A Region within a Lumenal Loop of *Saccharomyces cerevisiae* Ycf1p Directs Proteolytic Processing and Substrate Specificity

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Ycf1p, a member of the yeast multidrug resistance-associated protein (MRP) subfamily of ATP-binding cassette proteins, is a vacuolar membrane transporter that confers resistance to a variety of toxic substances such as cadmium and arsenite. Ycf1p undergoes a *PEP4*-dependent processing event to yield N- and C-terminal cleavage products that remain associated with one another. In the present study, we sought to determine whether proteolytic cleavage is required for Ycf1p activity. We have identified a unique region within lumenal loop 6 of Ycf1p, designated the loop 6 insertion (L6_ins), which appears to be necessary and sufficient for proteolytic cleavage, since L6_ins can promote processing when moved to new locations in Ycf1p or into a related transporter, Bpt1p. Surprisingly, mutational results indicate that proteolytic processing is not essential for Ycf1p transport activity. Instead, the L6_ins appears to regulate substrate specificity of Ycf1p, since certain mutations in this region lower cellular cadmium resistance with a concomitant gain in arsenite resistance. Although some of these L6_ins mutations block processing, there is no correlation between processing and substrate specificity. The activity profiles of the Ycf1p L6_ins mutants are dramatically affected by the strain background in which they are expressed, raising the possibility that another cellular component may functionally impact Ycf1p activity. A candidate component may be a new full-length MRP-type transporter (*NFT1*), reported in the *Saccharomyces* Genome Database as two adjacent open reading frames, *YKR103w* and *YKR104w*, but which we show here is present in most *Saccharomyces* strains as a single open reading frame.

Many proteins are initially synthesized as precursors that undergo proteolytic processing events to generate their mature form. Such proteins include the vacuolar proteases and mating pheromones in *Saccharomyces cerevisiae*, which require proteolytic cleavage to generate biologically active molecules (22, 41). Posttranslational processing can also cleave certain transcription factors, such as sterol regulatory element-binding protein (SREBP) or Notch, from a membrane tether so that they can function in the nucleus (8). In other instances, the role of processing is less clear. Presenilin, a component of the γ secretase required for the proteolytic processing of the amyloid precursor protein, is a multispanning membrane protein that itself is proteolytically cleaved into two fragments (50, 51). Although both the N- and C-terminal cleavage products of presenilin have been shown to be necessary for activity, it is uncertain whether processing is required for activity (27, 34).

Ycf1p, a member of the yeast ATP-binding cassette (ABC) transporter superfamily, also undergoes proteolytic processing and is the only known example of an ABC transporter that is cleaved (31, 47). Ycf1p localizes to the vacuolar membrane, where it functions as a glutathione conjugate transporter to detoxify the cell of a variety of compounds, including the heavy metals cadmium and arsenite (16, 28, 29, 43, 47). We have previously shown that the cleavage of Ycf1p depends on the master vacuolar protease, Pep4p, and therefore is likely to occur upon arrival of Ycf1p at the vacuolar membrane (31). Ycf1p is comprised of a typical ABC core region (MSD1, NBD1, L1, MSD2, and NBD2) and an additional N-terminal extension (MSD0 and L0) that is a diagnostic feature of certain members of the multidrug resistance-associated protein (MRP) subfamily to which Ycf1p belongs (see Fig. 2A). The posttranslational cleavage of Ycf1p liberates two fragments, one containing the N-terminal extension plus a small segment of the C-terminal core, and the other containing the remainder of the C-terminal core (31, 49). It does not appear that the fragments created by posttranslational processing represent inactive degradation products since the cleavage products interact and both contain regions that have been shown to be critical for Ycf1p function (12, 13, 31).

In the present study we identify a 17-amino-acid insertion in loop 6 of MSD1, designated the loop 6 insertion (L6_ins), and show that this region is necessary and sufficient to promote proteolytic cleavage. A major goal of the present study was to determine whether the proteolytic cleavage of Ycf1p modulates transport function. Notably, mutational analysis of L6_ins indicates that the activity of Ycf1p is independent of its posttranslational processing status. Instead, we found that mutations within L6_ins can affect Ycf1p substrate specificity, even though the L6_ins lies on the luminal face of the vacuolar membrane where the substrate is released after its transport. Surprisingly, we detected dramatic differences in resistance to toxic substances of two different laboratory strains both bearing the same L6_ins mutation. These data suggest that an additional component present in some strains, but absent in others, may be important for Ycf1p activity. We report here the discovery of a new full-length yeast MRP-type transporter gene (*NFT1*) comprised of two adjacent open reading frames (ORFs), *YKR103w* and *YKR104w*, that represents a candidate gene which may account for the observed strain differences.
The plates used for the spot tests were prepared by adding the indicated concentrations of CdSO₄ or AsNaO₂ (Sigma, St. Louis, Mo.) to the minimal plate medium immediately prior to pouring the plates. A fresh batch of cadmium plates was made for each experiment, since toxicity tended to vary somewhat from batch to batch and to decrease with age of the plates.

