

# The Yeast Cadmium Factor Protein (YCF1) Is a Vacuolar Glutathione *S*-Conjugate Pump\*

(Received for publication, May 31, 1995, and in revised form, October 30, 1995)

Ze-Sheng Li†, Mark Szczypka§, Yu-Ping Lu†, Dennis J. Thiele§¶, and Philip A. Rea‡||

From the †Plant Science Institute, Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6018 and §Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan 48109-0606

The yeast cadmium factor gene (*YCF1*) from *Saccharomyces cerevisiae*, which was isolated according to its ability to confer cadmium resistance, encodes a 1,515-amino acid ATP-binding cassette (ABC) protein with extensive sequence homology to the human multidrug resistance-associated protein (MRP1) (Szczypka, M., Wemmie, J. A., Moye-Rowley, W. S., and Thiele, D. J. (1994) *J. Biol. Chem.* 269, 22853–22857). Direct comparisons between *S. cerevisiae* strain DTY167, harboring a deletion of the *YCF1* gene, and the isogenic wild type strain, DTY165, demonstrate that *YCF1* is required for increased resistance to the toxic effects of the exogenous glutathione *S*-conjugate precursor, 1-chloro-2,4-dinitrobenzene, as well as cadmium. Whereas membrane vesicles isolated from DTY165 cells contain two major pathways for transport of the model compound *S*-(2,4-dinitrophenyl)glutathione (DNP-GS), an MgATP-dependent, uncoupler-insensitive pathway and an electrically driven pathway, the corresponding membrane fraction from DTY167 cells is more than 90% impaired for MgATP-dependent, uncoupler-insensitive DNP-GS transport. Of the two DNP-GS transport pathways identified, only the MgATP-dependent, uncoupler-insensitive pathway is subject to inhibition by glutathione disulfide, vanadate, verapamil, and vinblastine. The capacity for MgATP-dependent, uncoupler-insensitive conjugate transport *in vitro* strictly copurifies with the vacuolar membrane fraction. Intact DTY165 cells, but not DTY167 cells, mediate vacuolar accumulation of the fluorescent glutathione-conjugate, monochlorobimane-GS. Introduction of plasmid borne, epitope-tagged gene encoding functional *YCF1* into DTY167 cells alleviates the 1-chloro-2,4-dinitrobenzene-hypersensitive phenotype concomitant with restoration of the capacity of vacuolar membrane vesicles isolated from these cells for MgATP-dependent, uncoupler-insensitive DNP-GS transport. On the basis of these findings, the *YCF1* gene of *S. cerevisiae* is inferred to encode an MgATP-energized, uncoupler-insensitive vacuolar glutathione *S*-conjugate transporter. The energy requirements, kinetics, substrate specificity, and inhibitor profile of *YCF1*-mediated transport demonstrate that the vacuolar glutathi-

one conjugate pump of yeast bears a strong mechanistic resemblance to the *MRP1*-encoded transporter of mammalian cells and the cognate, but as yet molecularly undefined, function of plant cells.

Animal and plant cells have the capacity to eliminate a broad range of lipophilic toxins from the cytosol after their conjugation with glutathione (Ishikawa, 1992; Martinoia *et al.*, 1993; Li *et al.*, 1995). This process, mediated by a GS-*X* pump,<sup>1</sup> a novel MgATP-dependent transporter that catalyzes the efflux of glutathione *S*-conjugates and glutathione disulfide (GSSG) from the cytosol via the plasma membrane and/or endomembranes, is thought to constitute a terminal phase of xenobiotic detoxification.

The metabolism and detoxification of xenobiotics comprises three main phases (Ishikawa, 1992). Phase I is a preparatory step in which toxins are oxidized, reduced, or hydrolyzed to introduce or expose functional groups of the appropriate reactivity. Cytochrome P450s and mixed function oxidases are examples of phase I enzymes. In phase II, the activated derivative is conjugated with GSH, glucuronic acid, or glucose. In the case of the GSH-dependent pathway, *S*-conjugates of GSH are formed by cytosolic glutathione *S*-transferases. In the final phase, phase III, of the GSH-dependent pathway, GS-conjugates are eliminated from the cytosol by the GS-*X* pump.

