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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 lumenal 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.

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TABLE 1. Yeast strains used in this study

Strain	Relevant genotype	Reference or source
BY4741	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 his3Δ1*	7a
F744	*MAT***a**	Σ1278b derivative from G. Fink via J. Boeke
MS10	*MAT***a** *ura3-52 leu2-3,112 ade2-101*	M. Rose
MS1907	*MAT***a** *ura3-52 leu2-3,112 ade2-101 pep4*::*LEU2*	M. Rose
NKY276	*MAT***a** *lys2 ura3 ho*::*LYS2*	SK1 derivative from N. Kleckner via J. Boeke
SM1058	*MAT***a** *trp1 leu2 ura3 his4 can1*	32
SM3794	*MAT***a** *trp1 leu2 ura3 his4 can1 bpt1*Δ::*KanMX4*	38
SM3851	*MAT***a** *trp1 leu2 ura3 his4 can1 ycf1*Δ	38
SM4516	SM3851 [2μ *URA3*]	Transformant of SM3851 with pSM217
SM4517	SM3851 [2μ *URA3 YCF1*]	Transformant of SM3851 with pSM1752
SM4518	SM3851 [2μ *URA3 YCF1-GFP*]	Transformant of SM3851 with pSM1753
SM4522	SM3794 [2μ *URA3 BPT1-GFP*]	Transformant of SM3794 with pSM1490
SM4523	MS10 [2μ *URA3 YCF1-GFP*]	Transformant of MS10 with pSM1753
SM4524	MS1907 [2μ *URA3 YCF1-GFP*]	Transformant of MS1907 with pSM1753
SM4527	MS10 [2μ *URA3*]	Transformant of MS10 with pSM217
SM4528	MS1907 [2μ *URA3*]	Transformant of MS1907 with pSM217
SM4539	MS10 [*CEN URA3 YCF1-GFP*]	Transformant of MS10 with pSM1762
SM4540	MS1907 [*CEN URA3 YCF1-GFP*]	Transformant of MS1907 with pSM1762
SM4543	SM3851 [2μ *URA3 ycf1*(Δ*L6*)*-GFP*]	Transformant of SM3851 with pSM1775
SM4590	SM3794 [2μ *URA3 BPT1*::*L6-GFP*]	Transformant of SM3794 with pSM1784
SM4643[b,c]	*MAT***a** *trp1 leu2 ura3 his4 can1 YCF1-GFP*	Integrant in SM3851 using pSM1817
SM4644[b,c]	*MAT***a** *trp1 leu2 ura3 his4 can1 ycf1*(Δ*L6*)*-GFP*	Integrant in SM3851 using pSM1826
SM4648	SM3851 [2μ *URA3 ycf1*(Δ*L6*, *L12*::*L6*)*-GFP*]	Transformant of SM3851 with pSM1832
SM4656	SM3851 [2μ *URA3 ycf1*(Δ*L6-1*)*-GFP*]	Transformant of SM3851 with pSM1833
SM4657	SM3851 [2μ *URA3 ycf1*(Δ*L6-2*)*-GFP*]	Transformant of SM3851 with pSM1834
SM4658	SM3851 [2μ *URA3 ycf1*(Δ*L6-3*)*-GFP*]	Transformant of SM3851 with pSM1835
SM4672	SM3851 [2μ *URA3 ycf1*(*L6-2*→*A*)*-GFP*]	Transformant of SM3851 with pSM1850
SM4678	SM3851 [2μ *URA3 ycf1*(*L6-2*→*L6-1*)*-GFP*]	Transformant of SM3851 with pSM1851
SM4680	SM3851 [2μ *URA3 ycf1*(*L6-2*→*E*)*-GFP*]	Transformant of SM3851 with pSM1854
SM4697[b,c]	*MAT***a** *trp1 leu2 ura3 his4 can1 ycf1*(*L6-2*→*L6-1*)*-GFP*	Integrant in SM3851 using pSM1853
SM4717[b,c]	*MAT***a** *trp1 leu2 ura3 his4 can1 ycf1*(*L6-2*→*A*)*-GFP*	Integrant in SM3851 using pSM1859
SM4718[b,c]	*MAT***a** *trp1 leu2 ura3 his4 can1 ycf1*(*L6-2*→*E*)*-GFP*	Integrant in SM3851 using pSM1860
SM4719[a]	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 his3Δ1 ycf1*::*KanMX4*	Research Genetics (Huntsville, Ala.)
SM4720[b,d]	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 his3Δ1 YCF1-GFP*	Integrant in SM4719 using pSM1817
SM4721[b,d]	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 his3Δ1 ycf1*(Δ*L6*)*-GFP*	Integrant in SM4719 using pSM1826
SM4722[b,d]	*MAT***a** *leu2Δ0 met15*Δ0 ura3Δ0 his3*Δ1 ycf1*(*L6-2*→*L6-1*)*-GFP*	Integrant in SM4719 using pSM1853
SM4723[b,d]	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 his3Δ1 ycf1*(*L6-2*→*A*)*-GFP*	Integrant in SM4719 using pSM1859
SM4724[b,d]	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 his3Δ1 ycf1*(*L6-2*→*E*)*-GFP*	Integrant in SM4719 using pSM1860
SM4726[b,d]	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 his3Δ1 ycf1*(Δ*L6-1*)*-GFP*	Integrant in SM4719 using pSM1863
SM4727[b,d]	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 his3Δ1 ycf1*(Δ*L6-2*)*-GFP*	Integrant in SM4719 using pSM1864
SM4728[b,d]	*MAT***a** *leu2Δ0 met15Δ0 ura3Δ0 his3Δ1 ycf1*(Δ*L6-3*)*-GFP*	Integrant in SM4719 using pSM1865
SM4729[b,c]	*MAT***a** *trp1 leu2 ura3 his4 can1 ycf1*(Δ*L6-1*)*-GFP*	Integrant in SM3851 using pSM1863
SM4730[b,c]	*MAT***a** *trp1 leu2 ura3 his4 can1 ycf1*(Δ*L6-2*)*-GFP*	Integrant in SM3851 using pSM1864
SM4731[b,c]	*MAT***a** *trp1 leu2 ura3 his4 can1 ycf1*(Δ*L6-3*)*-GFP*	Integrant in SM3851 using pSM1865
SM4757	SM3851 [2μ *URA3 ycf1*(Δ*L6, L12*::*L6-2*)*-GFP*]	Transformant of SM3851 with pSM1876

