The yeast cadmium factor (Ycf1p) is a vacuolar protein involved in resistance to Cd\textsuperscript{2+} and to exogenous glutathione S-conjugate precursors in yeast. It belongs to the superfamily of ATP binding cassette transporters, which includes the human cystic fibrosis transmembrane conductance regulator and the multidrug resistance-associated protein. To examine the functional significance of conserved amino acid residues in Ycf1p, we performed an extensive mutational analysis. Twenty-two single amino acid substitutions or deletions were generated by site-directed mutagenesis in the nucleotide-binding domains, the proposed regulatory domain, and the fourth cytoplasmic loop. Mutants were analyzed phenotypically by measuring their ability to grow in the presence of Cd\textsuperscript{2+}. Expression and subcellular localization of the mutant proteins were examined by immunodetection in vacuolar membranes. For functional characterization of the Ycf1p variants, the kinetic parameters of glutathione S-conjugated leukotriene C\textsubscript{4} transport were measured. Our analysis shows that residues Ile\textsuperscript{711}, Leu\textsuperscript{712}, Phe\textsuperscript{713}, Glu\textsuperscript{927}, and Gly\textsuperscript{1413} are essential for Ycf1p expression. Five other amino acids, Gly\textsuperscript{663}, Gly\textsuperscript{756}, Asp\textsuperscript{777}, Gly\textsuperscript{1306}, and Gly\textsuperscript{1311}, are critical for Ycf1p function, and two residues, Glu\textsuperscript{709} and Asp\textsuperscript{921}, are unnecessary for Ycf1p biogenesis and function. We also identify several regulatory domain mutants in which Cd\textsuperscript{2+} tolerance of the mutant strain and transport activity of the protein are dissociated.

Transporters designated ABC\textsuperscript{1} proteins, or traffic ATPases, are present from micro-organisms to man (1, 2). Eukaryotic ABC proteins are often composed of two homologous halves, each containing a transmembrane domain (TMD) with six predicted membrane-spanning segments, and a NBF that contains the highly conserved Walker A (GXXGXXG(S/T)), Walker B (RX\textsubscript{214}w,yD) (3), and ABC “signature” (LSGXR/K/R) (4) motifs. Walker A and Walker B are common to a wide variety of nucleotide-binding proteins, whereas the signature region, just upstream of the Walker B motif, is distinctive to the ABC family. Midway between the Walker A and B regions lies the “center” region, which does not form a true consensus sequence, although certain residues are conserved among ABC proteins subsets (5). Certain ABC transporters have an additional R domain that serves regulatory functions (6), and others have a large N-terminal hydrophobic region (7). Eukaryotic ABC transporters include the human CFTR, multidrug resistance and MRP1 proteins, and the yeast a-factor transporter (Ste6p), pleiotropic drug resistance (Pdr5p), and Ycf1p proteins (1, 8–10).

Ycf1p is a vacuolar membrane protein that transports Cd\textsuperscript{2+} ions and several drugs as glutathione conjugates (GS-conjugates) with a requirement for ATP hydrolysis, playing a critical role in Cd\textsuperscript{2+} tolerance in the yeast Saccharomyces cerevisiae (11–13). Ycf1p has strong sequence similarity with CFTR and MRP1; in a comparison of all known yeast and human ABC transporters, these three proteins are classified in the same subgroup (5, 14). Ycf1p and MRP1 share with CFTR a region comparable with the R domain in CFTR, which mediates cAMP-dependent regulation of the chloride channel. The sequence identity of these proteins suggests that they probably have similar overall structures. Structure-function analysis of Ycf1p can thus provide valuable insights into the molecular mechanism of transport by ABC proteins.

We selected 22 amino acid residues of Ycf1p for extensive mutational analysis of the protein. Most of the selected residues are conserved in CFTR and MRP1 proteins, and the substitutions or deletions introduced are analogous to reported cystic fibrosis (CF)-associated mutations. Mutations were introduced by site-directed mutagenesis in NBF1, NBF2, the R domain, and ICL4 that joins TMD2 transmembrane segments X and XI. Mutants were analyzed phenotypically by assaying their Cd\textsuperscript{2+} tolerance. The mutant protein expression level was quantitated by immunodetection in vacuolar membranes; those showing expression were functionally characterized by measuring ATP-dependent transport of LTC\textsubscript{4} into vacuolar membrane vesicles. The kinetic parameters for LTC\textsubscript{4} uptake were determined for the wild type and mutants. The results presented here show that the mutations introduced in the highly conserved NBF domains are extremely deleterious for Ycf1p function. In addition, the mutational analysis identified a group of mutants located in the putative R domain in which Cd\textsuperscript{2+} tolerance was dissociated from the in vitro measured transport activity of the protein. We discuss a model for the regulatory role of the R-like domain in transporter function.