A remarkable feature of the last component of the GSH-dependent pathway, the GS-*X* pump, is the pharmacological and agronomic importance of the compounds it recognizes. Established transport substrates for the mammalian GS-*X* pump include the arachidonic acid metabolites, leukotriene C<sub>4</sub>, and prostaglandin A<sub>2</sub>, GS-conjugates of the anticancer drugs cisplatin, chlorambucil, and acrolein, GSSG, and the model compounds *S*-(2,4-dinitrophenyl)glutathione (DNP-GS) and monochlorobimane-GS (Ishikawa *et al.*, 1995). In plants, the GS-*X* pump is competent in the transport of GS-conjugates of the chloroacetanilide and triazine herbicides, metolachlor and sim-

\* This work was supported in part by United States Department of Agriculture Grant NRICGP 9303007 (to P. A. R.), Department of Energy/National Science Foundation/United States Department of Agriculture Triagency Plant Training Grant DE-FG02-94ER20162 (to the Plant Science Institute, University of Pennsylvania), and by National Institutes of Health, NIEHS Grant 1R01 ES06902 01A1 (to D. J. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† A Burroughs Wellcome Toxicology Scholar.

‡ To whom correspondence should be addressed. Tel: 215-898-0807; Fax: 215-898-8780; E-mail: parea@sas.upenn.edu.

<sup>1</sup> The abbreviations used are: GS-*X* pump, glutathione *S*-conjugate transporting ATPase; ABC transporter, ATP binding cassette transport protein; AMP-PNP, adenosine 5'-(β,γ-imino)triphosphate; CDNB, 1-chloro-2,4-dinitrobenzene; DNP-GS, *S*-(2,4-dinitrophenyl)glutathione; F-ATPase, mitochondrial H<sup>+</sup>-ATPase; FCCP, carbonylcyanide 4-trifluoromethoxyphenylhydrazone; GSSG, oxidized glutathione (glutathione disulfide); HA, 12CA5 epitope (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) of human influenza hemagglutinin; *MDR*, gene encoding multiple drug resistance ABC transporter (P-glycoprotein or putative P-glycoprotein homolog); metolachlor, 2-chloro-*N*-(2'-ethyl-6'-methylphenyl)-*N*-(2-methoxy-1-methylethyl)-acetamide; *MRP1*, gene encoding multidrug resistance-associated protein; P-ATPase, plasma membrane H<sup>+</sup>-ATPase; V-ATPase, vacuolar H<sup>+</sup>-ATPase; *YCF1*, gene encoding yeast cadmium factor; PMSF, phenylmethylsulfonyl fluoride; NBF, nucleotide binding fold; CFTR, cystic fibrosis transmembrane conductance regulator; GST, glutathione *S*-transferase; Mes, 4-morpholinoethanesulfonic acid.

etryn, respectively, as well as GSSG, DNP-GS, and monochlorobimane-GS (Martinoia *et al.*, 1993; Li *et al.*, 1995).

In spite of its relevance for understanding how cells combat chemotherapeutic agents and herbicides and the interest shown in this class of transporter because of its exclusive use of MgATP, rather than preformed transmembrane ion gradients, as a direct energy source for organic solute transport, the molecular identity of any GS-*X* pump has remained elusive. Critical, therefore, is the recent finding that the 190-kDa membrane glycoprotein encoded by the human multidrug resistance-associated protein gene (*MRP1*), implicated in the resistance of small cell lung cancer cell lines to a number of chemotherapeutic drugs (Cole *et al.*, 1992), catalyzes the MgATP-dependent transport of leukotriene C<sub>4</sub> and related glutathione *S*-conjugates (Leier *et al.*, 1994; Muller *et al.*, 1994; Zaman *et al.*, 1995).