[a] A derivative of the strain BY4741, provided by Research Genetics via J. Boeke (Johns Hopkins University School of Medicine, Baltimore, Md.).
[b] Strains constructed by the standard two-step integration-excision method by using the designated plasmid as described in Materials and Methods.
[c] Derived from SM3851, a derivative of the strain SM1058.
[d] Derived from SM4719, a derivative of the strain BY4741.

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 *Avr*II and then transformed into SM3851, a *ycf1*Δ derivative of strain SM1058, and SM4719, a *ycf1*Δ::*KanMX4* 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 (11).

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 $CdSO_4$ or $AsNaO_2$ (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 1:500 dilution in minimal medium and grown overnight at 30°C to an optical density at 600 nm (OD_{600}) of ca. 1.0. This overnight culture was diluted to an OD_{600} 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 $CdSO_4$ or $AsNaO_2$ 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 with GFP fused to the C terminus, we constructed the plasmid pSM1761 (*CEN LEU2 YCF1-GFP*) by recombinational cloning (33). A *Pvu*I restriction fragment from pSM1753 (2μ *URA3 YCF1-GFP*) (38) was recombined into *Bam*HI-*Xho*I-digested pRS315 (*CEN LEU2*) (39). A *URA3* version of pSM1761 was constructed

TABLE 2. Plasmids used in this study

Plasmid	Relevant genotype[a]	Reference or source
pJAW50	2μ *TRP1 YCF1*	48
pJAW53	YIp *5′1018nt-YCF1-hisG-URA3-hisG-3′1356nt-YCF1*	48
pRS306	YIp *URA3*	39
pRS315	*CEN LEU2*	39
pRS316	*CEN URA3*	39
pSM217	2μ *URA3*	10
pSM1490	2μ *URA3 BPT1-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 ycf1*(Δ*L6*)*-GFP*	This study
pSM1783	2μ *URA3 BPT1*::*Age*I*-GFP*	This study
pSM1784	2μ *URA3 BPT1*::*L6-GFP*	This study
pSM1785	2μ *URA3 YCF1-GFP* + (3′ region)	This study
pSM1817	YIp *URA3 YCF1-GFP*	This study
pSM1825	2μ *URA3 ycf1*(Δ*L6*)*-GFP* + (3′ region)	This study
pSM1826	YIp *URA3 ycf1*(Δ*L6*)*-GFP*	This study
pSM1831	2μ *URA3 ycf1*(Δ*L6*, *L12*::*Not*I)*-GFP*	This study
pSM1832	2μ *URA3 ycf1*(Δ*L6*, *L12*::*L6*)*-GFP*	This study
pSM1833	2μ *URA3 ycf1*(Δ*L6-1*)*-GFP*	This study
pSM1834	2μ *URA3 ycf1*(Δ*L6-2*)*-GFP*	This study
pSM1835	2μ *URA3 ycf1*(Δ*L6-3*)*-GFP*	This study
pSM1850	2μ *URA3 ycf1*(*L6-2→A*)*-GFP*	This study
pSM1851	2μ *URA3 ycf1*(*L6-2→L6-1*)*-GFP*	This study
pSM1853	YIp *URA3 ycf1*(*L6-2→L6-1*)*-GFP*	This study
pSM1854	2μ *URA3 ycf1*(*L6-2→E*)*-GFP*	This study
pSM1859	YIp *URA3 ycf1*(*L6-2→A*)*-GFP*	This study
pSM1860	YIp *URA3 ycf1*(*L6-2→E*)*-GFP*	This study
pSM1863	YIp *URA3 ycf1*(Δ*L6-1*)*-GFP*	This study
pSM1864	YIp *URA3 ycf1*(Δ*L6-2*)*-GFP*	This study
pSM1865	YIp *URA3 ycf1*(Δ*L6-3*)*-GFP*	This study
pSM1876	2μ *URA3 ycf1*(Δ*L6*, *L12*::*L6-2*)*-GFP*	This study

[a] YIp denotes yeast integrating plasmid.

by recombinational cloning of a *Pvu*I restriction fragment from pSM1761 with *Bam*HI-*Xho*I-digested pRS316 (*CEN URA3*) (39), yielding pSM1762 (*CEN URA3 YCF1-GFP*).

To create mutations within the Ycf1p processing site (Table 3), the region within L6 between amino acids 321 and 337 was deleted and mutated as follows. The entire region was deleted by recombinational cloning of a *YCF1* PCR product, generated from an oligonucleotide containing an engineered deletion of amino acids 321 to 337 (designated ΔL6), into *Age*I-digested pSM1753, thereby yielding pSM1775 (2μ *URA3 ycf1ΔL6-GFP*). Three smaller deletions were created within this region by recombinational cloning of various *YCF1* PCR products containing engineered deletions of amino acids 321 to 326 (ΔL6-1), 327 to 332 (ΔL6-2), or 333 to 337 (ΔL6-3) into *Age*I-digested pSM1775 (for the ΔL6-1 mutant) or into *Age*I-digested pSM1753 (for the ΔL6-2 and ΔL6-3 mutants). The resulting plasmids were pSM1833 (2μ *URA3 ycf1ΔL6-1-GFP*), pSM1834 (2μ *URA3 ycf1ΔL6-2-GFP*), and pSM1835 (2μ *URA3 ycf1ΔL6-3-GFP*).

