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A new pathway for vacuolar cadmium sequestration in *Saccharomyces cerevisiae*: YCF1-catalyzed transport of bis(glutathionato)cadmium

(ATP binding cassette transport protein/glutathione *S*-conjugate transporter/yeast cadmium factor protein/vacuolar membrane)

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ABSTRACT **The yeast cadmium factor (*YCF1*) gene encodes an MgATP-energized glutathione *S*-conjugate transporter responsible for the vacuolar sequestration of organic compounds after their *S*-conjugation with glutathione. However, while *YCF1* was originally isolated according to its ability to confer resistance to cadmium salts, neither its mode of interaction with Cd^{2+} nor the relationship between this process and organic glutathione-conjugate transport are known. Here we show through direct comparisons between vacuolar membrane vesicles purified from *Saccharomyces cerevisiae* strain DTY167, harboring a deletion of the *YCF1* gene, and the isogenic wild-type strain DTY165 that YCF1 mediates the MgATP-energized vacuolar accumulation of Cd·glutathione complexes. The substrate requirements, kinetics and Cd^{2+}/glutathione stoichiometry of cadmium uptake and the molecular weight of the transport-active complex demonstrate that YCF1 selectively catalyzes the transport of bis(glutathionato)cadmium ($Cd{\cdot}GS_2$). On the basis of these results—the Cd^{2+} hypersensitivity of DTY167, versus DTY165, cells, the inducibility of YCF1-mediated transport, and the rapidity and spontaneity of $Cd{\cdot}GS_2$ formation—this new pathway is concluded to contribute substantially to Cd^{2+} detoxification.**

A new class of ATP-binding cassette (ABC) transporter responsible for MgATP-energized transport of organic compounds after their conjugation with glutathione (GSH) has recently been discovered. Formerly designated the GS-*X* pump (1), this transporter, or family of transporters, has been implicated in the extrusion of a broad range of *S*-conjugated compounds from the cytosol.

To date, two closely related GS-*X* pumps have been identified molecularly. These are the human multidrug resistance-associated protein (MRP1) (2, 3) and the yeast cadmium factor (YCF1) protein (4, 5). MRP1 and YCF1 are 43% identical (63% similar) at the amino acid level, possess nucleotide binding folds with an equivalent spacing of conserved residues, and contain two subclass-specific structures, a central truncated cystic fibrosis transmembrane conductance regulator-like "regulatory" domain, rich in charged amino acids, and an ≈200-amino acid residue N-terminal extension (2, 4). MRP1 catalyzes the MgATP-energized transport of leukotriene C_4 and related GSH *S*-conjugates (GS-conjugates) across the plasma membrane of mammalian cells (3, 6, 7). YCF1 catalyzes the transport of organic GS-conjugates into the vacuole of *Saccharomyces cerevisiae* (5).

Given the participation of both of these integral membrane proteins in the transport of organic GS-conjugates and their implied role in the elimination and/or sequestration of cytotoxic drugs, it is intriguing that the *YCF1* gene was initially identified by screening a yeast genomic library for the ability of multicopy DNA fragments to confer resistance to cadmium salts in the growth medium (4). The question of how the vacuolar sequestration of organic GS-conjugates by YCF1 is related to Cd^{2+} resistance therefore arises. Specifically, is the detoxification of Cd^{2+} by YCF1 dependent on its interaction with GSH, as is the case for organic xenobiotics (5) and, if so, how does GSH exert its effects?

In this communication we address these questions to show that YCF1 is not only competent in the MgATP-energized transport of organic GS-conjugates but also Cd^{2+} after its complexation with GSH. Our findings demonstrate a new pathway for the vacuolar sequestration of Cd^{2+} in *S. cerevisiae*: YCF1-mediated transport of bis(glutathionato)cadmium ($Cd{\cdot}GS_2$).

MATERIALS AND METHODS

Yeast Strains. The two strains of *S. cerevisiae* used in these studies—DTY165 (*MATα ura3-52 his6 leu2-3,-112 his3-Δ200 trp1-901 lys2-801 suc2-Δ*) and the isogenic *ycf1Δ* mutant strain, DTY167 (*MATα ura3-52 his6 leu2-3,-112 his3-Δ200 trp1-901 lys2-801 suc2-Δ, ycf1::hisG*)—were manipulated as described (5, 8).

Isolation of Vacuolar Membrane Vesicles. Vacuolar membrane vesicles were prepared as described (5), except that the dithiothreitol (1 mM) and EGTA (1 mM) present in the standard membrane isolation medium (5, 9) were removed to prevent the attenuation of YCF1-dependent Cd^{2+} transport otherwise exerted by these compounds (see *Discussion*). Vesiculated vacuolar membranes were subjected to three cycles of 50-fold dilution into simplified suspension medium (1.1 M glycerol/5 mM Tris·Mes, pH 8.0), centrifugation at 100,000 × *g* for 35 min, and resuspension in the same medium before use.