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† To whom correspondence should be addressed. Tel.: 34 91 585 4616; Fax: 34 91 585 4657; E-mail: peraso@biomed.iib.ual.es.
‡ The abbreviations used are: ABC, ATP binding cassette transport protein; Cd-GS\textsubscript{2}, bis(glutathionato)Cd\textsuperscript{2+}; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; GS-conjugate, glutathione S-conjugate; GS-X pump, glutathione S-conjugate-transporting ATPase; HA, 12CA5 epitope from human influenza hemagglutinin; ICL, fourth cytoplasmic loop; LTC\textsubscript{4}, glutathione S-conjugated leukotriene C\textsubscript{4}; MES, 4-morpholinolinesulfonic acid; MIC, minimal inhibitory concentration; MRP, multidrug resistance-associated protein; NBF, nucleotide binding fold; probenecid, p-[diisopropylamino]benzoic acid; R, regulatory domain; SD, synthetic growth medium; TMD, transmembrane domain; YCF, yeast cadmium factor; kb, kilobase; HA, hemagglutinin.
YEAST STRAINS AND GROWTH MEDIA—A Ycf1p derivative of S. cerevisiae strain W303–1A (MATa, ycf1Δ:URA3, ade2–1, his3–11, 15, leu2–3, 112, trpl–1, ura3–1), constructed as described below, was used. In all experiments, growth was at 30 °C in SD medium (0.7% yeast nitrogen base without amino acids (U. S. Biological, Swampscott, MA), 2% glucose, pH 5.5) supplemented with required amino acids (100 μg/ml) and 2% glucose. All resistance assays were supplemented with drop-out mix (BIO 101, Joshua Way, Vista, CA).

Disruption of YCF1 Gene—A 1.1-kb BamHI-Smal fragment from pJJ242 (16) containing the URA3 gene, was used to replace a 4.7-kb BglII-Smal (blunt-ended) fragment of pBYCF1 (13), thus eliminating most of the promoter and coding sequence. A 3.5- kb SacI-MluI fragment containing the YCF1 disruption cassette was used to transform strain W303–1A to uracil auxotrophy. Gene disruption was verified by Southern hybridization (17).

Plasmid Constructions—A 7.1-kb fragment from pBYCF1, excised by SphiI (blunted with Klenow fragment) and SacI digestion, containing YCF1 gene was subcloned into Smal-SacI-digested pRS315 (18) to give pRS115YCF1. Digestion with XhoI-MluI, blunt-ended with Klenow fragment, and religating eliminated restriction sites between XhoI-Smal sites in the pRS115 polymerase, giving plasmid pBI1157. Blunt-ended ligation regenerated the MluI site. Plasmid pBI1197 was constructed by insertion of a 4.5-kb EcoRV-SalI fragment from pBYCF1 into pBluescript KS vector (Stratagene, La Jolla, CA), digestion with HindIII, and ligation to generate a 1.1-kb HindIII deletion internal to the EcoRV-SalI fragment.

Site-directed Mutagenesis—The following fragments from YCF1 were subcloned into pBluescript (19) for mutagenesis: (i) 1.4-kb SalI-SphI fragment (epitope tagging); (ii) 2.1-kb StuI-SalI fragment (mutations D777N, R1143C, Q1148P, Q1306E, G1311R, N1366K, G1441D); (iii) 1.1-kb HindIII fragment (remaining mutations). The StuI-SalI fragment was first subcloned into pSL301 (20) and recovered as SacI-SalI to be cloned into M13 mp19. Mutagenesis was performed by the Eckstein method (21) with the Sculptor™ in vitro Mutagenesis System (Amer sham) with the M13mp19 form and exchanged with the corresponding wild type segments of pBI1157, generating pBI1201. The YCF1 1.1-kb HindIII fragments containing the mutations generated were obtained from the replicative form of M13mp19 and inserted into pBI1197. The 4.5-kb EcoRV-SalI fragments from the resultant plasmids were exchanged with the corresponding wild type segments of YCF1-HA in pBI1201. Mutagenized StuI-SalI fragments were liberated from the M13mp19 form and exchanged with the corresponding fragments in pBI1201. All replacements of wild type by mutant fragments were confirmed by sequencing. The 7.1-kb ApaI-SacI fragment from pBI1201 containing the promoter and open reading frame was also cloned into pRS425 (22) to render the YCF1-HA gene on multicopy plasmid. The resulting plasmids were used to transform the Δycf1 strain using the lithium acetate procedure (23).

DNA Sequencing—DNA was sequenced by the dideoxy chain termination method (24) modified for single-stranded and double-stranded template with Sequenase, as described by the enzyme supplier (U. S. Biochemical Corp.).