MRP1 is a member of the ABC (ATP binding cassette) superfamily of transporters. Distributed throughout the major taxa, ABC transporters catalyze the MgATP-dependent transport of peptides, sugars, ions, and lipophiles across membranes. All are constituted of one or two copies each of two basic structural elements, a hydrophobic, integral membrane sector, containing approximately 6 transmembrane  $\alpha$ -helices, and a cytoplasmically oriented ATP-binding domain (nucleotide binding fold, NBF) (Hyde *et al.*, 1990; Higgins, 1995). The NBFs, a diagnostic feature of ABC transporters, are 30% identical between family members over a span of about 200 amino acid residues, and each encompasses a Walker A and Walker B box (Walker *et al.*, 1982) and an ABC signature motif (Higgins, 1995). The most thoroughly investigated ABC family members in eukaryotes include the mammalian P-glycoproteins (MDRs), some of which are implicated in drug resistance and others in lipid translocation (Ruetz and Gros, 1994); the pleiotropic drug resistance protein (PDR5) and STE6 peptide-mating pheromone transporter of yeast; the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel, mutation of which is associated with cystic fibrosis in man; the malarial, *Plasmodium falciparum*, chloroquine transporter (PFMDR1); and the major histocompatibility complex transporters responsible for peptide translocation and antigen presentation in T lymphocytes (reviewed in Balzi and Goffeau (1995) and Higgins (1995)).

Sequence comparisons between MRP1 and other ABC transporters reveal two major subgroups (Cole *et al.*, 1992; Szczyepka *et al.*, 1994). One consists of MRP1, the *Saccharomyces cerevisiae* cadmium factor (*YCF1*) gene, the

MgCl<sub>2</sub>, 25 mM KCl, 10 mM Tris-Mes (pH 6.9) containing 2 mg/ml bovine serum albumin, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM PMSF, pelleted by centrifugation at 37,000 × *g* for 25 min, and resuspended in suspension medium (1.1 M glycerol, 2 mM dithiothreitol, 1 mM Tris-EGTA, 2 mg/ml bovine serum albumin, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM PMSF, 5 mM Tris-Mes, pH 7.6) (Kim *et al.*, 1995). For the experiment shown in Fig. 4, 1 ml of partially purified vacuolar membrane vesicles (1.1–1.2 mg of protein), prepared by Ficoll flotation, were subjected to further fractionation by centrifugation through a 30-ml linear 10–40% (w/v) sucrose density gradient at 100,000 × *g* for 2 h. Successive fractions were collected from the top of the centrifuge tube and, after determining sucrose concentration refractometrically, the fractions were diluted with suspension medium. The diluted fractions were sedimented at 100,000 × *g* and resuspended in 100-μl aliquots of suspension medium for assay. For the immunoblots shown in Fig. 5 and the marker enzyme analyses shown in Table IV, crude microsomes were prepared by homogenization of spheroplasts in suspension medium and the sedimentation of total membranes at 100,000 × *g* for 35 min.

Microsomes and purified vacuolar membranes that were to be employed for SDS-polyacrylamide gel electrophoresis and immunoblotting were washed free of bovine serum albumin by three rounds of suspension in suspension medium *minus* bovine serum albumin and centrifugation at 100,000 × *g* for 35 min. The final membrane preparations were either used immediately or frozen in liquid nitrogen and stored at –85 °C.

**Measurement of Marker Enzyme Activities**—α-Mannosidase was determined according to Opheim (1978) using *p*-nitrophenyl-α-D-mannopyranoside as substrate. NADPH-cytochrome *c* reductase was estimated as FMN-promoted reduction of NADPH (Kubota *et al.*, 1977). GDPase was measured as the rate of liberation of P<sub>i</sub> from GDP (Yanagisawa *et al.*, 1990) in reaction buffer containing 0.05% (w/v) Triton X-100. V-ATPase, F-ATPase, and P-ATPase were assayed as bafilomycin A<sub>1</sub> (1 μM), azide (1 mM), and vanadate (100 μM) inhibited ATPase activity, respectively, at pH 8.0 (V-ATPase, F-ATPase) or pH 6.5 (P-ATPase) (Rea and Turner, 1990).