To examine growth inhibition by toxic compounds, cells were grown overnight to saturation in minimal medium and then subcultured at a 1:50 dilution in minimal medium and grown overnight at 30°C to an optical density of 0.1 (OD₆₀₀) of ca. 1.0. This overnight culture was diluted to an OD₆₀₀ of 0.1, which in turn was diluted in 10-fold increments. Aliquots (5 µl) of each 10-fold dilution were spotted onto SC-Ura or SC plates containing the indicated concentration of CdSO₄ or AsNaO₂ and incubated for 4 to 6 days at 30°C.

Plasmid constructions and DNA sequence analysis. Plasmids used in the present study are listed in Table 2. To analyze single copy (cen) Ycf1p-GFP by recombinational cloning (33). A PvuII restriction fragment from pSM1753 (2a URA3 YCF1-GFP) (38) was recombined into BamHI-Khol-digested pRS316 (CEN LEU2) (39). A URA3 version of pSM1761 was constructed.

Materials and Methods

Yeast strains, media, and growth conditions. Yeast strains used in the present study are listed in Table 1. The strains containing chromosomal forms of wild-type and mutant ycf1-GFP were generated by the standard two-step integration/ excision method. Each of the integrating plasmids (pSM1817, pSM1826, pSM1853, pSM1859, pSM1860, pSM1863, pSM1864, and pSM1865) was digested with AvrII and then transformed into SM3851, a ycf1Δ derivative of strain SM1058, and SM4719, a ycf1Δ:KanMX derivative of strain BY4741. Transformants for each strain were selected on SC-Ura plates. Excisants, leaving behind wild-type and mutant gene replacements, were selected on 5-fluoroorotic acid and confirmed by Southern blot analysis and PCR. The expression of wild-type and mutant forms of Ycf1p-green fluorescent protein (GFP) in each of the strains was confirmed by Western blot analysis (see Fig. 4B and C). The resulting strains are listed as integrants in the strain table (Table 1). All yeast transformations were performed as described previously (31).

Plate and liquid drop-out media were prepared as previously described (32). The plates used for the spot tests were prepared by adding the indicated concentrations of CdSO₄ or AsNaO₂ (Sigma, St. Louis, Mo.) to the minimal plate medium immediately prior to pouring the plates. A fresh batch of cadmium plates was made for each experiment, since toxicity tended to vary somewhat from batch to batch and to decrease with age of the plates.
by recombinational cloning of a PvuI restriction fragment from pSM1761 with BamHI-XhoI-digested pRS316 (CEN URA3) (39), yielding pSM1762 (CEN URA3 YCF1-GFP).

To create mutations within the Ycf1p processing site (Table 3), the region spanning the two ORFs was amplified using the 5' primer. Sequences of PCR products containing the engineered ORFs were obtained from the species were obtained from the Johns Hopkins University School of Medicine, Baltimore, Md.). The mouse anti-GFP polyclonal antibody was purchased from Chemicon (Temecula, Calif.). The horseradish peroxidase-conjugated secondary antibodies (donkey anti-rabbit immunoglobulin) and sheep anti-mouse immunoglobulin) used for immunoblotting were purchased from Amersham Pharmacia Biotech (Piscataway, N.J.).

**Antibodies.** The rabbit anti-GFP polyclonal antibody was a gift from R. Jensen (Johns Hopkins University School of Medicine, Baltimore, Md.). The mouse anti-GFP polyclonal antibody was purchased from Chemicon (Temecula, Calif.).

**Fluorescence microscopy.** To examine the localization of Ycf1p-GFP, cells were grown overnight to saturation in minimal medium and then subcultured at a 1:1,000 dilution in minimal medium and grown overnight at 30°C to an OD_{600} of ca. 0.7. Log-phase cells were examined at ×100 magnification on poly-l-lysine-coated slides by using a Zeiss Axioskop microscope equipped with fluorescence and Nomarski optics (Zeiss, Thornwood, N.Y.). Images were captured with a Cooke charge-coupled device camera and IP Lab spectrum software (Biovision Technologies, Inc., Exton, Pa.).

**RESULTS**

Proteolytic processing of Ycf1p required for its ability to confer resistance to cadmium? Ycf1p is proteolytically processed in a PEP4-dependent manner when it reaches the vacuolar membrane (31) (Fig. 1A). Our previous studies indicated that both the N- and the C-terminal cleavage products contain regions that contribute to the biological function of Ycf1p (31).