Cadmium Resistance Assays—Qualitative and quantitative determinations of Cd2+ resistance were performed. Cells were cultured for 2 days and suspended in water to an A600 of 0.4 (2 × 10^7 cells/ml) to be used as inoculum. For qualitative assays, 5 μl were dropped on plates alone or with CdCl2 at the indicated concentrations. Growth was scored after a 3-day incubation. For quantitative determination of the minimal inhibitory concentration (MIC), flat-bottom 96-well microtiter plates containing medium with CdCl2 concentrations ranging from 0 to 1 mM were inoculated to a final cell density of 6 × 10^5 cells/ml. Inoculum-free wells served as controls. The optical density (A600) optical density (A600) was determined after a 2-day incubation. Data were fitted to a sigmoidal dose-response equation using Prism 2.0 GraphPad Software. The MIC value is defined as the lowest Cd2+ concentration at which prominent inhibition of cell growth (90–95%) is observed.

Isolation of Vacuolar Membrane Vesicles—Vacuolar membrane vesicles were prepared from cultures grown to an A600 of 1.0. After washing cells with H2O, spheroplasts were obtained with Zymolyase 20T (U. S. Biological, Swampscott, MA) (25), and intact vacuoles were isolated by flotation centrifugation of spheroplast lysates on Ficoll 400 step gradients as described (26), using a shortened procedure with only one cycle of flotation centrifugation. The resulting vacuole fraction was vesiculated in 5 mM MgCl2, 25 mM KCl, 10 mM Tris-MES, pH 6.9, pelleted by centrifugation (37,000 × g, 25 min), and resuspended in buffer (1 mM glycerol, 2 mM dithiothreitol, 1 mM EGTA, 5 mM Tris-MES, pH 7.6) (11). Dithiothreitol and EGTA were omitted in vacuolar preparations used for transport assays. All buffers used contained a protease inhibitor mixture (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride).

Protein Determination of Ycf1p—Total protein was determined as described (27) using the Bio-Rad protein assay reagent and bovine IgG as standard. SDS-polyacrylamide gel electrophoresis on 7% gels was performed as described (28). Sample solubilization and Western blot analysis were performed as described (29, 30). Reversible protein staining with Ponceau S (31) and immunodetection of Ycf1p- HA using mouse anti-HA monoclonal antibody and a second antibody coupled to alkaline phosphatase (Bio-Rad) was as described (32). Western blots were scanned with a StudioscanII (Agfa-Gevaert, Leverkusen, Germany), and Adobe Photoshop and NIH Image 1.60/1.62 software were used to quantify the Ycf1p amounts. Standard default settings were used for all measurements, and all mutant enzymes were compared with an internal wild type control on the same gel.

Chemicals—[3H]LTC4 (165 Ci/mmol) was obtained from NEN Life Science Products. Unlabeled LTC4 was from Sigma. All other reagents were of analytical grade and purchased from Sigma, Roche Molecular Biochemicals, Amersham Pharmacia Biotech, or U. S. Biological.

RESULTS

Ycf1p-dependent Transport Activity in Vacuolar Membrane Vesicles—YCF1 has been identified as a gene necessary for the detoxification of Cd2+ (13) and shown to be a pump for GS-conjugates (GS-X pump) responsible for the vacuolar sequestration of organic GS-conjugates (11) and GS-cadmium complexes (Cd-GS2-) (12). We chose LTC4 as substrate of Ycf1p since human MRPI, a GS-X pump that transports LTC4 and structurally related conjugates in mammalian cells (33), complements Ycf1p function by restoring Cd2+ resistance in a Δycf1 deletion mutant (34). We measured [3H]LTC4 uptake into vacuolar membrane vesicles from a yeast strain harboring the wild type gene carried on either single or multiple copy plasmid. Incubation of the vacuolar vesicles with 50 nM [3H]LTC4 in the presence of ATP resulted in rapid LTC4 uptake (Fig. 1A). In vesicles from wild type cells expressing YCF1 from a single or a multicopy plasmid, the initial rates in the presence of ATP were 45 and 182 pmol/min/mg of protein, respectively, and 0.1 and 15 pmol/min/mg protein in the absence of ATP. LTC4 uptake in the presence of a nonhydrolyzable ATP analog, adenosine 5′-(3-thiotriphosphate), or into vacuolar preparations from a Δycf1 deletion mutant was negligible under these conditions (not shown). Ycf1p-dependent transport activity correlated with Cd2+ (Fig. 1B) and the amount of Ycf1p (Fig. 1C) of these strains. This indicates that LTC4 uptake is Ycf1p-dependent, allowing the use of quantitative transport assays as an indicator of Ycf1p function.