The entire L6-2 region from amino acids 327 to 332 was replaced with various residues as shown in Table 3 and as described below. The L6-2 region was replaced with six alanines or with a duplication of the amino acids in the L6-1 region (ERQDDH) by recombinational cloning of *YCF1* PCR products containing the engineered mutations into *Age*I-digested pSM1753. The resulting plasmids were pSM1850 (2μ *URA3 ycf1L6-2→A-GFP*) or pSM1851 (2μ *URA3 ycf1L6-2→L6-1-GFP*). The L6-2 region was also replaced with six glutamates by recombinational cloning of a *YCF1* PCR product containing the engineered mutation into *Age*I-digested pSM1850, thereby yielding pSM1854 (2μ *URA3 ycf1L6-2→E-GFP*).

To move the region required for Ycf1p cleavage from loop 6 to loop 12 of Ycf1p, first a *Not*I site was inserted after amino acid 984 in pSM1775 to yield pSM1831 (2μ *URA3 ycf1ΔL6*, *L12*::*NotI-GFP*). Next, the region from amino acids 321 to 337 was amplified from pJAW50 (2μ *TRP1 YCF1*) (48) and cloned into *Not*I-digested pSM1831 by homologous recombination, generating pSM1832 (2μ *URA3 ycf1ΔL6*, *L12*::*L6-GFP*). In the resulting plasmid, the region required for Ycf1p cleavage is deleted from its original location and transferred to the region between amino acids 984 and 985. A smaller region of the cleavage site (L6-2; residues 327 to 332) was also transferred to loop 12 by recombinational cloning to generate pSM1876 (2μ *URA3 ycf1ΔL6*, *L12*::*L6-2-GFP*).

The region required for Ycf1p cleavage site was also transferred to the analogous region in loop 6 of *BPT1*. First, an *Age*I restriction site was inserted after amino acid 325 in pSM1490 (2μ *URA3 BPT1-GFP*) (38) to yield pSM1783 (2μ *URA3 BPT1*::*Age*I*-GFP*). Next, the region from amino acids 321 to 337 in *YCF1* was amplified from pJAW50 and cloned into *Age*I-digested pSM1783 by homologous recombination, generating pSM1784 (2μ *URA3 BPT1*::*L6-GFP*). In the resulting plasmid, the potential Ycf1p cleavage site has been transferred to the region between amino acids 325 and 326 in Bpt1p.

To analyze chromosomal *ycf1* mutants, we constructed yeast integrating plasmids (YIp) to use for generating two-step gene replacements. First, the 1,092-bp region downstream of the *YCF1* ORF was amplified from pJAW53 (48) and cloned into *Nae*I-digested pSM1753 by recombinational cloning, generating pSM1785 (2μ *URA3 YCF1-GFP* + 3′ region), which is identical to pSM1753 except for the added 3′ downstream region. Next, a *Kpn*I-*Bam*HI fragment from pSM1785, containing *YCF1-GFP*, was subcloned into the same sites of pRS306 (YIp *URA3*) (39), yielding pSM1817 (YIp *URA3 YCF1-GFP*). The plasmid pSM1825 (2μ *URA3 ycf1ΔL6-GFP* + 3′ region) was generated by subcloning an *Age*I-*Kpn*I fragment from pSM1775 into the same sites of pSM1785. As described above, a *Kpn*I-*Bam*HI fragment from pSM1825, containing *ycf1ΔL6-GFP*, was subcloned into the same sites of pRS306, yielding pSM1826 (YIp *URA3 ycf1ΔL6-GFP*). Each of the smaller loop 6 deletions (ΔL6-1, ΔL6-2, and ΔL6-3) and the L6-2 mutations (L6-2→A, L6-2→L6-1, and L6-2→E) were also subcloned into integrating plasmids. *Age*I-*Kpn*I fragments from pSM1833, pSM1834, pSM1835, pSM1850, pSM1851, and pSM1854 were subcloned into the same sites of pSM1817 to generate pSM1863 (YIp *URA3 ycf1ΔL6-1-GFP*), pSM1864 (YIp *URA3 ycf1ΔL6-2-GFP*), pSM1865 (YIp *URA3 ycf1ΔL6-3-GFP*), pSM1859 (YIp *URA3 ycf1L6-2→A-GFP*), pSM1853 (YIp *URA3 ycf1L6-2→L6-1-GFP*), and pSM1860 (YIp *URA3 ycf1L6-2→E-GFP*), respectively.

All of the deletions and mutations described above were confirmed by DNA sequence analysis. Further details of the plasmid constructions have been described (30) and will be furnished upon request.

To sequence the junction of the ORFs *YKR103w* and *YKR104w*, a 626-bp region spanning the two ORFs was amplified by PCR from genomic DNA (colony PCR or purified genomic DNA) with 18mer oligonucleotide primers (24). The PCR products were sequenced by automated DNA sequencing by using the 5′ primer. Sequences of *YKR103w*/*104w* and related sequences in other *Saccharomyces* species were obtained from the *Saccharomyces* Genome Database (SGD) website (http://genome-www.stanford.edu/Saccharomyces/).

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 monoclonal antibody was purchased from Clontech (Palo Alto, 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.).

Immunoblot analysis. Cell extracts and immunoblots used to detect Ycf1p were prepared as previously described (15) except that samples were either heated at 65°C for 10 min prior to electrophoresis (Fig. 1A and 3B) or not heated at all, in order to minimize aggregation (Fig. 3A and 4). The primary antibodies used were rabbit anti-GFP (1:5,000) or mouse anti-GFP (1:670).

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-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

Is 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).