**TABLE 2. Plasmids used in this study**

| Plasmid | Relevant genotype | Reference or source |
|---------|------------------|---------------------|
| pJAW50  | 2µ TRPI YCF1     | 48                  |
| pJAW53  | YEp 3110Sm-YCF1-hisG-Ura3-hisG-3'135nt-YCF1 | 48                |
| pRS306  | YEp URA3        | 39                  |
| pRS315  | CEN LEU2        | 39                  |
| pRS316  | CEN URA3        | 39                  |
| pSM217  | 2µ URA3         | 10                  |
| pSM1490 | 2µ URA3 BPTI-GFP| 38                  |
| pSM1752 | 2µ URA3 YCF1    | 38                  |
| pSM1753 | 2µ URA3 YCF1-GFP| 38                 |
| pSM1761 | CEN LEU2 YCF1-GFP| This study             |
| pSM1762 | CEN URA3 YCF1-GFP| This study             |
| pSM1775 | 2µ URA3 ycfL6-GFP| This study             |
| pSM1783 | 2µ URA3 BPTI-Agel-GFP| This study           |
| pSM1784 | 2µ URA3 BPTI-L6-GFP| This study            |
| pSM1785 | 2µ URA3 YCF1-GFP (3') region | This study             |
| pSM1817 | YEp URA3 YCF1-FP| This study             |
| pSM1825 | 2µ URA3 ycfL6-GFP + (3') region | This study             |
| pSM1826 | YEp URA3 ycfL6-GFP| This study             |
| pSM1831 | 2µ URA3 ycfL6, L12::NotI-GFP| This study            |
| pSM1832 | 2µ URA3 ycfL6, L12::L6-GFP| This study            |
| pSM1833 | 2µ URA3 ycfL6(1)-GFP| This study             |
| pSM1834 | 2µ URA3 ycfL6(2)-GFP| This study             |
| pSM1835 | 2µ URA3 ycfL6(3)-GFP| This study             |
| pSM1850 | 2µ URA3 ycfL6-2-4-GFP| This study             |
| pSM1851 | 2µ URA3 ycfL6-2-4-GFP| This study             |
| pSM1853 | YEp URA3 ycfL6-2-4-GFP| This study             |
| pSM1854 | 2µ URA3 ycfL6-2-E-GFP| This study             |
| pSM1859 | YEp URA3 ycfL6-2-E-GFP| This study             |
| pSM1860 | YEp URA3 ycfL6-2-E-GFP| This study             |
| pSM1863 | YEp URA3 ycfL6-2-4-GFP| This study             |
| pSM1864 | YEp URA3 ycfL6-2-4-GFP| This study             |
| pSM1865 | YEp URA3 ycfL6-2-4-GFP| This study             |
| pSM1876 | 2µ URA3 ycfL6, L12::L6-GFP| This study             |

* YEp denotes yeast integrating plasmid.
We compared Ycf1p-dependent cadmium resistance in wild-type (PEP4) and pep4Δ strains (Fig. 1B). Because the latter is defective for Ycf1p processing, the ability of full-length unprocessed Ycf1p to confer resistance to cadmium could potentially be assessed in this mutant. Chromosomally expressed Ycf1p confers a low level of resistance to cadmium in a wild-type strain but not in a pep4Δ mutant (Fig. 1B, rows 1 and 2). An analogous difference in cadmium resistance between the wild-type and pep4Δ strains is apparent for strains expressing Ycf1p-GFP from a low-copy (CEN) plasmid (Fig. 1B, rows 3 and 4), although vacuolar localization was similar in both strains (Fig. 1C). These results initially raised the possibility that processing might be essential for the functional activity of Ycf1p. Interestingly, however, overexpression of Ycf1p from a multicopy plasmid (Fig. 1B, rows 5 and 6) suppressed the cadmium sensitivity observed in the pep4Δ strain, even though little or no processing was apparent (Fig. 1A). This result provided the first hint that processing may not, in fact, be a prerequisite for Ycf1p function. It should be noted that the extent of Ycf1p processing is the same in the presence or absence of cadmium (data not shown).

**Cleavage of Ycf1p requires a unique 17-amino-acid region of MSD1 that is necessary and sufficient for processing.** To more directly assess the functional requirement for processing, we sought to construct mutations within YCF1 that inhibited processing. Based on the gel mobility and the PEP4-dependent production of the Ycf1p proteolytic products (~38 and ~160 kDa) (31), we reasoned that the processing must occur within MSD1 that is necessary and sufficient for Ycf1p processing. Based on the gel mobility and the PEP4-dependent production of the Ycf1p proteolytic products (~38 and ~160 kDa) (31), we reasoned that the processing must occur within MSD1 that is necessary and sufficient for Ycf1p processing.

| L6ins sequence | Ycf1p allele | Phenotype |
|---------------|--------------|-----------|
| L6-1          | ERQQDD SSLQGF ENNH | WT |
| L6-2          | ERQQDD SSLQGF ENNH | WT (+GFP) |
| L6-3          | ERQQDD SSLQGF ENNH | ΔL6 |
| L6-4          | ERQQDD SSLQGF ENNH | ΔL6-1 |
| L6-5          | ERQQDD SSLQGF ENNH | ΔL6-2 |
| L6-6          | ERQQDD SSLQGF ENNH | ΔL6-3 |
| L6-7          | ERQQDD AAAAA ENNH | L6-2→A |
| L6-8          | ERQQDD EEEEE ENNH | L6-2→E |
| L6-9          | ERQQDD ERQQDD ENNH | L6-2→L6-1 |