The effect of LTC4 or ATP concentration on the rate of LTC4 uptake was measured in vacuolar vesicles prepared from a Δycf1 strain expressing the epitope-tagged YCF1-HA from a single copy plasmid. The uptake was saturated with respect to...
Structure-Function Analysis of Ycf1p

Vaccular membrane vesicles (10 μg of protein) were incubated with 50 nM [3H]LTC4, as described under “Experimental Procedures.” Additions were made 5 min before initiation of the uptake reaction. Before addition glutathione (GSH) andCd2+ to the uptake medium, a mixture of 20 mM GSH and 10 mM CdSO4 was incubated in 0.2 M phosphate buffer, pH 8.0, at 45 °C for 24 h to allow Cd · GS to form (12). Azido-phenacyl-GS was added from a Me2SO stock solution. Gramicidin D and carbonyl cyanide m-chlorophenyl hydrazone (CCCP) were added from ethanol stock solutions. Me2SO or ethanol alone (<2%) had no effect on transport activity. The rate for the wild-type strain (100%) was 50 pmol · min⁻¹ · mg of protein⁻¹. Values are the means of two independent experiments differing less than 10%. DIDS, 4,4’-dioxideoanatoctol-bene-2,2’-disulfonate.

| Addition                  | LTC4 uptake % of control |
|---------------------------|--------------------------|
| Control                   | 100                      |
| GSH (1 mM)                | 85                       |
| GSH (1 mM) + Cd2⁺ (0.5 mM)| 55                       |
| Azido-phenacyl-GS (100 μM)| 37                       |
| Decyl-GS (100 μM)         | 40                       |
| Gramicidin D (10 μM) + KCl (50 mM) | 93                 |
| CCCP (100 μM)             | 96                       |
| DIDS (1 mM)               | 0                        |
| Probenecid (5 mM)         | 4                        |

LTC4 and ATP concentrations with an apparent $K_m$ of 0.82 ± 0.14 μM for LTC4 and 55 ± 9.7 μM for ATP. The $V_{max}$ was 1.85 ± 0.12 nmol·min⁻¹·mg of protein⁻¹ (Table III). The results revealed that Ycf1p affinity is higher than LTC4 than for other substrates previously assessed, such as S-(2,4-dinitrophenyl) glutathione ($K_m$ = 14.1 μM) (11) or Cd-GS2 ($K_m$ = 39.1 μM) (12) and with the same order of magnitude as the LTC4 affinity for its physiological transporter, MRP1 ($K_m$ = 0.11 μM) (33).

A large variety of GS-conjugates bearing aliphatic or aromatic S-substitutes inhibit transport activity by ATP-dependent GS-conjugate carriers in membrane vesicles (11, 35, 36). We examined inhibition of LTC4 uptake by different glutathione-related compounds. Glutathione (1 mM GSH) had a weak inhibitory effect, concurring with the low affinity of Ycf1p for this compound (37), whereas 100 μM GS-conjugates such as S-decyglutathione or S-azido-phenacyl glutathione inhibited LTC4 transport by 60–63% (Table I). LTC4 uptake was also inhibited by 45% with 500 μM Cd2⁺ in the presence of 1 mM GSH. Agents that dissipate the H⁻ electrochemical gradient established by the vacuolar H⁻-ATPase did not decrease LTC4 transport significantly. In contrast, DIDS and probenecid, potent inhibitors of anion transporters (36), completely abolished LTC4 uptake at the concentrations used. These results show that the energy-dependent LTC4 uptake is not linked to the vacuolar electrochemical H⁻ gradient and has properties similar to those of GS-conjugate transport by other GS-X pumps.

Selection of Amino Acids for Site-directed Mutagenesis—Individual Ycf1p amino acids were selected for site-directed mutagenesis with the following criteria. In the NBF domains and ICL4 loop, residues highly conserved in ABC proteins were selected. In the less well conserved R-like domain, the selected amino acids were conserved at least in Ycf1p and CFTR. In all cases, the amino acid changes introduced are those described as CF-associated mutations (38). Alignment of the conserved regions of the N- and C-terminal NBF domains and the ICL4 sequence of CFTR, MRP1, and Ycf1p are shown in Fig. 2A; Fig. 2B shows all substitutions introduced in Ycf1p and their localization within the protein. We generated 11 mutations in Ycf1p NBF1 and NBF2 domains. Two other mutations, R1143C and Q1148P, are localized in a protein region homologous to the ICL4 in MRP1 and CFTR. ICL4 is suggested to couple activity of the NBF domains to channel gating in...
The R domain of CFTR contains several consensus phosphorylation sites for cyclic AMP-dependent protein kinase A and for protein kinase C, thought to be important for regulation of its activity (40). The N-terminal part of this domain has been highly conserved during evolution, whereas the C-terminal two-thirds is poorly conserved (41). As mutagenesis of the putative protein kinase A phosphorylation site in the R-like domain of Ycf1p abolishes Cd\textsuperscript{2+} resistance, Ycf1p is proposed to require a similar domain for normal function, but the manner in which this region participates in Ycf1p activity has not been established (13). To determine the role of the R domain, we introduced six mutations in the highly conserved N-terminal part of the domain (L817S, D821G, L825T, L826S, G835R, and I840P) and three in the less conserved C-terminal part (Y855L, A910G, and E927K). The activity of the Ycf1p variants was evaluated by measuring Cd\textsuperscript{2+} tolerance and Ycf1p-mediated LTC\textsubscript{4} transport in vacuolar membrane vesicles.