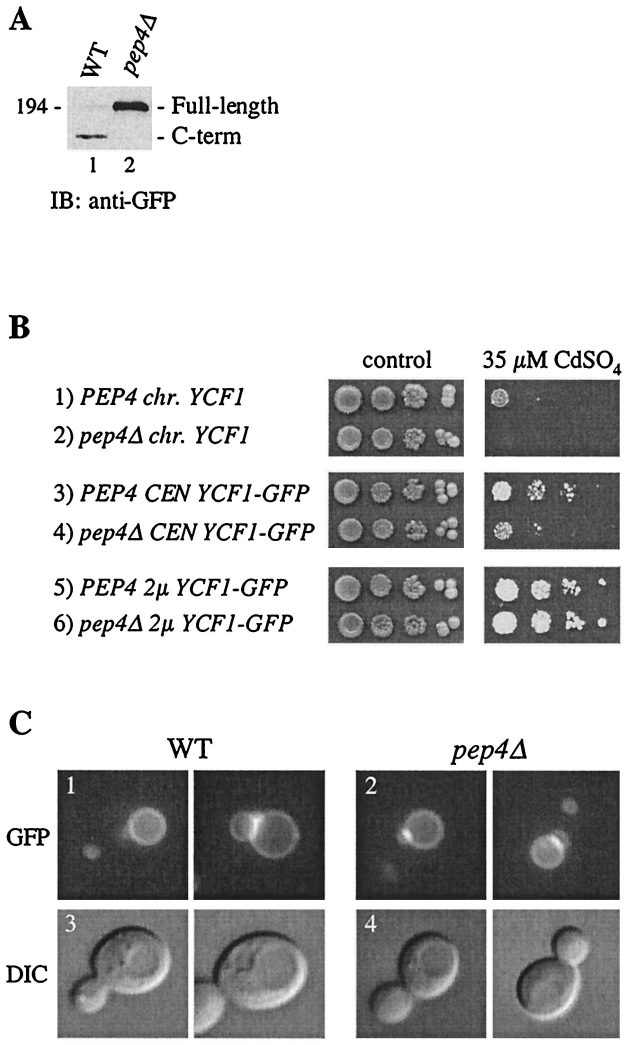


FIG. 1. Ycf1p processing and resistance of cells to cadmium is impaired when *PEP4* is deleted. (A) The steady-state level of Ycf1p-GFP in wild-type (WT) and *pep4*Δ strains was examined by immunoblot analysis. Crude yeast cell extracts (0.4 OD_{600} cell equivalents per lane) were resolved by sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis and transferred to nitrocellulose. Ycf1p-GFP was detected by using rabbit anti-GFP polyclonal antibodies. Unprocessed Ycf1p-GFP is indicated as full-length and the C-terminal cleavage product as C-term. A molecular weight marker is indicated. The strains used were SM4523 (lane 1) and SM4524 (lane 2). (B) Cadmium resistance was tested by spotting an aliquot (5 μl) of cells at 0.1 OD_{600}, and serial 10-fold dilutions thereof, onto plates containing no $CdSO_4$ (control) or 35 μM $CdSO_4$, followed by incubation at 30°C for 2 or 5 days, respectively. The strains used in rows 1 to 6 are SM4527, SM4528, SM4539, SM4540, SM4523, and SM4524, respectively. (C) Vacuolar membrane localization of Ycf1p-GFP in wild-type (1 and 3) and *pep4*Δ (2 and 4) cells by fluorescence microscopy. In the differential interference contrast (DIC) images (panels 3 and 4), the vacuole appears as an indentation. In the corresponding GFP panels (panels 1 and 2), the bright areas of fluorescence correspond to a region where two vacuolar lobes intersect. The strains used were SM4523 (wild type) and SM4524 (*pep4*Δ).

However, the functional significance of the cleavage event per se has not been determined. Here, we examined whether Ycf1p requires proteolytic processing for its activation, or instead, if unprocessed Ycf1p remains functional.

TABLE 3. Summary of sequences and phenotypes of Ycf1p variants in SM1058 strain background

Row	$L6_{ins}$ sequence[a]			Ycf1p allele	Phenotype[c]		
	L6-1	L6-2	L6-3		Processing[b]	Cd^r	As^r
1	ERQDDH	SSLQGF	ENNHP	WT	+	+	+
2	ERQDDH	SSLQGF	ENNHP	WT (+GFP)	+	+	−
3	------	------	-----	ΔL6	−	+	−
4	------	SSLQGF	ENNHP	ΔL6-1	+	+	−
5	ERQDDH	------	ENNHP	ΔL6-2	−	+	−
6	ERQDDH	SSLQGF	-----	ΔL6-3	+	+/−	+
7	ERQDDH	AAAAAA	ENNHP	L6-2→A	+	+/−	+
8	ERQDDH	EEEEEE	ENNHP	L6-2→E	+/−	+/−	+
9	ERQDDH	ERQDDH	ENNHP	L6-2→L6-1	−/+	+/−	+

[a] The sequences correspond to mutations within the $L6_{ins}$ 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.

We compared Ycf1p-dependent cadmium resistance in wild-type (*PEP4*) and *pep4*Δ 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 *pep4*Δ mutant (Fig. 1B, rows 1 and 2). An analogous difference in cadmium resistance between the wild-type and *pep4*Δ 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 *pep4*Δ 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 or near loop 6 (L6) in MSD1, which is lumenally disposed (Fig. 2A). Interestingly, analysis of the L6 region in an alignment between all yeast MRP subfamily members identifies an insertion of ca. 17 amino acids that is unique to Ycf1p and is designated here as the L6 insertion ($L6_{ins}$) (Fig. 2B). Deletion of the $L6_{ins}$ ($\Delta L6_{ins}$) completely blocks processing of Ycf1p (Fig. 3A, compare lanes 2 and 3). Thus, we conclude that the $L6_{ins}$ is required for proteolytic processing of Ycf1p. Although additional regions of Ycf1p could impact proteolytic processing, this possibility has not yet been further investigated.