**Measurement of DNP-GS Uptake**—Unless otherwise indicated, [<sup>3</sup>H]DNP-GS uptake was measured at 25 °C in 200-μl reaction volumes containing 3 mM ATP, 3 mM MgSO<sub>4</sub>, 5 μM gramicidin-D, 10 mM creatine phosphate, 16 units/ml creatine kinase, 50 mM KCl, 1 mg/ml bovine serum albumin, 400 mM sorbitol, 25 mM Tris-Mes (pH 8.0), and 66.2 μM [<sup>3</sup>H]DNP-GS (8.7 mCi/mmol) (Li *et al.*, 1995). Gramicidin D was included in the uptake medium to abolish the H<sup>+</sup> electrochemical potential difference (Δμ<sub>H<sup>+</sup></sub>) that would otherwise be established by the V-ATPase in medium containing MgATP. Uptake was initiated by the addition of vacuolar membrane vesicles (10–15 μg of membrane protein), brief mixing of the samples on a vortex mixer and allowed to proceed for 1–60 min. Uptake was terminated by the addition of 1 ml of ice-cold wash medium (400 mM sorbitol, 3 mM Tris-Mes, pH 8.0) and vacuum filtration of the suspension through prewetted Millipore HA cellulose nitrate membrane filters (pore diameter, 0.45 μm). The filters were rinsed twice with 1 ml of ice-cold wash medium and air-dried, and radioactivity was determined by liquid scintillation counting in BCS mixture (Amersham Corp.). Nonenergized [<sup>3</sup>H]DNP-GS uptake and extravascular solution trapped on the filters were enumerated by the same procedure except that ATP and Mg<sup>2+</sup> were omitted from the uptake medium.

**Fluorescence Microscopy**—Cells were grown in YPD medium for 24 h at 30 °C to an OD<sub>600 nm</sub> of approximately 1.4, and 100-μl aliquots of the suspensions were transferred to 15-ml volumes of fresh YPD medium containing 100 μM *syn*-(ClCH<sub>2</sub>,CH<sub>2</sub>)-1,5-diazabicyclo-[3.3.0]octa-3,6-dione-2,8-dione (monochlorobimane) (Kosower and Pazhenchevsky, 1980). After incubation for 6 h, the cells were pelleted by centrifugation, washed twice with YPD medium lacking monochlorobimane, and viewed without fixation under an Olympus BH-2 fluorescence microscope equipped with a BP-490 UV excitation filter, AFC-0515 barrier filter, and Nomarski optics attachment.

**Electrophoresis and Immunoblotting**—Membrane samples were subjected to one-dimensional SDS-polyacrylamide gel electrophoresis on 7–12% (w/v) concave exponential gradient gels after delipidation with acetone:ethanol (Parry *et al.*, 1989). The separated polypeptides were electrotransferred to 0.45-μm nitrocellulose filters at 60 V for 4 h at 4 °C in a Mini Trans-Blot transfer cell (Bio-Rad) and reversibly stained with Ponceau-S (Rea *et al.*, 1992). The filters were blocked and incubated overnight with mouse anti-HA monoclonal antibody (20 μg/ml) (Boehringer-Mannheim). Immunoreactive bands were visualized by reaction with horseradish peroxidase-conjugated goat anti-mouse IgG (1/1000 dilution) (Boehringer-Mannheim) and incubation in buffer con-

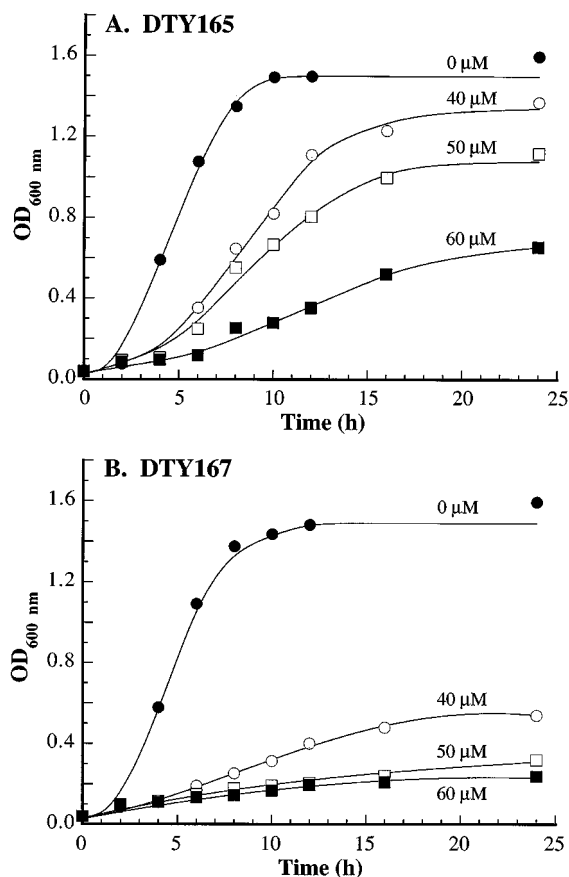


FIG. 1. Differential sensitivities of DTY165 (wild type) (A) and DTY167 (*ycf1Δ* mutant) cells (B) to growth inhibition by CDNB. Cells were grown at 30 °C for 24 h to an OD<sub>600 nm</sub> of approximately 1.4 in YPD medium before inoculation of aliquots into 15 ml volumes of the same medium containing 0–60 μM CDNB. OD<sub>600 nm</sub> was measured at the times indicated.

