using the strains in B was determined by Western blotting whole cell extracts. D, sucrose gradient fractions from the strains in B were Western-blotted with antibodies against Yor1p, Sec61p, and Pma1p. wt, wild type.
to the efficiency of export. Further analysis of these newly identified signals in Yor1p will provide insight into the mechanism of ER export of polytopic membrane proteins, an issue important in diseases like cystic fibrosis and hyperinsulinemic hypoglycemia where modulation of ER export could be a potential therapy.

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