To determine whether $L6_{ins}$ is sufficient for processing, we inserted this 17-amino-acid region into a different lumenal

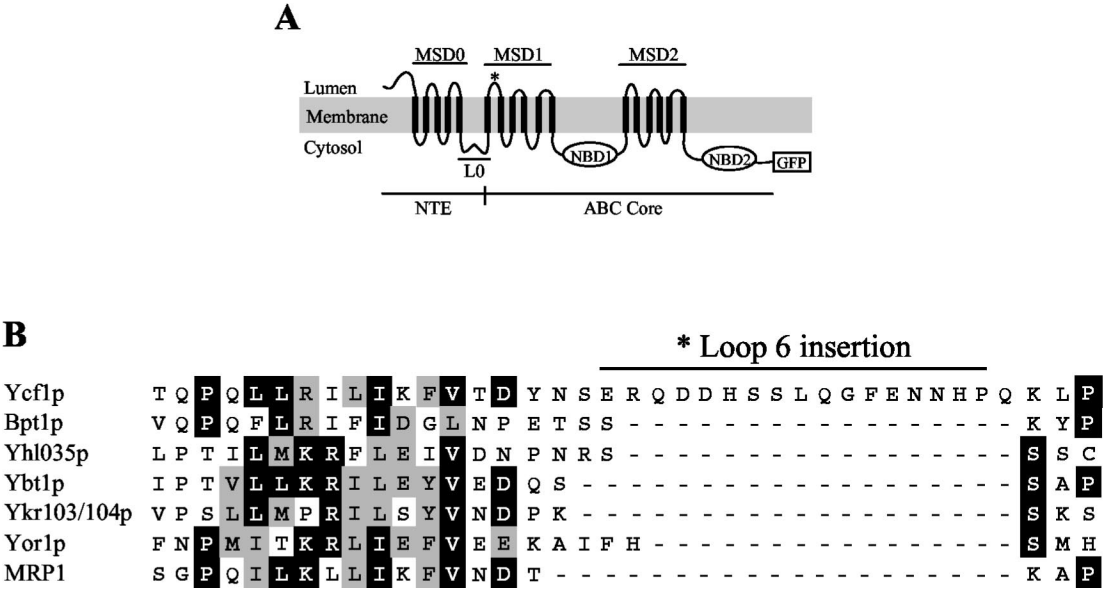


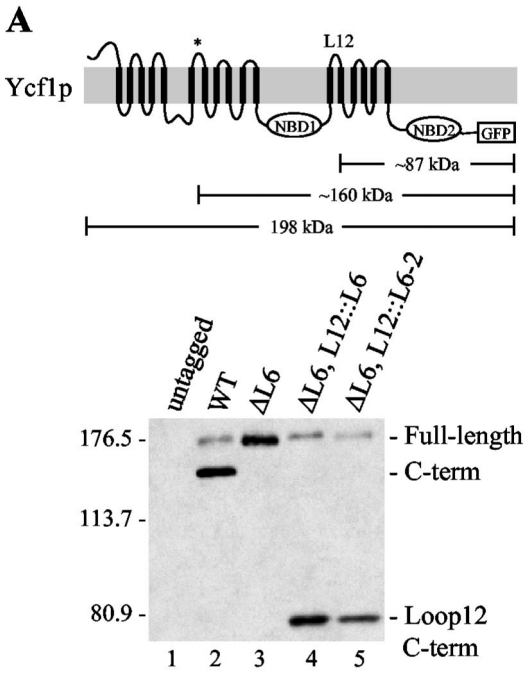
FIG. 2. Among the yeast MRPs, Ycf1p contains a unique insertion within loop 6 of MSD1. (A) The proposed topology of Ycf1p is shown. The N-terminal extension (NTE) contains a membrane-spanning domain (MSD0) and a cytosolic linker region (L0). The ABC core region contains two membrane-spanning domains (MSD1 and MSD2) and two nucleotide-binding domains (NBD1 and NBD2), and is separated by a linker (L1) between NBD1 and MSD2 (for simplicity sake, L1 is omitted in this and subsequent figures). The proposed cleavage site within loop 6 is indicated by an asterisk. The GFP coding sequence, fused immediately before the stop codon of Ycf1p, is indicated. (B) The lumenally disposed loop 6 (L6; residues 303 to 341) of Ycf1p was aligned to the five other yeast MRP subfamily members (Bpt1p, Yhl035p, Ybt1p, Ykr103/104p, and Yor1p) and its closest human homologue, MRP1, by using CLUSTALW software (46). Black boxes represent amino acid identity, and gray boxes indicate amino acid similarity as determined with the Boxshade server (www.ch.embnet.org/software/box_form.html). The unique L6 insertion in Ycf1p corresponds to residues 321 to 337 and is absent in all of the other proteins as indicated by the gap in this region.

loop of Ycf1p. Strikingly, movement of this region within Ycf1p, from L6 in MSD1 to the middle of loop 12 (L12) in MSD2 ($\Delta L6_{ins}$, L12::$L6_{ins}$), creates a novel processing site within Ycf1p, suggesting that $L6_{ins}$ 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 L6-2) from $L6_{ins}$ is transferred to the middle of L12 (Fig. 3A, lane 5).

We sought to determine whether $L6_{ins}$ 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 the $L6_{ins}$ is necessary and sufficient for proteolytic processing.

Mutations within the $L6_{ins}$ have variable effects on the processing of Ycf1p. To further define the minimal region required for proteolytic processing, we divided the $L6_{ins}$ 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 *ycf1*Δ 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 (ΔL6-1) or the last five (ΔL6-3) amino acids of the $L6_{ins}$ had no effect on Ycf1p cleavage (Fig. 4, lanes 4 and 6). However, deletion of the middle six amino acids (ΔL6-2) severely impaired processing (Fig. 4, lane 5).