We compared Ycf1p-dependent cadmium resistance in wild-type (PEP4) and pep4Δ strains (Fig. 1B). Because the latter is defective for Ycf1p processing, the ability of full-length unprocessed Ycf1p to confer resistance to cadmium could potentially be assessed in this mutant. Chromosomally expressed Ycf1p confers a low level of resistance to cadmium in a wild-type strain but not in a pep4Δ mutant (Fig. 1B, rows 1 and 2). An analogous difference in cadmium resistance between the wild-type and pep4Δ strains is apparent for strains expressing Ycf1p-GFP from a low-copy (CEN) plasmid (Fig. 1B, rows 3 and 4), although vacuolar localization was similar in both strains (Fig. 1C). These results initially raised the possibility that processing might be essential for the functional activity of Ycf1p. Interestingly, however, overexpression of Ycf1p from a multicopy plasmid (Fig. 1B, rows 5 and 6) suppressed the cadmium sensitivity observed in the pep4Δ strain, even though little or no processing was apparent (Fig. 1A). This result provided the first hint that processing may not, in fact, be a prerequisite for Ycf1p function. It should be noted that the extent of Ycf1p processing is the same in the presence or absence of cadmium (data not shown).

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|---------------|--------------|-----------|
| L6-1          | ERQQDD SSLQGF ENNH | WT |
| L6-2          | ERQQDD SSLQGF ENNH | WT (+GFP) |
| L6-3          | ERQQDD SSLQGF ENNH | ΔL6 |
| L6-4          | ERQQDD SSLQGF ENNH | ΔL6-1 |
| L6-5          | ERQQDD SSLQGF ENNH | ΔL6-2 |
| L6-6          | ERQQDD SSLQGF ENNH | ΔL6-3 |
| L6-7          | ERQQDD AAAAA ENNH | L6-2→A |
| L6-8          | ERQQDD EEEEE ENNH | L6-2→E |
| L6-9          | ERQQDD ERQQDD ENNH | L6-2→L6-1 |

A The sequences correspond to mutations within the L6ins of Ycf1p (amino acids 321 to 337). Deletions are indicated by a dash (-).

B The processing phenotypes (scored as +, +/-, -/+ , and –) are based on the immunoblot analysis in Fig. 4.

C The cadmium (Cd r) and arsenite (As r) resistance phenotypes reflect the results shown in Fig. 5.
loop of Ycf1p. Strikingly, movement of this region within Ycf1p, from L6 in MSD1 to the middle of loop 12 (L12) in MSD2 (\(L_6^{\text{ins}}, L_{12}::L_6^{\text{ins}}\)), creates a novel processing site within Ycf1p, suggesting that \(L_6^{\text{ins}}\) carries the information necessary for both recognition and cleavage (Fig. 3A, lane 4). Ycf1p is also processed within or near L12 when an even more minimal segment (six amino acids, referred to below as \(L_6^{-2}\)) from \(L_6^{\text{ins}}\) is transferred to the middle of L12 (Fig. 3A, lane 5).

We sought to determine whether \(L_6^{\text{ins}}\) confers processing when inserted into a different ABC protein, by transferring it to L6 of Bpt1p, the closest yeast homologue of Ycf1p (38, 44). Notably, the normally unprocessed Bpt1p is now cleaved (Fig. 3B, compare lanes 2 and 3). Taken together, the results shown in Fig. 3A and B indicate that \(L_6^{\text{ins}}\) is necessary and sufficient for proteolytic processing.

Mutations within the \(L_6^{\text{ins}}\) have variable effects on the processing of Ycf1p. To further define the minimal region required for proteolytic processing, we divided the \(L_6^{\text{ins}}\) into three subregions, L6-1, L6-2, and L6-3 (see Table 3 for residues within these intervals) and generated deletion and substitution mutations in these subregions. Results from this analysis are shown in Fig. 4 and are summarized in Table 3. Immunoblot analysis was used to monitor the processing of mutant versions of Ycf1p expressed either from a multicopy plasmid (Fig. 4A) or from a chromosomally integrated copy of wild-type or mutant GFP-tagged YCF1 (Fig. 4B and C). The integrants were generated by a two-step gene replacement into two different ycf1Δ strain backgrounds (derived from our laboratory strain, SM1058, or from the parental strain for the yeast knockout collection, BY4741). The processing phenotype for each mutant expressed from the chromosome is identical for the two strain backgrounds and essentially identical to that observed when the mutants are overexpressed from multicopy plasmids (Fig. 4, compare B and C to A). Deletion of the first six (\(\Delta L_6^{-1}\)) or the last five (\(\Delta L_6^{-3}\)) amino acids of the \(L_6^{\text{ins}}\) had no effect on Ycf1p cleavage (Fig. 4, lanes 4 and 6). However, deletion of the middle six amino acids (\(\Delta L_6^{-2}\)) severely impaired processing (Fig. 4, lane 5).