Purification of Cadmium·Glutathione Complexes. Singly radiolabeled $^{109}Cd{\cdot}GS_n$ and doubly radiolabeled $^{109}Cd{\cdot}[^3H]GS_n$ complexes were prepared by sequential gel-filtration and anion-exchange chromatography of the reaction products generated by incubating 20 mM $^{109}CdSO_4$ (78.4 mCi/mmol; 1 Ci = 37 GBq) with 40 mM GSH or 40 mM

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Abbreviations: GSH, glutathione; GS-conjugate, GSH *S*-conjugates; Cd·GS, mono(glutathionato)cadmium; $Cd{\cdot}GS_2$, bis(glutathionato)cadmium; CDNB, 1-chloro-2,4-dinitrobenzene; DNP-GS, *S*-(2,4-dinitrophenyl)glutathione; MALD-MS, matrix-assisted laser desorption mass spectrometry; MRP1, human multidrug resistance-associated protein; YCF1, yeast cadmium factor protein; GSSG, oxidized GSH; HMW, high molecular weight; LMW, low molecular weight.

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[^{3}H]GSH (240 mCi/mmol) in 15 ml 10 mM phosphate buffer (pH 8.0) containing 150 mM KNO_3 at 45°C for 24 h. For gel-filtration, 2 ml aliquots of the reaction mixture were applied to a column (40 × 1.5 cm interior diameter) packed with water-equilibrated Sephadex G-15, eluted with deionized water, and ^{109}Cd and/or ^{3}H in the fractions was measured by liquid scintillation counting. The fractions encompassed by each of the two $^{109}Cd{\cdot}GS_n$ peaks identified were pooled, lyophilized, and redissolved in 4 ml of loading buffer (5 mM Tris·Mes, pH 8.0). For anion-exchange chromatography, 0.5 ml aliquots of the resuspended lyophilizates from gel-filtration chromatography were applied to a Mono-Q HR5/5 column (Pharmacia) equilibrated with the same buffer. Elution was with a linear gradient of NaCl (0.5 ml/min; 0–500 mM) dissolved in loading buffer. The individual fractions corresponding to the major peaks of ^{109}Cd obtained from the Mono-Q column (one each for the peaks resolved by gel-filtration chromatography) were pooled, lyophilized, and resuspended in 4 ml deionized water after liquid scintillation counting. Buffer salts were removed before transport measurements or mass spectrometry by passing the samples down a column (120 × 1.0 cm interior diameter) packed with water-equilibrated Sephadex G-15.

Measurement of $^{109}Cd^{2+}$ Uptake. MgATP-energized, uncoupler-insensitive $^{109}Cd^{2+}$ uptake by vacuolar membrane vesicles was measured at 25°C in 200 μl reaction volumes containing 3 mM ATP, 3 mM $MgSO_4$, 5 μM gramicidin-D, 10 mM creatine phosphate, 16 units/ml creatine phosphate kinase, 50 mM KCl, 400 mM sorbitol, and 25 mM Tris·Mes (pH 8.0), and the indicated concentrations of $^{109}CdSO_4$, GSH, or ^{109}Cd- and/or ^{3}H-labeled purified $Cd{\cdot}GS_n$ complexes as described (5) except that the wash media contained 100 μM $CdSO_4$ in addition to sorbitol (400 mM) and Tris·Mes (3 mM, pH 8.0).

Pretreatment of DTY165 Cells with Cd^{2+} or 1-Chloro-2,4-Dinitrobenzene (CDNB). For studies of the inducibility of *YCF1* expression and YCF1-dependent transport, DTY165 cells were grown in yeast extract/peptone/dextrose (YPD) medium (8) for 24 h at 30°C to an OD_{600} of 1.0–1.2, pelleted by centrifugation and resuspended in fresh YPD medium containing $CdSO_4$ (200 μM) or CDNB (150 μM). After washing in distilled water, total RNA was extracted and vacuolar membrane vesicles were prepared from the pretreated cells. Control RNA and membrane samples were prepared from DTY165 cells treated in an identical manner except that $CdSO_4$ and CDNB were omitted from the second incubation cycle.

RNase Protection Assays. Cd^{2+} and CDNB-elicited increases in *YCF1* mRNA levels were assayed by RNase protection using 18S rRNA as an internal control. *YCF1*-specific probe was generated by PCR amplification of the full-length *YCF1*::*HA* gene, encoding human influenza hemagglutinin 12CA5 (HA) epitope-tagged YCF1, using plasmid pYCF1-HA (5) as template. The forward, *YCF1*-specific, primer and backward primer, containing the HA-tag coding sequence, had the sequences 5′-AAACTCGAGATGGCTG-GTAATCTTGTTTC-3′ and 5′-GCCTCTAGATCAAGCG-TAGTCTGGGACGTCGTATGGGTAATTTTCAT-TGA-3′, respectively. 18S rRNA-specific probe was synthesized by PCR of *S. cerevisiae* genomic DNA using sense and antisense primers with the sequences 5′-AGATTAAGCCAT-GCATGTCT-3′ and 5′-TGCTGGTACCAGACTTGC-CCTCC-3′, respectively. Both PCR products were individually subcloned into pCRII vector (Invitrogen) to generate plasmids pCR-YCF1 and pCR-Y18S. After linearization of pCR-YCF1 and pCR-Y18S with *Afl*I and *Nco*I, a 320-nucleotide *YCF1*-specific RNA probe and 220-nucleotide 18S rRNA-specific probe were synthesized using T7 RNA polymerase and SP6 RNA polymerase, respectively. Aliquots of total RNA, prepared as described (10), from control, $CdSO_4$-, or CDNB-pretreated DTY165 cells were hybridized with a mixture of ^{32}P-labeled *YCF1* antisense probe (1×10^6 cpm) and 18S rRNA antisense probe (5×10^2 cpm), and RNase protection (11) was assayed using an RPAII kit (Ambion).