**Mutant ycf1 Strains Growth on Cadmium Medium—**Yeast transformants carrying the ycf1 mutant alleles either in the wild type strain (Fig. 3), with MIC ranging from 111 to 172 \( \mu \text{M} \), with 200 \( \mu \text{M} \) the MIC of the wild type strain (Table II). These mutations are all located in the NBF domains except for E927K, in the R domain. Strains expressing ycf1 mutations L817S, L825T, L826S, G835R, I840P, Y855L, A910G, R1143P, and Q1148P were more sensitive to Cd\textsuperscript{2+} than the wild type strain (Fig. 3), with MIC ranging from 111 to 172 \( \mu \text{M} \), with 200 \( \mu \text{M} \) the MIC of the wild type strain (Table II). These mutations are all located in the R-like domain and ICL4. Only two substitutions, E709Q and D821G in NBF1 and R domains, respectively, did not modify Ycf1p in vivo activity, since a wild type growth pattern was observed in yeast expressing these mutant enzymes.

**Effect of ycf1 Mutations on Subcellular Protein Localization—**Ycf1p is an integral protein of the vacuolar membrane (11). Intracellular sorting of the protein or its turnover may be affected by mutations, contributing to the changes observed in Cd\textsuperscript{2+} resistance of the mutants. We estimated the relative amount of mutant Ycf1p in the vacuolar membrane by quantitative immunoblotting (Table II). Four of the mutant proteins, I711S, ΔL712, A7F713, G756D, D777N, E927K, G1306E, G1311R, N1366K and G1413D, gave significantly lower yields of protein expression compared with the wild type control, which may explain their decreased capacity for Cd\textsuperscript{2+} resistance.

**FIG. 2. Location of the mutations introduced into YCF1.** A, alignment of the highly conserved regions of the NBF domains, Walker A (center), ABC (signature), and Walker B, and the intracellular loop ICL4 of CFTR, MRP1, and Ycf1 proteins. The amino acid sequences are numbered according to their positions in the proteins. Residues associated with CF mutations in CFTR are shown by arrows, and residues mutated in Ycf1p are boxed. B, the amino acid substitutions introduced by site-directed mutagenesis and their location are indicated in the predicted model for the domain structure of Ycf1p based on the structural model proposed in (7). ICL, cytoplasmic loop. Domains are numbered according to CFTR domains. In the additional TMD\textsubscript{4} domain, four transmembrane segments have been represented, but it is predicted to contain 4–6 segments.

CFTR (39), and a cluster of CF mutations have been found in this loop.

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Structural-Function Analysis of Ycf1p

FIG. 3. Cadmium resistance of wild type and mutant ycf1 strains. Cells of a Δycf1 yeast strain harboring the centromeric plasmid pRS315 alone (Δycf1) or carrying the wild type YCF1-HA gene (wt) or the ycf1 mutant alleles created by site-directed mutagenesis (indicated by the position of the mutated residue) were suspended in water to an A600 of 0.4, and 5 μl were spotted onto SD drop-out plates containing the indicated CdCl2 concentrations. Growth was scored after a 3-day incubation at 30 °C for Cd2+ concentrations. Growth was scored after a 3-day incubation at 30 °C for Cd2+ plates and a 1-day incubation for the control plate. The amino acid changes introduced in the positions indicated are shown in Fig. 2.