To determine whether the specific sequence of the \(L_6^{-2}\) region (SSLQGF) is critical for processing, we replaced this subregion with various residues, mutated singly and in combination (Table 3, rows 7 to 9). Single-residue alanine replacements within the \(L_6^{-2}\) region had no effect on processing of Ycf1p (data not shown). Furthermore, replacement of the entire \(L_6^{-2}\) region with six alanines (\(L_6^{-2}A\)) also had no effect on processing, suggesting that a specific sequence per se is not critical for processing (Fig. 4, lane 7). However, further mutagenesis indicates that the particular sequence content of the \(L_6^{-2}\) region does influence processing. Replacement of the \(L_6^{-2}\) region with six glutamates (\(L_6^{-2}E\)) resulted in inefficient processing of Ycf1p and a lower overall steady-state level of protein, presumably reflecting degradation, compared to the other mutants (Fig. 4, lane 8). We also replaced the \(L_6^{-2}\) region with the six amino acids from the neighboring region (\(L_6^{-2}L_6^{-1}\)), thereby creating a duplication of \(L_6^{-1}\). Duplication of the \(L_6^{-1}\) region significantly impaired Ycf1p processing (Fig. 4, lane 9). Thus, the \(\Delta L_6^{\text{ins}}, \Delta L_6^{-2},\) and \(L_6^{-2}L_6^{-1}\) mutants, in which proteolytic processing is defective, provide use-
ful tools for determining whether processing and function of Ycf1p are at all correlated (Table 3). The L6-2\textsuperscript{3} mutant is particularly advantageous, since it allows us to address the importance of proteolytic processing, without having changed the overall size of L6. Importantly, each Ycf1p mutant expressed from a multicopy plasmid or from the chromosome properly localizes to the vacuolar membrane (data not shown), indicating that any defects in proteolytic processing or activity are not due to mislocalization.

L6\textsubscript{ins} ycf1 mutants affect substrate specificity when expressed at chromosomal levels. Previous studies have shown that ycf1\Delta cells are sensitive to toxic compounds such as cadmium and arsenite (16, 43, 47) and that Ycf1p directly mediates the transport of cadmium-glutathione complexes (28). We initially examined the ability of the Ycf1p processing mutants to confer resistance to cadmium when the mutant proteins are overexpressed from multicopy plasmids. Under these conditions, the deletion of the entire L6\textsubscript{ins} (or portions thereof) did not affect cadmium resistance, regardless of the status of Ycf1p processing (data not shown). Furthermore, moving the processing site to L12 also had no effect on cadmium resistance (data not shown) even though this created a novel functional processing site in Ycf1p (Fig. 3A). These results were not completely surprising considering our earlier observation that Ycf1p expressed from a multicopy plasmid suppressed the cadmium sensitivity of a pep4\textsuperscript{H9004} mutant (Fig. 1B), suggesting that overexpression of mutant forms of Ycf1p may mask differences that are more subtle.

To circumvent any overexpression artifacts, we assessed the cadmium and arsenite resistance of the Ycf1p mutant proteins expressed from the chromosome. The results from the cadmium spot test show that strains bearing wild-type or GFP-tagged Ycf1p and some of the mutants (Fig. 5, rows 2, 3, and

FIG. 3. The L6\textsubscript{ins} is necessary and sufficient for processing within loops 6 and 12 of Ycf1p and within loop 6 of Bpt1p. Schematics (top parts of panels) are shown for Ycf1p (A) and Bpt1p (B). The location of the proposed native cleavage site (*) in Ycf1p and the engineered cleavage sites in Ycf1p L12 and Bpt1p L6 are indicated, as well as the predicted molecular weight of the C-terminal fragments resulting from cleavage in these regions. Immunobots show the steady-state level of wild-type and mutant Ycf1p-GFP (A) and Bpt1p-GFP (B). Ycf1p-GFP and Bpt1p-GFP were detected by using anti-GFP monoclonal and polyclonal antibodies, respectively. Unprocessed (full-length) and C-terminal cleavage products (C-term) are indicated. Panel A, lanes 1 to 5: SM4517, SM4518, SM4543, SM4648, and SM4757, respectively. Panel B, lanes 1 to 3: SM4516, SM4522, and SM4590, respectively. Abbreviations: WT, wild type; ΔL6, L6 insertion deleted (amino acids 321 to 337); ΔL6\textsubscript{ins} L12::L6\textsubscript{ins} (L6\textsubscript{ins} deleted and amino acids 321 to 337 transferred to L12 of Ycf1p between amino acids 984 and 985); ΔL6\textsubscript{ins} L12::L6-2 (L6\textsubscript{ins} deleted and amino acids 327 to 332 transferred to L12 of Ycf1p between amino acids 984 and 985); EV (empty vector); Bpt1p::L6, Ycf1p amino acids 321 to 337 inserted into Bpt1p between amino acids 325 and 326.
4 to 6, respectively) confer a wild-type level of resistance to cadmium, while the other mutants (Fig. 5, rows 7 to 10) confer a significantly lower level of cadmium resistance (see also Table 3). Notably, each of these phenotypic groups consists of some mutants that are processed normally and some that are not (Fig. 5, right column, and Table 3), clearly indicating that the degree of cadmium resistance and the processing status are unrelated.