taining H<sub>2</sub>O<sub>2</sub> (0.03% w/v), diaminobenzidine (0.6 mg/ml) and NiCl<sub>2</sub> (0.03% w/v) (Rea *et al.*, 1992).

**Protein Estimations**—Protein was estimated by a modification of the method of Peterson (1977).

**Chemicals**—[<sup>3</sup>H]DNP-GS (specific activity, 8.7 mCi/mmol) and monochlorobimane-GS were synthesized enzymatically and purified by a modification of the procedure of Kunst *et al.* (1989) (Li *et al.*, 1995). Metolachlor-GS was synthesized by general base catalysis and purified by reverse-phase fast protein liquid chromatography (Li *et al.*, 1995).

GSH and CDNB were purchased from Fluka; AMP-PNP, aprotinin, ATP, creatine kinase (type I from rabbit muscle, 150–250 units/mg of protein), creatine phosphate, FCCP, GSSG, gramicidin D, leupeptin, PMSF, verapamil, and vinblastine were from Sigma; monochlorobimane was from Molecular Probes; cellulose nitrate membranes (0.45-μm pore size, HA filters) were from Millipore; [<sup>3</sup>H]glutathione [(*glycine*-2-<sup>3</sup>H)-L-Glu-Cys-Gly; 44 Ci/mmol] was from DuPont NEN; and metolachlor was a gift from CIBA-Geigy, Greensboro, NC. All other reagents were of analytical grade and purchased from Fisher, Fluka, or Sigma.

## RESULTS

**Sensitivity to CDNB**—If the *YCF1* gene product were to participate in the detoxification of *S*-conjugable xenobiotics, mutants deleted for this gene would be expected to be more sensitive to the toxic effects of these compounds than wild type cells. This is what was found (Fig. 1).

The isogenic wild type strain DTY165 and the *ycf1Δ* mutant strain, DTY167, were indistinguishable during growth in YPD medium lacking CDNB: both strains grew at the same rate after a brief lag. However, the addition of CDNB to the culture medium caused a greater retardation of the growth of DTY167 cells (Fig. 1B) than DTY165 cells (Fig. 1A). Inhibitory concen-

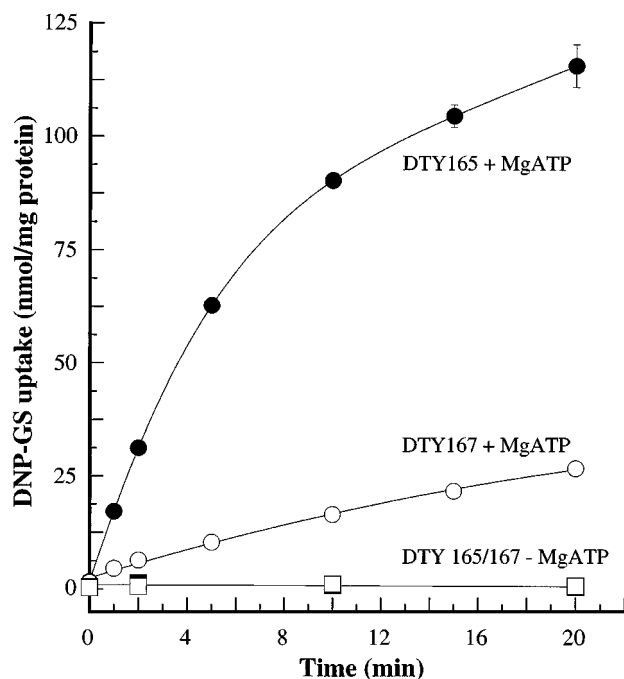


FIG. 2. Time course of [ $^3\text{H}$ ]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells. Uptake was measured in the absence ( $-MgATP$ ) or presence of 3 mM MgATP ( $+MgATP$ ) in reaction media containing 66.2  $\mu\text{M}$  [ $^3\text{H}$ ]DNP-GS, 10 mM creatine phosphate, 16 units/ml creatine kinase, 50 mM KCl, 0.1% (w/v) bovine serum albumin, 400 mM sorbitol, and 25 mM Tris-Mes (pH 8.0) at 25  $^{\circ}\text{C}$ . Values shown are means  $\pm$  S.E. ( $n = 3$ ).