**TABLE II**

**Characteristics of ycf1 mutants**

| Mutation  | Localization | CdCl2 MIC (μM) | Relative amount of Ycf1p% | LTC4 uptake (pnmol·min⁻¹·mg⁻¹) |
|-----------|--------------|----------------|---------------------------|--------------------------------|
| Wild type |              | 200            | 100                       | 54                             |
| Δycf1     |              | 26             | 0                         | <1                             |
| G663V     | NBF1 (A)     | 27             | 90                        | <1                             |
| E709Q     | NBF1 (C)     | 200            | 100                       | 53                             |
| Φ711S     | NBF1 (C)     | 27             | 0                         | ND                             |
| ΔL712     | NBF1 (C)     | 26             | 0                         | ND                             |
| G756D     | NBF1 (S)     | 30             | 0                         | ND                             |
| G756D     | NBF1 (S)     | 25             | 100                       | <1                             |
| D777N     | NBF1 (B)     | 30             | 110                       | 18                             |
| L817S     | R             | 113            | 100                       | 45                             |
| D821G     | R             | 200            | 102                       | 53                             |
| L825T     | R             | 130            | 108                       | 64                             |
| L825T     | R             | 165            | 97                        | 38                             |
| G835R     | R             | 120            | 80                        | 32                             |
| L840P     | R             | 111            | 7                         | 36                             |
| Y855L     | R             | 130            | 103                       | 92                             |
| A910G     | R             | 158            | 93                        | 83                             |
| E927K     | R             | 32             | 0                         | ND                             |
| R1143C    | ICL4          | 137            | 49                        | 39                             |
| Q1148P    | ICL4          | 172            | 70                        | 44                             |
| G1306E    | NBF2 (A)     | 38             | 220                       | <1                             |
| G1311R    | NBF2 (A)     | 30             | 89                        | <1                             |
| N1366K    | NBF2 (C)     | 36             | 25                        | 16                             |
| G1413D    | NBF2 (S)     | 24             | 0                         | ND                             |

a. Conserved segments within the NBF domains are indicated: A, Walker A; B, Walker B; C, center region; S, ABC signature.

* MICs for Cd2+ were determined as described under “Experimental Procedures.” Values are the average of independent duplicate experiments.

**TABLE III**

| Mutation  | VT concentrations (μM) | LTC4 Uptake (pnmol·min⁻¹·mg⁻¹) |
|-----------|------------------------|-------------------------------|
| Wild type | 0                      | 54                            |
| Δycf1     | 50                     | 92                            |
| G663V     | 100                    | 15                            |

**CdCl2 (μM)**

0 50 100

detoxification, although an alteration in protein activity cannot be excluded (see below). The remaining mutant proteins, G663V, E709Q, G756D, D777N, L817S, D821G, L825T, L826S, G835R, Y855L, A910G, G1306E, and G1311R, reached the vacuolar membrane at levels close to those of the wild type control or even higher, as was the case for G1306E (Table II). Within this group, only E709Q and D821G mutants exhibited wild type phenotype for Cd2+ resistance, in agreement with their wild type Ycf1p amount in the vacuole. In the other cases, an alteration in protein activity may account for Cd2+ toxicity.

LTC4 Uptake in Vacuolar Membrane Vesicles from ycf1 Mutants—To measure the magnitude of change introduced by the mutations on the transport activity of the protein, we measured LTC4 uptake into vacuolar membrane vesicles by the mutant proteins. A group of mutants was found to have significantly altered LTC4 uptake (Table II). Of these, four mutations (G663V, G756D, G1306E, and G1311R) completely abolished transport activity, whereas six of the Ycf1p variants (D777N, L826S, G835R, I840P, R1143C, and N1366K) showed a partial reduction in LTC4 uptake with values ranging from 30% (N1366K) to 72% (R1143C) of the wild type control. Two mutant proteins, Y855L and A910G, showed values above wild type, whereas the transport rate of the five remaining mutant enzymes was found to be essentially wild type. In all cases in which LTC4 uptake was detectable, the kinetic transport parameters were determined (Table III). The apparent Km for LTC4 or ATP of all mutant enzymes was not significantly changed, with the exception of the D777N variant, which had a 25-fold increase in the Km for ATP; thus, transport alterations were due to Vmax modifications. In mutants exhibiting impaired transport activity, the changes in LTC4 transport paralleled the observed defective Cd2+ detoxification. Nevertheless, mutant enzymes showing either wild type (E709Q, L817S, D821G, and L825T) or above wild type Vmax values (Y855L and A910G) all had diminished ability to detoxify Cd2+ ions, except for E709Q and D821G (Fig. 3 and Table II). These results indicate that Leu117, Leu225, Tyr265, and Ala310 are nonessential residues for correct intracellular sorting and LTC4 transport activity of Ycf1p but are important for Cd2+ resistance. When transport activity was corrected for the relative amount of Ycf1p in the vacuolar membrane, it became evident that not only mutants expressing the Y855L and A910G mutations, but also those carrying I840P and R1143C changes, showed a gain-of-function phenotype with respect to LTC4 uptake when Cd2+...
two mutants could thus be due to their defect in protein
in two different vacuolar membrane preparations isolated independently. Changes over 30% are considered to be significant.

pre1pre2

undetectable even in a

proximal mutations I711S and

D

(HisP) NBF domain points to other residues as the putative
resolution of the
tested in any ABC protein. Moreover, recent crystal structure
this particular change on transporter activity has not been

overexpression, as shown for other membrane proteins (49),
cannot be discarded. The inability of mutations I711S, AL712,
and ΔP713 to confer Cd2\(^{2+}\) resistance is clearly caused by a
biogenesis defect of the corresponding proteins, as is also the
case for mutations E927K and G1413D. Two other mutants
showing reduced protein expression, I840P and R1143C, were
also affected in LTC4 transport and have been included in
group 4. In the case of mutants R1143P and N1366K, protein
biogenesis was altered, but they retained fully active LTC4
transport activity.