Strikingly different results were observed when arsenite resistance was examined. First, wild-type YCF1-GFP, when chromosomally integrated, was unable to confer resistance to arsenite compared to untagged chromosomal YCF1, although both GFP-tagged and untagged Ycf1p confer cadmium resistance (Fig. 5, compare rows 2 and 3). Thus, the C-terminal cytosolically disposed GFP tag may influence Ycf1p transport activity for some, but not all substrates. Second, the mutants that show reduced cadmium resistance display an unanticipated gain of function for arsenite resistance compared to the Ycf1p-GFP parental construct (Fig. 5, compare rows 7 to 10 with row 3, and Table 3). Together, these findings for cadmium and arsenite resistance of chromosomal ycf1 mutants suggest that proteolytic processing of L6 is not a prerequisite for Ycf1p to function but that the L6 region appears to be involved in substrate specificity, either directly or indirectly.

**Strain differences suggest that Ycf1p may interact with a second component to transport certain substrates.** We also examined the cadmium and arsenite resistance of the Ycf1p mutant proteins expressed from the chromosome in a different strain background, BY4741, which is the parental strain for the yeast knockout collection and a derivative of the strain S288C (17) (Fig. 6). Notably, the ycf1 null mutant is sensitive to both substrates. The activity of wild-type Ycf1p (untagged and GFP tagged) and mutant forms of Ycf1p-GFP expressed from the chromosome in the SM1058 strain background is shown. An aliquot (5 μl) of cells at 0.1 OD₆₀₀, and serial 10-fold dilutions thereof, were spotted onto control plates (no CdSO₄ or AsNaO₂) and plates containing 30 μM CdSO₄ or 1.5 mM AsNaO₂ and then incubated at 30°C for 2 to 6 days. The strains used in rows 1 to 10 are SM3851, SM1058, SM4643, SM4644, SM4729, SM4730, SM4731, SM4717, SM4718, and SM4697, respectively. The indicated processing phenotypes were determined by the immunoblot analysis shown in Fig. 4B (see also Table 3).

![Immunoblot analysis](https://example.com/immunoblot.png)

**FIG. 5.** Mutations within the L₆ ins affect substrate specificity when Ycf1p is expressed from the chromosome in the SM1058 strain background. The activity of wild-type Ycf1p (untagged and GFP tagged) and mutant forms of Ycf1p-GFP expressed from the chromosome in the SM1058 strain background is shown. An aliquot (5 μl) of cells at 0.1 OD₆₀₀, and serial 10-fold dilutions thereof, were spotted onto control plates (no CdSO₄ or AsNaO₂) and plates containing 30 μM CdSO₄ or 1.5 mM AsNaO₂ and then incubated at 30°C for 2 to 6 days. The strains used in rows 1 to 10 are SM3851, SM1058, SM4643, SM4644, SM4729, SM4730, SM4731, SM4717, SM4718, and SM4697, respectively. The indicated processing phenotypes were determined by the immunoblot analysis shown in Fig. 4B (see also Table 3). NA, not applicable.

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Strikingly different results were observed when arsenite resistance was examined. First, wild-type YCF1-GFP, when chromosomally integrated, was unable to confer resistance to arsenite compared to untagged chromosomal YCF1, although both GFP-tagged and untagged Ycf1p confer cadmium resistance (Fig. 5, compare rows 2 and 3). Thus, the C-terminal cytosolically disposed GFP tag may influence Ycf1p transport activity for some, but not all substrates. Second, the mutants that show reduced cadmium resistance display an unanticipated gain of function for arsenite resistance compared to the Ycf1p-GFP parental construct (Fig. 5, compare rows 7 to 10 with row 3, and Table 3). Together, these findings for cadmium and arsenite resistance of chromosomal ycf1 mutants suggest that proteolytic processing of L6 is not a prerequisite for Ycf1p to function but that the L6 region appears to be involved in substrate specificity, either directly or indirectly.

**Strain differences suggest that Ycf1p may interact with a second component to transport certain substrates.** We also examined the cadmium and arsenite resistance of the Ycf1p mutant proteins expressed from the chromosome in a different strain background, BY4741, which is the parental strain for the yeast knockout collection and a derivative of the strain S288C (17) (Fig. 6). Notably, the ycf1 null mutant is sensitive to both compounds (Fig. 6, row 1). However, we were surprised to find that, in contrast to the results presented in Fig. 5 for the SM1058 strain background, neither the GFP tag nor any of the L6 deletion or point mutations affects cadmium or arsenite resistance when expressed chromosomally in the BY4741 strain background. There was no phenotype for any of the L6 mutants, even with higher concentrations of cadmium or arsenite (data not shown). Based on these results, we predict that an additional component(s) that differs between the two strain backgrounds is important for influencing the ability of Ycf1p to confer resistance to certain substrates.