Matrix-Assisted Laser Desorption Mass Spectrometry (MALD-MS). The $^{109}Cd{\cdot}GS_n$ complexes purified by gel-filtration and anion-exchange chromatography were adjusted to a final concentration of 2–5 mM (as Cd) with deionized water, mixed with an equal volume of sinapinic acid (10 mg/ml) dissolved in acetonitrile/H_2O/trifluoroacetic acid (70:30:0.1%, vol/vol) and applied to the ion source of a PerSeptive Biosystems (Cambridge, MA) Voyager RP Biospectrometry Workstation. The instrument, which was equipped with a 1.3-m flight tube and variable two-stage ion source set at 30 kV, was operated in linear mode. Mass/charge (m/z) ratio was measured by time-of-flight after calibration with external standards.

Protein Assays. Protein was estimated by a modification of the method of Peterson (12).

Chemicals. [^{3}H]GSH [(*glycine*-2-^{3}H)-L-Glu-Cys-Gly; 4.4 Ci/mmol] was from DuPont/NEN, and $^{109}CdSO_4$ (78.44 Ci/mmol) was from Amersham. All other reagents were of analytical grade and purchased from Fisher, Fluka, Research Organics, or Sigma or synthesized as described (5).

RESULTS

***ycf1*Δ Mutants Are Defective in GSH-Dependent Cd^{2+} Transport.** Physiological (1 mM) concentrations of GSH (13) promoted Cd^{2+} uptake by vacuolar membrane vesicles purified from the wild-type strain DTY165 but not the *ycf1*Δ mutant strain DTY167 (Fig. 1). Addition of Cd^{2+} (80 μM) to GSH-containing media elicited MgATP-dependent, uncoupler-insensitive $^{109}Cd^{2+}$ uptake rates of 4.5 and 0.8 nmol/mg per min by DTY165 and DTY167 membranes, respectively (Fig. 1 *A* and *B*). Uptake by DTY165 membranes was diminished more than 9-fold by the omission of GSH (Fig. 1*A*) whereas uptake by DTY167 membranes was slightly stimulated (Fig. 1*B*).

GSH maximally stimulated uptake within minutes ($t_{1/2} < 5$ min) of the addition of Cd^{2+} to the uptake medium (data not shown) and uptake was sigmoidally dependent on Cd^{2+} concentration, achieving half-maximal velocity at 120 μM (Fig. 1*C*).

Specific Requirement for GSH. The stimulatory action of GSH was abolished by the omission of MgATP from the assay medium (Fig. 1 and Table 1), and 1 mM concentrations of oxidized GSH (GSSG), *S*-methylglutathione, cysteinylglycine, cysteine, or glutamate did not promote MgATP-dependent, uncoupler-insensitive Cd^{2+} uptake by vacuolar membrane vesicles from either strain (Table 1).

Purification of Transport-Active Complex. To determine the mode of action of GSH and the form in which Cd^{2+} is transported, reaction mixtures initially containing Cd^{2+} and GSH were fractionated and YCF1-dependent uptake was assayed.

Incubation of $^{109}Cd^{2+}$ with GSH and gel-filtration of the mixture on Sephadex G-15 yielded two major ^{109}Cd-labeled peaks: a low molecular weight peak [LMW-mono(glutathionato)cadium (Cd·GS)] and a high molecular weight peak (HMW-Cd·GS) (Fig. 2*A*). When rechromatographed on Mono-Q, LMW-Cd·GS and HMW-Cd·GS eluted at 0 (Fig. 2*C*) and 275 mM NaCl, respectively (Fig. 2*B*). Of these two ^{109}Cd-labeled components, HMW-Cd·GS, alone, underwent YCF1-dependent transport. MgATP-dependent, uncoupler-insensitive HMW-^{109}Cd·GS uptake by DTY165 membranes increased as a single Michaelian function of concentration (K_m, 39.1 ± 14.1 μM; V_{max}, 157.2 ± 30.4 nmol/mg/10 min) (Fig. 3*A*). By contrast, uptake of LMW-^{109}Cd·GS by DTY165 membranes was negligible at all of the concentrations ex-