To determine whether the specific sequence of the L6-2 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 L6-2 region had no effect on processing of Ycf1p (data not shown). Furthermore, replacement of the entire L6-2 region with six alanines (L6-2→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 L6-2 region does influence processing. Replacement of the L6-2 region with six glutamates (L6-2→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 L6-2 region with the six amino acids from the neighboring region (L6-2→L6-1), thereby creating a duplication of L6-1. Duplication of the L6-1 region significantly impaired Ycf1p processing (Fig. 4, lane 9). Thus, the $\Delta L6_{ins}$, ΔL6-2, and L6-2→L6-1 mutants, in which proteolytic processing is defective, provide use-



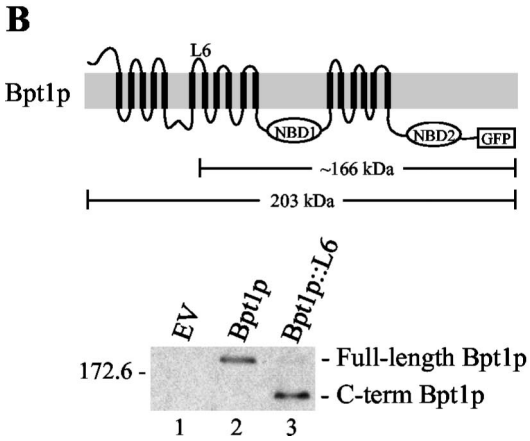


FIG. 3. The $L6_{ins}$ is necessary and sufficient for processing within loops 6 and 12 of Ycf1p and within loop 6 of Bpt1p. Schematics (top parts of panels) are shown for Ycf1p (A) and Bpt1p (B). The location of the proposed native cleavage site (*) in Ycf1p and the engineered cleavage sites in Ycf1p L12 and Bpt1p L6 are indicated, as well as the predicted molecular weight of the C-terminal fragments resulting from cleavage in these regions. Immunoblots 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; $\Delta L6_{ins}$, L6 insertion deleted (amino acids 321 to 337); $\Delta L6_{ins}$, L12::$L6_{ins}$ ($L6_{ins}$ deleted and amino acids 321 to 337 transferred to L12 of Ycf1p between amino acids 984 and 985); $\Delta L6_{ins}$, L12::L6-2 ($L6_{ins}$ 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.

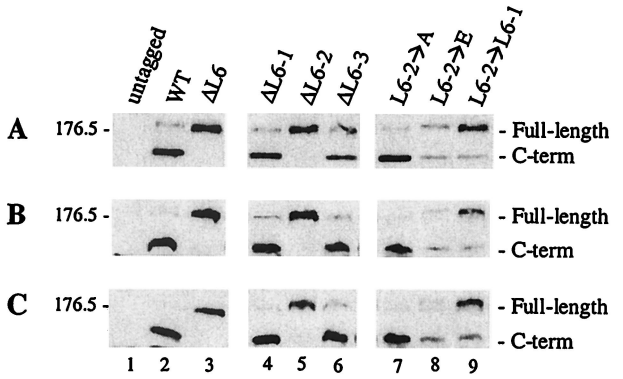


FIG. 4. Mutations within the $L6_{ins}$ have variable effects on Ycf1p processing. The steady-state protein level of wild-type and mutant Ycf1p-GFP expressed from multicopy plasmids in SM1058 (A) or from the chromosome in the SM1058 (B) and BY4741 (C) strain backgrounds was determined by immunoblot analysis as in Fig. 1A, except that panels B and C contain 0.8 OD_{600} cell equivalents per lane. Ycf1p-GFP was detected by using either mouse anti-GFP monoclonal antibodies (A) or rabbit anti-GFP polyclonal antibodies (B and C). The sequence for each mutant is shown in Table 3. The strains used in lanes 1 to 9 were SM4517, SM4518, SM4543, SM4656, SM4657, SM4658, SM4672, SM4680, and SM4678 (A); SM1058, SM4643, SM4644, SM4729, SM4730, SM4731, SM4717, SM4718, and SM4697 (B); and BY4741, SM4720, SM4721, SM4726, SM4727, SM4728, SM4723, SM4724, and SM4722 (C), respectively.

ful tools for determining whether processing and function of Ycf1p are at all correlated (Table 3). The L6-2→L6-1 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.

$L6_{ins}$ *ycf1* mutants affect substrate specificity when expressed at chromosomal levels. Previous studies have shown that *ycf1*Δ 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 2μ plasmids. Under these conditions, the deletion of the entire $L6_{ins}$ (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 2μ plasmid suppresses the cadmium sensitivity of a *pep4*Δ 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