Group 3 includes mutant enzymes that, although correctly located,
evitably impaired Ycf1p Cd2\(^{2+}\) tolerance and LTC4 transport
function. Mutations of this group localized in the NBF
domains completely abolished (G663V, G756D, G1306E, and
G1311R) or severely damaged (D777N) Ycf1p function,
whereas mutations in the R-like domain (L826S and G835R)
had a milder effect. Such detrimental mutations include sub-
stitutions in the conserved Walker A, Walker B, and signature
motifs of NBF1 and the Walker A motif of NBF2, corroborating
their importance in the ABC transporter function. Mutant
D777N shows a much lower affinity for ATP than the wild type
Ycf1p. This residue has been proposed, based on HisP crystal
structure (44), to interact with magnesium during ATP hydro-
lysis. Identical changes at corresponding positions in the signa-
ture region of NBF1 and NBF2, G756D, and G1413D produced
quite distinct phenotypes. Mutant protein G756D was present
in the vacuole at wild type levels but inactive, whereas G1413D
was undetectable. The equivalent substitutions to several of
three NBF mutations in other ABC proteins, CFTR (50),
multidrug resistance protein (51, 52), Ste6p (53), or HisP (54),
also give rise to normally expressed although nonfunctional
mutant proteins. In contrast, the analogous changes to L826S
and G835R in the N-terminal part of the R-like domain have
only been characterized in CFTR, resulting in aberrant pro-
cessing (55).

Group 4 is of particular interest and includes mutants in
which Cd2\(^{2+}\) resistance and LTC4 transport activity were disso-
ciated. Cd2\(^{2+}\) tolerance of some R domain or ICL4 mutants
were reduced as compared with the wild type strain, but their trans-
port activity was near wild type, with variations lower than
30% (L817S and L825T) or even increased above wild type
(Y855L and A910G). The gain-of-function effect also became

resistance was affected. The decreased Cd2\(^{2+}\) resistance of these
two mutants could thus be due to their defect in protein
biogenesis.

**DISCUSSION**

We performed a mutational analysis of Ycf1p, a yeast hom-
olog of both human CFTR and MRP1 transporters, generating
22 mutations by site-directed mutagenesis. Mutant phenotypes
were characterized by measuring growth in the presence of
Cd2\(^{2+}\) ions, the relative amount of mutant protein in the vacu-
olar membrane, and Ycf1p-dependent transport in vacuolar
membrane vesicles.

For the sake of discussion, the mutants described here can be
divided into four groups. Group 1 includes mutants E709Q and
D821G, which showed essentially wild type phenotype for the
three parameters tested. These residues can be assumed to be
relatively unimportant for Ycf1p biogenesis and function.
Interestingly, the Glu\(^{709}\) residue lies in the position that corre-
sponds to Glu\(^{504}\) of CFTR, predicted to be the NBF1 catalytic
carboxylate residue involved in ATP hydrolysis by comparison
with the β subunit of F1-ATPase (42, 43). Although CFTR muta-
tion E504Q is associated to CF, to our knowledge the effect of
this particular change on transporter activity has not been
tested in any ABC protein. Moreover, recent crystal structure
resolution of the *Salmonella typhimurium* histidine permease
(HisP) NBF domain points to other residues as the putative
activating base (44).

Group 2 consists of mutant enzymes defective in biogenesis.
Mutants of particular interest in this group are those located in
the center region of NBF1 (I711S, AL712, ΔP713) and NBF2
(N1366K). The equivalent CFTR changes, ΔF508 and N1303K,
are frequent CF-associated mutations that have been described
as processing mutations (6, 45). In fact, mutant protein ΔF508 is
retained in the endoplasmic reticulum membrane and de-
graded by the ubiquitin-proteasome system (46, 47). The intro-
duction into YCF1 of the analogous mutation, ΔP713, or the
proton-motive mutations I711S and AL712 led to mutant proteins
undetectable even in a pre1pre2 mutant strain defective in the
benzymotysip-like activity of the proteasome (data not shown).
These results suggest that an alternative pathway is used in
yeast to eliminate aberrant Ycf1p proteins. Other groups (13, 48)
found that mutant ΔP713 protein expressed in a multicopy
plasmid was mislocalized, although a mislocalization due to