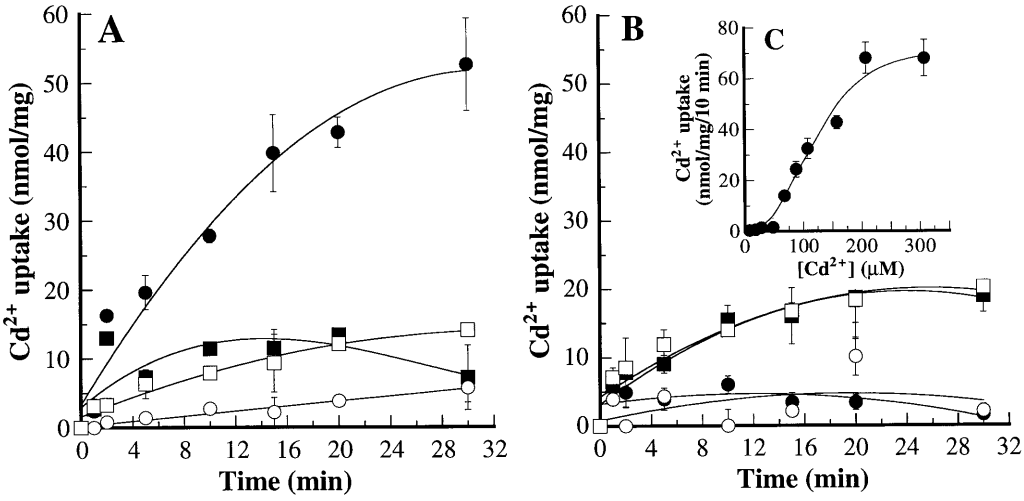

FIG. 1. Uptake of Cd^{2+} into vacuolar membrane vesicles purified from DTY165 and DTY167 cells. Uptake of $^{109}Cd^{2+}$ by DTY165 membranes (*A*) or DTY167 membranes (*B*) was measured in the absence of MgATP plus (○) or minus GSH (1 mM) (□) or in the presence of MgATP (3 mM) plus (●) or minus (■) GSH. $^{109}CdSO_4$ and gramicidin-D were added at concentrations of 80 μM and 5 μM, respectively. (*C*) Rate of $^{109}Cd^{2+}$ uptake by DTY165 membranes plotted as a function of the total concentration of Cd^{2+} ([Cd^{2+}]) added to uptake media containing 1 mM GSH, 3 mM MgATP, and 5 μM gramicidin-D. Values shown are means ± SE (n = 3–6).

amined (Fig. 3*B*). Vacuolar membranes from DTY167 cells transported neither HMW-^{109}Cd·GS nor LMW-^{109}Cd·GS (Fig. 3).

Cd·GS_2 Is the Transport-Active Complex. The transport-active complex, HMW-Cd·GS, was identified as Cd·GS_2 by three criteria. (*i*) The average Cd/GS molar ratio of the transported species, estimated from the $^{109}Cd/^{3}H$ ratios of the HMW-Cd·GS peaks obtained after chromatography of reaction mixtures initially containing $^{109}Cd^{2+}$ and [^{3}H]GSH on Sephadex G-15 and Mono-Q were 0.44 ± 0.09 and 0.49 ± 0.17, respectively (Table 2). (*ii*) DTY165 membranes accumulated ^{109}Cd and [^{3}H]GS in a molar ratio of 0.49 ± 0.01 when incubated in media containing HMW-^{109}Cd·[^{3}H]GS, MgATP, and gramicidin-D (Table 2). (*iii*) The principal ion peak detected after MALD-MS of HMW-Cd·GS had an m/z ratio of 725.4 ± 0.7, consistent with the molecular weight of Cd·GS_2 (724.6 Da, Fig. 4). The transport-inactive complex, LMW-Cd·GS, on the other hand, was tentatively identified as Cd·GS on the basis of its smaller apparent molecular size (Fig. 2*A*), failure to bind Mono-Q (Fig. 2*C*), and Cd/GS ratio of 0.67 ± 0.04 and 0.86 ± 0.07 after chromatography on Sephadex G-15 and Mono-Q (Table 2), respectively.

While an m/z ratio of 725 for HMW-Cd·GS would be equally compatible with the transport of Cd·GSSG, this is refuted by two findings: (*i*) GSSG, alone, does not promote YCF1-dependent uptake (Table 1), and (*ii*) the transport-active complex is probably a mercaptide. Pretreatment of HMW-Cd·GS with 2-mercaptoethanol inhibits MgATP-dependent, uncoupler-insensitive Cd^{2+} uptake by DTY165 membranes by more then 80% (Table 2), and *S*-methylation abolishes the stimulatory action of GSH (Table 1).

Table 1. Effects of different GSH-related compounds on uncoupler-insensitive ^{109}Cd uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells

	^{109}Cd uptake, nmol/mg/10 min			
	DTY165		DTY167	
Compound	− MgATP	+ MgATP	− MgATP	+ MgATP
Cd^{2+}	5.8 ± 2.4	5.6 ± 1.5	4.3 ± 1.3	4.6 ± 2.1
Cd^{2+} + GSH	4.2 ± 1.2	37.4 ± 4.5	3.3 ± 1.1	8.3 ± 2.7
Cd^{2+} + GSSG		5.1 ± 3.2		3.8 ± 2.3
Cd^{2+} + GS-CH_3		4.5 ± 1.9		3.7 ± 3.1
Cd^{2+} + Cys-Gly		5.6 ± 3.2		6.9 ± 1.4
Cd^{2+} + Cys		7.0 ± 1.2		3.9 ± 1.0
Cd^{2+} + Glu		5.7 ± 1.1		5.2 ± 1.3

GSH, oxidized glutathione (GSSG), *S*-methylglutathione (GS-CH_3), cysteinylglycine, cysteine, and glutamate were added at concentrations of 1 mM. MgATP, $^{109}CdSO_4$, and gramicidin-D were added at concentrations of 3 mM, 80 μM, and 5 μM, respectively. Values shown are means ± SE (n = 3–6).