**Identification of a new MRP in yeast.** Several ABC transporters appear to interact with other membrane transporters or channels. Examples include the human MRP-type protein SUR1, which interacts with the KᵦT₆ channel KᵦT₆.2 (1), and partial molecules of the yeast ABC transporter Ste6p, which complement mutations in full-length Ste6p possibly via heterooligomer formation (4, 5, 7). Full-length ABC transporters may also interact as homodimers or heterodimers, as has been proposed for human MRPI (36) or yeast Ycf1p and Bpt1p (38). Thus, when considering candidate genes that may represent the putative second component responsible for the strain differences described above, we examined the possibility that another ABC transporter may be involved. We focused on YKR103w and YKR104w, reported in the SGD as two adjacent ORFs that code for partial ABC transporters, separated by a nonsense codon, followed by 33 sense codons (Fig. 7A). We hypothesized that the nonsense codon might represent a mutation in S288C (the strain used to sequence the genome and the strain from which BY4741 is derived), whereas in SM1058 (also known as EG123 and whose lineage is distinct from S288C [40]), YKR103/104w may actually code a single “full-length” MRP-type transporter homologous throughout its length to Ycf1p and Bpt1p (6). Expression of the full-length versus truncated transporter, then, might influence the activity of Ycf1p.

To examine the possibility that YKR103/104w varies between strains, we used PCR amplification of genomic DNA, followed by DNA sequence analysis to confirm that, in the S288C-
designated L6ins, that is unique to Ycf1p and is in the vicinity of the yeast MRP subfamily, revealed a 17-amino-acid insertion, with sequence alignment between Ycf1p and other members of the MRP subfamily consisting of six full-length transporters: YCF1, YBT1, YOR1, YHL035c, and NFT1. Interestingly, other S. cerevisiae strains, whose lineages derive independently of S288C (SK1 and 21278b), and other species of yeast from the wild (S. paradoxus and S. mikatae), also encode the full-length NFT1 gene (Fig. 7B). The fact that the NFT1 gene product is full-length in SM1058 and truncated in BY4741 may account for the different activities of some of the Ycf1p mutants in these two strain backgrounds; however, proof of this hypothesis awaits further analysis.

**DISCUSSION**

Identification of a sequence within MSD1 that is necessary and sufficient for Ycf1p processing. Previous studies by us and others have shown that the MRP-type ABC transporter Ycf1p resides in the vacuolar membrane (28, 31, 47), where it undergoes PEP4-dependent processing. An important goal in the present study was to generate mutations within Ycf1p that block cleavage, permitting us to assess the connection between processing and the transport activity of Ycf1p.

The estimated size of the Ycf1p cleavage products, together with sequence alignment between Ycf1p and other members of the yeast MRP subfamily, revealed a 17-amino-acid insertion, designated L6ins, that is unique to Ycf1p and is in the vicinity of the cleavage site (Fig. 2B). L6ins lies within a predicted luminal loop (L6) of Ycf1p. Deletion of L6ins resulted in accumulation of full-length Ycf1p and, strikingly, its transfer to the vacuolar membrane (28, 31, 47), where it underlies the activity of wild-type Ycf1p (untagged and GFP tagged) and mutant forms of Ycf1p-GFP expressed from the chromosome in the BY4741 strain background. The activity of YCF1, YBT1, YOR1, and YHL035c is necessary and sufficient for Ycf1p processing.

Nevertheless, it is possible that cleavage occurs within or near the L6ins sequence, is not proteolytically processed either in yeast or insect cells, but is cleaved in a manner that is analogous to that which occurs in yeast cells. However, it is possible that cleavage occurs within or near the L6ins in yeast cells, indicating that posttranslational proteolytic processing may be inherent to the biology of Ycf1p. Notably, human MRPI, a close homolog of Ycf1p but missing the L6ins sequence, is not proteolytically processed either in mammalian or Sf21 insect cells (20, 35).

Proteolytic processing is unrelated to the function of Ycf1p. Since Ycf1p is not processed in a pep4Δ mutant, and because the pep4Δ mutant is sensitive to cadmium, it seemed possible that the processing site mutants might also be sensitive to cadmium. However, mutations in L6ins of Ycf1p (discussed below) showed that the ability of Ycf1p to confer resistance to cadmium is completely unrelated to its processing phenotype. Thus, the sensitivity of the pep4Δ mutant to cadmium must not due to lack of Ycf1p processing but instead may be caused by one of the many pleiotropic phenotypes associated with the pep4Δ mutant (21, 45); such pleiotropic effects could indirectly alter Ycf1p function or cellular cadmium levels.

A subset of mutations in L6ins resulted in Ycf1p proteins...
uncovered a function for Ycf1p processing, it is possible that a role may be revealed under as-yet-unexamined physiological condition(s).

**Mutations within L6ins affect substrate specificity.** Resistance to both cadmium and arsenite is Ycf1p dependent, as evidenced by the sensitivity of a ycf1Δ strain to both compounds (Fig. 5) (16, 43, 47). Surprisingly, we found that certain L6ins mutations in Ycf1p can differentially affect cellular resistance to these compounds, at least in our laboratory strain background SM1058. One group confers resistance to cadmium but not arsenite, while another group confers resistance to arsenite and reduced resistance to cadmium (Fig. 5 and Table 3). Furthermore, each group contains some ycf1 mutants that are proteolytically processed and some that are not. One explanation for the two distinct classes is that mutant Ycf1p can exist in two conformations (“A” and “B”) in which different domains or distinct regions within the active site of Ycf1p interact to bind, recognize, or transport various substrates. According to this scenario, the first group of mutant proteins (Ycf1p-GFP, ΔL6ins, ΔL6-1, and ΔL6-2) confer cadmium resistance but not arsenite resistance because they assume conformation “A.” The other mutant proteins (ΔL6-3, L6-2–A, E, or L6-1) assume conformation “B,” conferring arsenite resistance and reduced cadmium resistance.