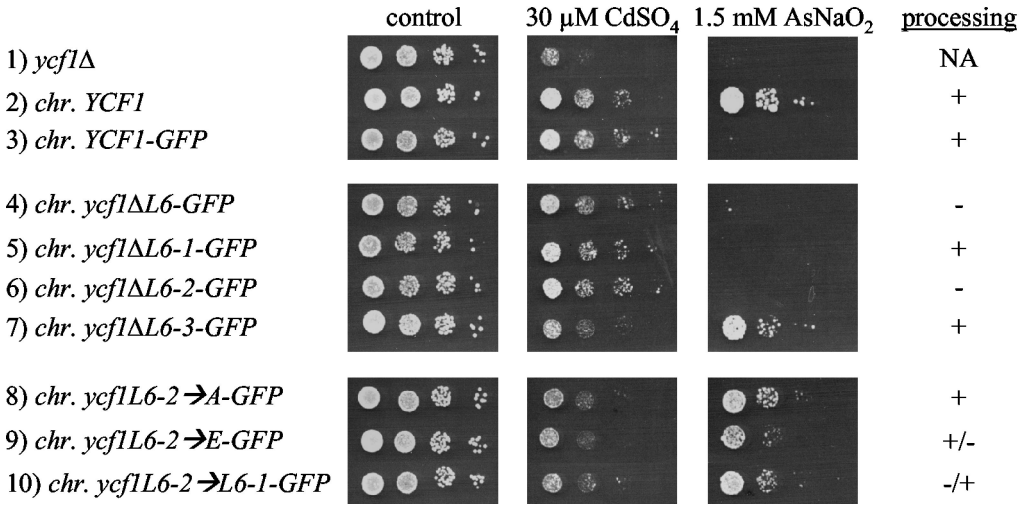


FIG. 5. Mutations within the $L6_{ins}$ affect substrate specificity when Ycf1p is expressed from the chromosome in the SM1058 strain background. The activity of wild-type Ycf1p (untagged and GFP tagged) and mutant forms of Ycf1p-GFP expressed from the chromosome in the SM1058 strain background is shown. An aliquot (5 μl) of cells at 0.1 OD_{600}, and serial 10-fold dilutions thereof, were spotted onto control plates (no $CdSO_4$ or $AsNaO_2$) and plates containing 30 μM $CdSO_4$ or 1.5 mM $AsNaO_2$ 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.

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 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 K_{ATP} channel $K_{IR}6.2$ (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 MRP1 (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-

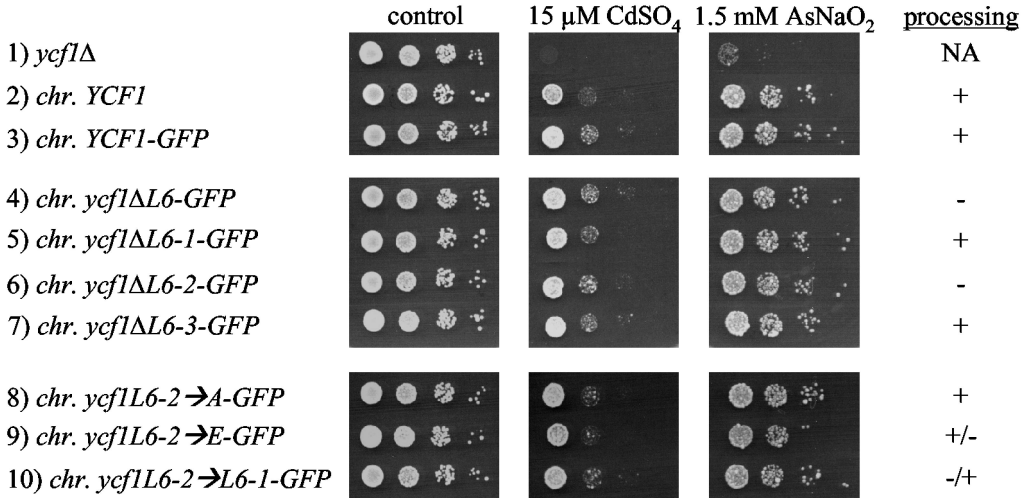


FIG. 6. Mutations within the $L6_{ins}$ do not affect the substrate specificity when Ycf1p is expressed from the chromosome in the BY4741 strain background. The activity of wild-type Ycf1p (untagged and GFP tagged) and mutant forms of Ycf1p-GFP expressed from the chromosome in the BY4741 strain background is shown. An aliquot (5 μl) of cells at 0.1 OD_{600}, and serial 10-fold dilutions thereof, were spotted onto control plates (no $CdSO_4$ or $AsNaO_2$) and plates containing 15 μM $CdSO_4$ or 1.5 mM $AsNaO_2$ and then incubated at 30°C for 2 to 6 days. The strains used in rows 1 to 10 were SM4719, BY4741, SM4720, SM4721, SM4726, SM4727, SM4728, SM4723, SM4724, and SM4722, respectively. The processing phenotypes were determined by the immunoblot analysis shown in Fig. 4C. NA, not applicable.

derived strain BY4741, *YKR103/104w* are separate ORFs, with a nonsense codon (TAG) at codon 1219 of *YKR103w*. In contrast, we found that in our laboratory strain SM1058, codon 1219 (TAT) encodes a tyrosine residue. Thus, in SM1058, the combined *YKR103/104w* ORF encodes a new full-length MRP-type transporter, which we have designated *NFT1* (Fig. 7B). In at least some *S. cerevisiae* strain backgrounds, then, the yeast MRP subfamily consists of six full-length transporters: *YCF1*, *BPT1*, *YBT1*, *YOR1*, *YHL035c*, and *NFT1*.

Interestingly, other *S. cerevisiae* strains, whose lineages derive independently of S288C (SK1 and Σ1278b), 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

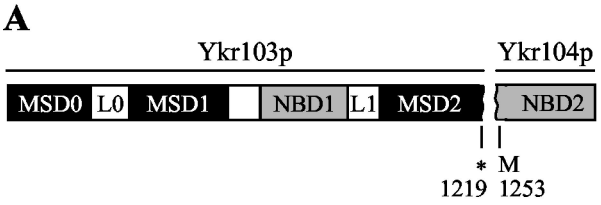
Identification of a sequence within MSD1 that is necessary and sufficient for Ycf1p processing. Previous studies by us and others have shown that the MRP-type ABC transporter Ycf1p resides in the vacuolar membrane (28, 31, 47), where it undergoes *PEP4*-dependent processing. An important goal in the 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 $L6_{ins}$, that is unique to Ycf1p and is in the vicinity of the cleavage site (Fig. 2B). $L6_{ins}$ lies within a predicted lumenal loop (L6) of Ycf1p. Deletion of $L6_{ins}$ resulted in accumulation of full-length Ycf1p and, strikingly, its transfer to L12 of Ycf1p or L6 of another MRP protein, Bpt1p, resulted in novel cleavage (Fig. 3). These findings indicate that the $L6_{ins}$ is necessary and sufficient to direct proteolytic processing, at least when present in the lumenal loop of a vacuolar MRP-type membrane protein, where it is accessible to vacuolar proteases. Cleavage could occur within the $L6_{ins}$ or somewhere in its vicinity. For instance, the addition of the $L6_{ins}$ to L12 of Ycf1p and L6 of Bpt1p could expose a nearby protease-sensitive site that is normally occluded in these loops.