Cd·GS_2 Transport Is Directly Energized by MgATP. Purification of Cd·GS_2 enabled the energy requirements of YCF1-

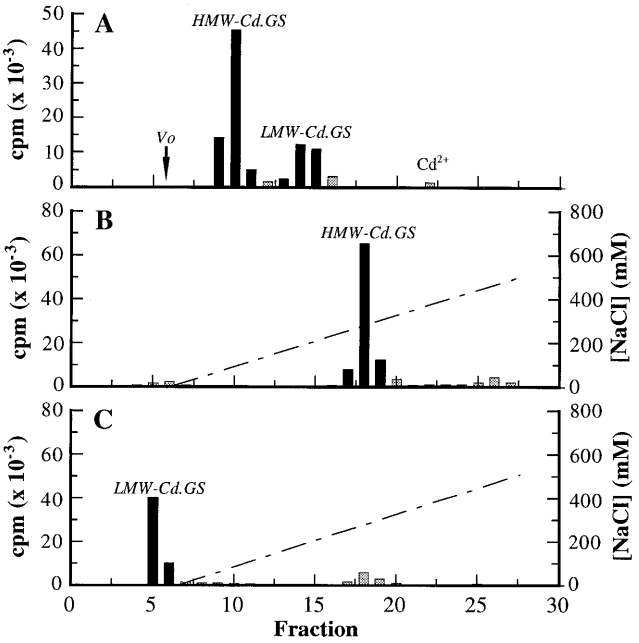

FIG. 2. Purification of cadmium·glutathione complexes by gel-filtration (*A*) and anion-exchange chromatography (*B* and *C*). $^{109}CdSO_4$ (20 mM) was incubated with 40 mM GSH at 45°C for 24 h, and the mixture was chromatographed on Sephadex G-15 to resolve a high molecular weight ^{109}Cd-labeled component (HMW-^{109}Cd·GS) from a low molecular weight component (LMW-^{109}Cd·GS) (A). The peaks corresponding to HMW-^{109}Cd·GS and LMW-^{109}Cd·GS were then chromatographed on Mono-Q and eluted with a linear NaCl gradient (- — -) (*B* and *C*). ^{109}Cd (cpm × 10^{-3}) was determined on 5 μl aliquots of the column fractions by liquid scintillation counting. Solid bars denote fractions subjected to further manipulations.

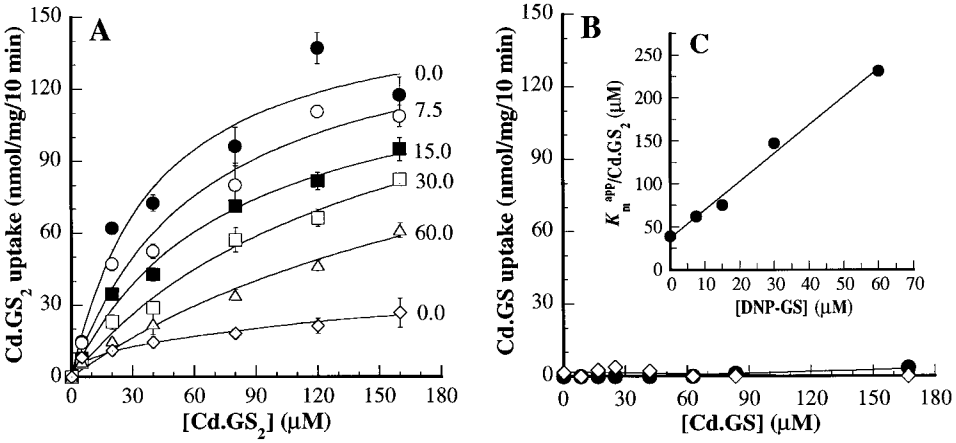

FIG. 3. Kinetics of MgATP-dependent, uncoupler-insensitive $^{109}Cd{\cdot}GS_2$ (HMW-$^{109}Cd{\cdot}GS$; *A*) and $^{109}Cd{\cdot}GS$ (LMW-$^{109}Cd{\cdot}GS$; *B*) uptake. *S*-(2,4-dinitrophenyl)glutathione (DNP-GS) was added at the concentrations (μM) indicated to DTY165 membranes (●, ○, ■, □, △) or DTY167 membranes (◇). A secondary plot of the apparent Michaelis constants for $Cd{\cdot}GS_2$ uptake ($K_m^{app}/Cd{\cdot}GS_2$) as a function of DNP-GS concentration is shown (*C*). The kinetic parameters for $Cd{\cdot}GS_2$ transport by DTY165 membranes were K_m, 39.1 ± 14.1 μM, V_{max}, 157.2 ± 30.4 nmol/mg/10 min, and $K_{i(DNP\text{-}GS)}$, 11.3 ± 2.1 μM. Kinetic parameters were computed by nonlinear least squares analysis (14). Values shown are means ± SE (n = 6).

dependent transport to be examined directly and confirmed that more than 83% of the MgATP-dependent, uncoupler-insensitive Cd^{2+} transport measured using DTY165 membranes was mediated by YCF1. Agents that dissipate both the ΔpH and $\Delta\psi$ components of the H^+-electrochemical gradient established by the vacuolar H^+-ATPase [V-ATPase; carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), gramicidin-D] or directly inhibit the V-ATPase, itself (bafilomycin A_1), decreased MgATP-dependent $Cd{\cdot}GS_2$ uptake by vacuolar membrane vesicles from DTY165 cells by 22% (Table 3). Ammonium chloride, which abolishes ΔpH while leaving $\Delta\psi$ unaffected, on the other hand, inhibited uptake by only 15% (Table 3). From these results and the inability of uncouplers to markedly increase the inhibitions caused by V-ATPase inhibitors, alone (Table 3), $Cd{\cdot}GS_2$ uptake by wild-type membranes is inferred to proceed via a YCF1-dependent, MgATP-energized pathway that accounts for most of the transport measured and a YCF1-independent pathway, primarily driven by the H^+-gradient established by the V-ATPase, that makes a minor contribution to total uptake.