**TABLE III**

Effect of ycf1 mutations on the kinetic parameters of LTC4 uptake in vacuolar membrane vesicles

| Mutation | Kn (LTC4) | Km (ATP) | Vmax | Vmax (normalized) |
|----------|-----------|----------|------|-----------------|
| Wild type | 0.82 ± 0.14 | 55 ± 10 | 0.05  | 1.48            |
| E709Q    | 0.72 ± 0.18 | 55 ± 10 | 0.05  | 1.48            |
| D777N    | 0.81 ± 0.20 | 1383 ± 246 | 0.07  | 1.48            |
| L817S    | 0.73 ± 0.14 | 42 ± 16 | 0.05  | 1.48            |
| L825T    | 0.81 ± 0.28 | 55 ± 10 | 0.05  | 1.48            |
| L826S    | 0.67 ± 0.21 | 60 ± 10 | 0.05  | 1.48            |
| G835R    | 0.79 ± 0.15 | 60 ± 11 | 0.05  | 1.48            |
| R1143C   | 0.63 ± 0.15 | 50 ± 9  | 0.05  | 1.48            |
| Q1415P   | 0.76 ± 0.26 | 48 ± 7  | 0.05  | 1.48            |
| N1366K   | 0.85 ± 0.13 | 59 ± 6  | 0.05  | 1.48            |

\( ^{a} \) Apparent Kn for LTC4 at 4 mM ATP. The initial rate of LTC4 uptake in vacuolar membrane vesicles was assayed with LTC4 concentrations from 0.1 to 3 \( \mu \text{M} \), as described under “Experimental Procedures.”

\( ^{b} \) Data were fitted to the Michaelis-Menten equation using Prism 2.0 GraphPad Software. Values are the average ± S.D. from two determinations in two different vacuolar membrane preparations isolated independently. Changes over 30% are considered to be significant.

\( ^{c} \) Apparent Kn for ATP at 50 nM LTC4. The initial rate of LTC4 uptake in vacuolar membrane vesicles was assayed with ATP concentrations from 0.35 to 6 mM, as described under “Experimental Procedures.”

\( ^{d} \) The uptake values were normalized on the basis of the amount of mutant protein detected in the vacuolar membrane fractions relative to the wild-type control.
evident for I840P and R1143C mutants when transport activity was corrected for the relative amount of Ycf1p. A similar gain-of-function phenotype has been observed in CFTR R domain mutants H620Q and A800G (55).

Several hypotheses can be considered to explain this striking phenotype. A simple explanation would be that the limiting step in Cd\(^{2+}\) detoxification is not transport into the vacuole but an earlier event such as complexation with GSH. In this case, an increase in GS-X pump activity would not lead to an increase in Cd\(^{2+}\) tolerance. This hypothesis is discarded based on the finding that Ycf1p overexpression led to an increase in LTC\(_4\) transport and to a parallel increase in Cd\(^{2+}\) tolerance of the yeast strain (Fig. 1). Affinity or specificity changes of the transporter do not seem probable, since the \(K_M\) for LTC\(_4\) or ATP were not modified in these mutants, and LTC\(_4\) uptake in mutant vacuolar vesicles was inhibited by Cd\(^{2+}\) plus GSH or azidoephosphoryl-GS to the same extent as in wild type vesicles (data not shown). In all these mutants, only an increase in the \(V_{max}\) of LTC\(_4\) transport was detected. The observed gain-of-function phenotype for LTC\(_4\) transport of some R domain and ICL4 loop mutants may be due to the release from an inhibitory interaction between these two protein regions, leading to the observed increase in the \(V_{max}\) in CFTR and other ABC proteins, it has been speculated that the intracellular loops may link the NBFs to the TMDs to couple ATP binding and hydrolysis to the transporter activity (39, 56, 57). This coupling may be modulated by the R-like domain in Ycf1p. Intragenic suppressor analysis of R domain and/or ICL4 loop mutations will shed light on the proposed domain interaction. This hypothesis would account for the augmented LTC\(_4\) uptake in vacuolar membrane vesicles but would not explain the reduced Cd\(^{2+}\) resistance observed \(in vivo\) in group 4 mutants. The necessary functional coupling between Cd-GS\(_2\) synthesis and their efficient transport into the vacuole may have been lost in these mutants. It is tempting to speculate that the R domain may interact \(in vivo\) both with ICL4 and with a protein that would couple synthesis of GS-conjugates to transport. Interaction of the R domain with such a protein would lead to a disruption in its interaction with ICL4, with an increase of the transport activity of the protein. The similarity in properties of many of the mutants studied to those of their counterparts in mammalian ABC proteins leads to the expectation that the mechanistic information gathered for yeast Ycf1p may be extrapolated to their human homologs.

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