It is somewhat surprising that substrate specificity can be affected by alterations in a region of the Ycf1p transporter on the side of the membrane where the substrate is released, as opposed to the side where the substrate encounters the transporter. Perhaps the L6ins mutations in Ycf1p selectively affect substrate release rather than substrate binding. Alternatively, the differential transport of cadmium and arsenite observed for some of the ycf1 luminal loop mutations may relate to the mode of substrate transport, i.e., cadmium is transported by Ycf1p as a complex with glutathione (28) while, at least for MRP1, arsenite may be cotransported with glutathione (37). Another recent study also identified mutations that altered the substrate specificity of Ycf1p; in this case the mutations mapped to cytosolic and membrane-spanning regions of Ycf1p (12). Similarly, for human MRP1, residues within several substrate specificity city (18, 25, 42, 53–55). Taken together, the studies on yeast and human MRP transporters provide evidence that the recognition, binding, and/or transport of particular substrates can involve regions on both sides of the membrane, as well as regions spanning the membrane.

**Strain variation in the phenotype of ycf1 mutants.** In the course of our Ycf1p studies, we used two differing strain backgrounds: (i) SM1058, also designated EG123, which is our standard laboratory strain and has a poorly defined lineage (40), and (ii) BY4741, an S288C derivative that is the parental strain for the yeast deletion collection (17). Quite surprisingly, we found that none of the L6ins Ycf1p mutants used in the present study affected cellular cadmium or arsenite resistance in BY4741 (Fig. 6), in contrast to the results in the SM1058 strain background discussed above. Although the L6ins mutant phenotypes were indistinguishable from wild-type in BY4741, the Ycf1pΔ mutation in this background remained sensitive to these compounds, indicating that resistance is still Ycf1p dependent.

These results suggest that another cellular component(s),
which may be directly or indirectly involved in YCF1-mediated resistance to toxic compounds such as cadmium or arsenite, may be different in the two strains. There are several possibilities for such a component. It could act (i) directly on Ycf1p, for instance, to form a heterodimer; (ii) indirectly, perhaps altering general properties of how Ycf1p functions; or (iii) at a step different from Ycf1p, for instance, to influence the intracellular level of toxic compounds or their targets. Phenotypic variation among yeast strains is not uncommon and has been used as a starting point to identify novel components for a number of cellular processes, including pseudohyphal growth, flocculation, and cell polarity (23, 26). Clearly, an important next step will be to determine whether the yef1 “modifier” segregates as a single, or multiple, gene trait.

Discovery of a new ABC transporter in yeast, Nft1p. One possible cellular component that we considered might contribute to the strain differences discussed above could be another MRP protein. We investigated two uncharacterized ORFs, YKR103w and YKR104w. We had previously observed in an analysis of yeast ABC proteins that these adjacent sequences in the yeast genome appear to encode two pieces of a single full-length MRp protein (6, 44). We discovered that in the strain SM1058, YKR103/104w is one continuous ORF rather than two ORFs separated by a nonsense codon, as in the strains BY4741 and S288C (Fig. 7). Because YKR103/104w actually represents a single gene encoding a new full-length MRp-type transporter, we have named this gene NFT1. Interestingly, possession of a full-length NFT1 gene appears to be the “wild-type” situation for yeast, since other S. cerevisiae laboratory strains (S288C and SK1) that differ in lineage from S288C also possess the full-length NFT1 gene, as do other Saccharomyces species (S. paradoxus and S. mikatae). Differences between S288C and other S. cerevisiae strains, as well as other Saccharomyces species, have been reported for a variety of genes, such as the aquaporin genes (9). It is possible that the manner in which S288C was cultivated in the laboratory led to an unintentional selection against the presence of full-length NFT1.

The observed strain differences in cadmium and arsenite resistance of certain yef1 L6 mutations expressed in SM1058 versus BY4741 could potentially be attributed to one strain background expressing the full-length MRp transporter (Nft1p) and the other expressing one or both of the partial molecules (Ykr103p and Ykr104p). At least two models can be envisioned to explain how NFT1 or YKR103/104w might account for the strain differences we observed with certain Ycf1p mutant proteins. First, the expression of Ykr103p and/or Ykr104p in the BY4741 strain background could positively influence the activity of the Ycf1p mutants, perhaps by forming a heterodimer with Ycf1p. Such partial-molecule complementation has been observed for mutations in another ABC transporter, Ste6p (4, 7). Another possibility is that the full-length transporter, Nft1p, could negatively influence the activity of Ycf1p in the SM1058 strain background, thereby revealing the mutant phenotypes. Although it is tempting to speculate that Nft1p is involved in Ycf1p-mediated cadmium and arsenite resistance, it is indeed possible that neither Nft1p nor Ykr103p and/or Ykr104p are affecting the activity of Ycf1p and that the strain differences are due to another cellular component(s).

Elucidation of the function of Nft1p is currently under investigation.

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