Table 2. Molar Cd/GS ratios of LMW-Cd·GS and HMW-Cd·GS complexes fractionated by Sephadex G-15 and Mono-Q chromatography (Fig. 2) before and after MgATP-dependent, uncoupler-insensitive uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells

	^{109}Cd uptake, nmol/mg/10 min		Molar ratio Cd/GS	
Fraction	DTY165	DTY167	Before uptake	After uptake
Sephadex G-15				
HMW-Cd·GS			0.44 ± 0.09	
LMW-Cd·GS			0.67 ± 0.04	
Mono-Q				
HMW-Cd·GS	66.3 ± 2.7	5.6 ± 2.6	0.49 ± 0.17	0.49 ± 0.01
LMW-Cd·GS	4.4 ± 0.8	3.9 ± 1.4	0.86 ± 0.07	
After 2-ME				
HMW-Cd·GS	11.9 ± 2.4	4.4 ± 3.0		

Cd/GS ratios were estimated from the $^{109}Cd/[^3H]$ radioisotope ratios of samples prepared from $^{109}CdSO_4$ and [^{3}H]GSH. HMW-$^{109}Cd{\cdot}[^3H]GS$ was pretreated with 2-mercaptoethanol (2-ME) by heating a 1:4 mixture of HMW-$^{109}Cd.[^3H]GS$ with 2-ME at 60°C for 10 min before measuring $^{109}Cd^{2+}$ uptake. Uptake was measured using 50 μM concentrations (as Cd) of the complexes indicated in standard uptake medium containing 5 μM gramicidin-D. Values shown are means ± SE (n = 3–6).

$Cd{\cdot}GS_2$ Competes with DNP-GS for Transport. As would be predicted if $Cd{\cdot}GS_2$ and the model organic GS-conjugate, DNP-GS, follow the same transport pathway, the K_i for inhibition of MgATP-dependent, uncoupler-insensitive $Cd{\cdot}GS_2$ uptake by DNP-GS (11.3 ± 2.1 μM; Fig. 3 *A* and *C*) coincided with the K_m for DNP-GS transport (14.1 ± 7.4 μM, ref. 5).

Pretreatment with Cd^{2+} or CDNB Increases *YCF1* Expression. RNase protection assays of *YCF1* expression in DTY165 cells and measurements of MgATP-dependent, uncoupler-insensitive $^{109}Cd{\cdot}GS_2$ and [^{3}H]DNP-GS uptake by vacuolar membranes prepared from the same cells after 24 h of growth in media containing $CdSO_4$ (200 μM) or the cytotoxic DNP-GS precursor, CDNB (150 μM), demonstrated a parallel increase in all three quantities. *YCF1*-specific mRNA levels were increased by 1.9- and 2.5-fold by pretreatment of DTY165 cells with $CdSO_4$ and CDNB, respectively (Fig. 5).

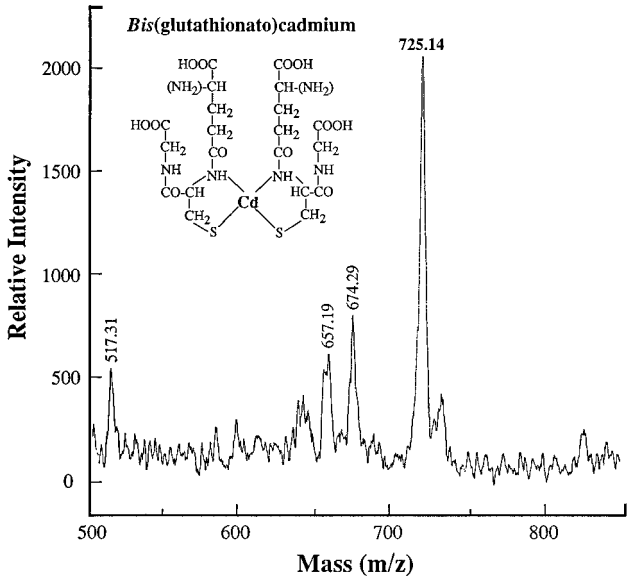

FIG. 4. Matrix-assisted laser desorption mass spectrometry of HMW-Cd·GS. MALD-MS was performed on Sephadex G-15-, Mono-Q-purified HMW-Cd·GS as described. The molecular structure inferred from a mean m/z ratio of 725.4 ± 0.7 (n = 9) and average Cd/GS stoichiometry of 0.5 ($Cd{\cdot}GS_2$, molecular weight 724.6 Da) is shown.