Interestingly, a previous study showed that *YCF1*, when heterologously expressed in Sf21 insect cells, also undergoes proteolytic processing to yield approximately the same size C-terminal product as that observed in its native yeast cell environment (35). Since Ycf1p localizes to the plasma membrane in Sf21 cells, rather than to the lysosome, this cleavage may not be truly analogous to that which occurs in yeast cells. Nevertheless, it is possible that cleavage occurs within or near the $L6_{ins}$ even in insect cells, indicating that posttranslational proteolytic processing may be inherent to the biology of Ycf1p. Notably, human MRP1, a close homologue of Ycf1p but missing the $L6_{ins}$ 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 *pep4*Δ mutant, and because the *pep4*Δ mutant is sensitive to cadmium, it seemed possible that the processing site mutants might also be sensitive to cadmium. However, mutations in $L6_{ins}$ 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 *pep4*Δ 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 *pep4*Δ mutant (21, 45); such pleiotropic effects could indirectly alter Ycf1p function or cellular cadmium levels.

A subset of mutations in $L6_{ins}$ resulted in Ycf1p proteins



B

S288C (SGD)	1213	IIMKAS*LDAGLAG
BY4741	1213	IIMKAS*LDAGLAG
SM1058	1213	IIMKASYLDAGLAG
SM4643	1213	IIMKASYLDAGLAG
SK1	1213	IIMKASYLDAGLAG
Σ1278b	1213	IIMKASYLDAGLAG
S. paradoxus	1212	VIMKASYMDAGLAG
S. mikitae	1244	IVLKAPYLDAGLAG

FIG. 7. Identification of a new full-length MRP type transporter in yeast. (A) The partial ABC transporters Ykr103p and Ykr104p are represented schematically, with subdivisions indicating the predicted domains based on sequence alignments with Ycf1p. Ykr103p is comprised of MSD0, L0, MSD1, NBD1, L1, and the majority of MSD2 (with the possible exception of the last transmembrane span according to Kyte and Doolittle hydropathy analysis), and Ykr104p is comprised of NBD2. The abbreviations for the domains are as described in Fig. 2. The numbers shown by the stop codon (*) for Ykr103p and a potential initiating methionine (M) for Ykr104p correspond to the amino acid positions in a hypothetical full-length ABC transporter encoded by the two ORFs *YKR103w* and *YKR104w*. (B) The amino acid sequence of the Ykr103p and Ykr104p junction is shown for different *S. cerevisiae* strains and for the homologues found in different *Saccharomyces* species, as determined by DNA sequence analysis (BY4741, SM1058, SM4643, SK1, and Σ1278b) or obtained from the SGD website (*S. paradoxus* and *S. mikatae*). The sequence deposited in SGD for S288C and our sequence results for BY4741 both contain a stop codon (*) at amino acid 1219, whereas other *S. cerevisiae* strains, as well as additional "wild" yeast strains, contain a tyrosine (Y) and thus code for a full-length ABC transporter. The region shown for the homologues in *S. paradoxus* and *S. mikatae* was chosen based on sequence alignments with *YKR103*/*104w*. The GenBank accession numbers for *NFT1* in *S. cerevisiae* strains EG123, Σ1278b, and SK1 are AY230264, AY230265, and AY230266, respectively.

that cannot be processed but retain the ability to confer the wild-type level of cadmium resistance (Fig. 5 and Table 3). Thus, proteolytic processing of Ycf1p is clearly not required to activate Ycf1p. Proteolytic processing does not appear to "deactivate" Ycf1p either, since Ycf1pΔL6$_{ins}$ (unprocessed) does not confer a greater resistance to cadmium than wild-type Ycf1p (processed). Thus, processing appears to represent a "neutral" event in terms of Ycf1p activity. Full functionality of split molecules is not unprecedented for ABC transporters, and indeed the natural coexpression of independently encoded ABC modules in bacteria and humans (19, 52), or the artificial coexpression of half, quarter–three-quarter, and other combinations of partial molecules for a variety of eukaryotic ABC proteins (i.e., Ste6p, MRP1, and MRP2) also promotes normal function (2, 3, 7, 14). However, Ycf1p is the only full-length ABC transporter known to undergo processing as a normal part of its life cycle in vivo. Although our studies have not uncovered a function for Ycf1p processing, it is possible that a role may be revealed under as-yet-unexamined physiological condition(s).

Mutations within L6$_{ins}$ affect substrate specificity. Resistance to both cadmium and arsenite is Ycf1p dependent, as evidenced by the sensitivity of a *ycf1*Δ strain to both compounds (Fig. 5) (16, 43, 47). Surprisingly, we found that certain L6$_{ins}$ 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, ΔL6$_{ins}$, 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 L6$_{ins}$ 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* lumenal 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 membrane spans appear to affect substrate specificity (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 L6$_{ins}$ 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 L6$_{ins}$ mutant phenotypes were indistinguishable from wild-type in BY4741, the *ycf1*Δ 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 *ycf1* "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 (Σ1278b and SK1) that differ in lineage from S288C also possess the full-length *NFT1* gene, as do other *Saccharomyces* species (*S. paradoxus* and *S. mikitae*). 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 *ycf1* 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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