Table 3. Effects of uncouplers and V-ATPase inhibitors on uptake of $Cd{\cdot}GS_2$ by vacuolar membrane vesicles purified from DTY165 and DTY167 cells

Addition	$Cd{\cdot}GS_2$ uptake, nmol/mg/10 min	
	DTY165	DTY167
Control	105.8 ± 12.4 (100)	17.3 ± 2.7 (100)
Gramicidin-D	77.8 ± 6.4 (73.5)	9.8 ± 2.0 (56.6)
FCCP	62.2 ± 11.4 (58.8)	10.2 ± 1.6 (59.0)
NH_4Cl	89.8 ± 8.2 (84.8)	10.0 ± 1.7 (57.8)
NH_4Cl + gramicidin-D	69.8 ± 12.0 (66.0)	8.8 ± 2.2 (50.9)
Bafilomycin A_1	81.8 ± 6.0 (76.6)	12.8 ± 3.6 (74.0)
Bafilomycin A_1 + gramicidin-D	70.2 ± 12.2 (66.4)	7.2 ± 2.4 (41.6)

Uptake was measured in standard uptake medium containing 50 μM purified $^{109}Cd{\cdot}GS_2$. Bafilomycin A_1, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), gramicidin-D, and NH_4Cl were added at concentrations of 0.5 μM, 5 μM, 5 μM, and 1 mM, respectively. Values outside parentheses are means ± SE (n = 3–6); values inside parentheses are rates of uptake expressed as percentage of control.

The same pretreatments increased MgATP-dependent, uncoupler-insensitive $^{109}Cd{\cdot}GS_2$ uptake into vacuolar membrane vesicles by 1.4- and 1.7-fold and [^{3}H]DNP-GS uptake by 1.6- and 2.8-fold (Fig. 5).

DISCUSSION

These investigations provide an indication of the mechanism by which *YCF1* confers Cd^{2+} resistance in *S. cerevisiae* and its relationship to the transport of organic GS-conjugates by demonstrating that the integral membrane protein encoded by this gene specifically catalyzes the MgATP-energized uptake of $Cd{\cdot}GS_2$ by vacuolar membrane vesicles. The codependence of $Cd{\cdot}GS_2$ and organic GS-conjugate transport on YCF1 is evident at multiple levels. (*i*) The *ycf1*Δ mutant strain, DTY167, is hypersensitive to Cd^{2+} and CDNB in the growth medium, and both hypersensitivities are alleviated by transformation with plasmid-borne *YCF1* (4, 5). (*ii*) Vacuolar membrane vesicles purified from DTY167 cells are grossly impaired for MgATP-energized, uncoupler-insensitive organic GS-conjugate and GSH-promoted Cd^{2+} uptake. (*iii*) $Cd{\cdot}GS_2$ and organic GS-conjugate compete for the same uptake sites on YCF1. (*iv*) Factors that increase *YCF1* expression elicit a parallel increase in $Cd{\cdot}GS_2$ and organic GS-conjugate transport. Thus, a number of ostensibly disparate observations—the strong association between cellular GSH levels and Cd^{2+} resistance (e.g., ref. 15), the markedly increased sensitivity of vacuole deficient *S. cerevisiae* strains to Cd^{2+} toxicity (M.S. and D.J.T., unpublished results), and the coordinate regulation of the yeast *YCF1* and *GSH1* genes, the latter of which encodes γ-glutamylcysteine synthetase (16, 17)—are now explicable in terms of a model in which YCF1 catalyzes the GSH-dependent vacuolar sequestration of Cd^{2+}.

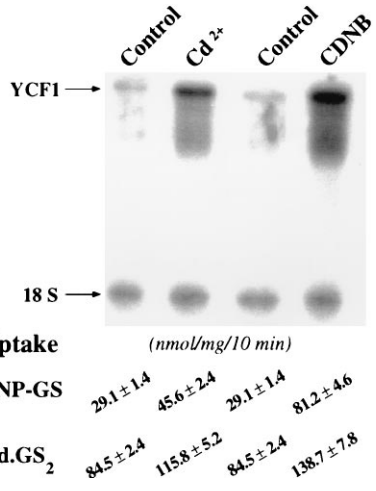

FIG. 5. Induction of *YCF1* expression and YCF1-dependent $Cd{\cdot}GS_2$ and DNP-GS transport by pretreatment of DTY165 cells with $CdSO_4$ (Cd^{2+}, 200 μM) or CDNB (150 μM) for 24 h. *YCF1*-specific mRNA and 18S rRNA were detected in the total RNA extracted from control or pretreated cells (10 μg/lane) by RNase protection. Uptake of $^{109}Cd{\cdot}GS_2$ (50 μM) or [^{3}H]DNP-GS (66.2 μM) by vacuolar membrane vesicles was measured in standard uptake medium containing 5 μM gramicidin-D. Values shown are means ± SE (n = 3).

While Tommasini *et al*. (18) have recently confirmed our earlier findings (5) by showing that YCF1 is a vacuolar GS-conjugate transporter, and gone on to demonstrate that transformation with human *MRP1* suppresses the *ycf1*Δ mutation, their results differ from ours in their inability to detect MgATP-energized, GSH-dependent Cd^{2+} uptake by microsomes from *MRP1*-transformed yeast. Because of this and their failure to inhibit DNP-GS transport by the inclusion of GSH (1 mM) and Cd^{2+} (100–300 μM) in the uptake medium, these authors attribute the Cd^{2+} resistance conferred by *MRP1* to a two-phase process: the initial transport of Cd^{2+} into the vacuole by H^+/Cd^{2+} antiport and its subsequent complexation with an unknown binding molecule transported by MRP1. Two alternative conclusions therefore follow. Either MRP1 and YCF1 do not have precisely the same transport mechanism or Tommasini *et al*.'s inability to measure GSH-promoted Cd^{2+} transport has a methodological basis. Of these, the second explanation is the more likely. First, the idea that MRP1 and YCF1 use identical mechanisms for the uptake of organic GS-conjugates but different mechanisms for Cd^{2+} seems unnecessarily complicated when both transporters must encounter the same microenvironment in yeast. Second, the two-phase mechanism does not explain how *MRP1* phenocopies *YCF1* and how DNP-GS inhibits YCF1-dependent $Cd{\cdot}GS_2$ transport in a simple competitive manner. Third, inspection of the procedures employed by Tommasini *et al* (18)—methods originally developed in our laboratory for a different purpose (9)—reveals a critical technical error: systematic contamination of the Cd^{2+} uptake media with approximately 140 μM concentrations of dithiothreitol and EDTA. We know from preliminary experiments that these concentrations are more than sufficient to cause a near total abolition of GSH-dependent Cd^{2+} uptake by YCF1 while leaving organic GS-conjugate uptake unaffected. Dithiothreitol reacts with Cd^{2+} to form a transport-inactive dimercaptide (Z.-S.L. and P.A.R., unpublished results); EDTA chelates free Cd^{2+} to quench its reaction with GSH.

Further verification of the one-phase model is provided by the functional equivalence of YCF1 to MRP1 in all other regards. At the biochemical level, YCF1 specifically catalyzes the transport of $Cd{\cdot}GS_2$. This is the most straightforward explanation of the mass and 1:2 Cd/GS ratio of the transport-active complex and the finding that the concentration-dependence of uptake assumes a Michaelian, rather than sigmoidal, function with a moderately low K_m when $Cd{\cdot}GS_2$ is purified from the other components present in a mixture of Cd^{2+} and GSH. Analogously, MRP1 catalyzes the transport of bis(glutathionato)platinum ($Pt{\cdot}GS_2$) (19). MALD-MS and ^{1}H-NMR spectroscopy of the transport-active complex formed when the anticancer drug, cisplatin, is combined with GSH indicate that Pt spontaneously complexes with GSH in a 1:2 ratio. Two GSH molecules coordinate each Pt^{2+} ion via their cysteinyl sulfur and amino nitrogen atoms to generate the transport-active complex. At the cellular level, YCF1 and MRP1 confer resistance to and are induced by a similar spectrum of xenobiotics. *YCF1* confers cross-resistance to organic xenobiotics and heavy metals (4, 5). Enhanced expression of *MRP1* in mammalian cells is associated with cross-

resistance to cisplatin and heavy metals, such as cadmium and arsenite, as well as GSH-conjugable drugs (7, 20). Expression of *YCF1* is increased by exposure of cells to GSH-conjugable xenobiotics and Cd^{2+}. Expression of *MRP1* is increased by exposure of cells to arsenite, Cd^{2+} and Zn^{2+} (20).

The existence of phytochelatins (PCs), peptides consisting of repeating units of γ-glutamylcysteine followed by a C-terminal glycine [$(\gamma\text{-Glu-Cys})_n$-Gly], in the fission yeast, *Schizosaccharomyces pombe* (21) and the isolation and characterization of the heavy metal tolerance (*HMT1*) gene, a six transmembrane span-single nucleotide binding fold ("single half") ATP binding cassette (ABC) transporter, responsible for vacuolar uptake of PC and Cd·PC, from the same organism (21, 22), prompts two related questions. Do similar mechanisms of Cd^{2+} sequestration operate in *S. cerevisiae*? Is YCF1 competent in the transport of Cd·PC as well as $Cd{\cdot}GS_2$? The first question has not been resolved but PCs are more widespread in fungi than was once thought. Although heavy metal detoxification in fungi has been largely attributed to metallothioneins (23), recent studies of *Candida glabrata*, *S. cerevisiae*, and *Neurospora crassa* also implicate PCs. Exposure of *C. glabrata* to copper salts stimulates metallothionein formation but exposure to cadmium salts stimulates PC formation (24). In both *S. cerevisiae* and *N. crassa*, PCs have been detected and in the latter synthesis is not only activated by Cd^{2+} but also Zn^{2+} (25). Interestingly, in all three organisms, PC_2 is the dominant PC species found. Hence, the second question or a modification thereof. Given the structural resemblance between $Cd{\cdot}PC_2$ [$Cd{\cdot}(\gamma\text{-Glu-Cys})_2$-Gly] and $Cd{\cdot}GS_2$, might YCF1 be able to transport either complex? The answer to this question is no: YCF1 does not transport PC_2. PC_2 fractions purified from *S. pombe* show no evidence of YCF1-dependent transport by vacuolar membrane vesicles isolated from *S. cerevisiae* irrespective of whether the PC preparations are complexed with Cd^{2+} or not (Z.-S.L. and P.A.R., unpublished results). The novelty of YCF1-mediated $Cd{\cdot}GS_2$ transport as a mechanism for the vacuolar sequestration of Cd^{2+} is therefore substantiated.

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