trations of CDNB resulted in a slower, more linear, growth rate for at least 24 h for both strains, but DTY167 underwent growth retardation at lower concentrations than did DTY165. The optical densities of the DTY167 cultures were diminished by 65, 82, 85, and 91% by 40, 50, 60, and 70  $\mu\text{M}$  CDNB, respectively, after 24 h of incubation (Fig. 1B), whereas the corresponding diminutions for the DTY165 cultures were 14, 31, 59, and 92% (Fig. 1A). The increase in sensitivity to CDNB conferred by deletion of the YCF1 gene was similar to that seen with cadmium (data not shown).

**Impaired Vacuolar DNP-GS Transport**—Vacuolar membrane vesicles purified from DTY165 cells exhibited high rates of MgATP-dependent [ $^3\text{H}$ ]DNP-GS uptake (Fig. 2). Providing that creatine phosphate and creatine kinase were included in the uptake media to ensure ATP regeneration, addition of 3 mM MgATP increased the initial rate of DNP-GS uptake by 122-fold to a value of 12.2 nmol/mg/min. The same membrane fraction from DTY167 cells, although capable of similar rates of MgATP-independent DNP-GS uptake, was only 17-fold stimulated by MgATP and capable of an initial rate of uptake of only 1.7 nmol/mg/min (Fig. 2).

**Selective Impairment of Uncoupler-Insensitive Transport**—Direct comparisons between vacuolar membrane vesicles from DTY165 and DTY167 cells demonstrated that deletion of the YCF1 gene selectively abolished MgATP-energized,  $\Delta\bar{\mu}_{\text{H}^+}$ -independent DNP-GS transport.

Agents that dissipate both the pH ( $\Delta\text{pH}$ ) and electrical ( $\Delta\psi$ ) components of the  $\Delta\bar{\mu}_{\text{H}^+}$  established by the V-ATPase (FCCP, gramicidin D) or directly inhibit the V-ATPase, itself (bafilomycin  $\text{A}_1$ ), decreased MgATP-dependent DNP-GS uptake by vacuolar membrane vesicles from DTY165 cells from  $77.7 \pm 1.0$  nmol/mg/10 min to between  $43.2 \pm 1.0$  and  $47.4 \pm 1.7$  nmol/mg/10 min (Table I). Ammonium chloride, which abolishes  $\Delta\text{pH}$  while leaving  $\Delta\psi$  unaffected, on the other hand, did not inhibit DNP-GS uptake (Table I). On the basis of these characteristics,

TABLE I

Effects of MgATP, MgAMP-PNP, protonophores, ionophores and V-ATPase inhibitors on [ $^3\text{H}$ ]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells

Uptake was measured for 10 min in standard uptake medium (see "Materials and Methods") containing 66.2  $\mu\text{M}$  [ $^3\text{H}$ ]DNP-GS plus the compounds indicated. MgATP (3 mM) was present throughout unless otherwise indicated. MgAMP-PNP, bafilomycin  $\text{A}_1$ , FCCP, gramicidin D, and  $\text{NH}_4\text{Cl}$  were added at concentrations of 3 mM, 0.5  $\mu\text{M}$ , 5  $\mu\text{M}$ , 5  $\mu\text{M}$  and 1 mM, respectively. Values outside parentheses are means  $\pm$  SE ( $n = 3-6$ ); values inside parentheses are rates of uptake expressed as percentage of control.

Additions	DNP-GS uptake	
	DTY165	DTY167
	nmol/mg/10 min	
Control	$77.7 \pm 1.0$ (100)	$15.4 \pm 0.4$ (100)
$-MgATP$	$2.2 \pm 0.4$ (2.8)	$1.5 \pm 0.6$ (9.7)
MgAMP-PNP ( $-MgATP$ )	$2.5 \pm 0.5$ (3.2)	$1.4 \pm 0.3$ (9.1)
FCCP	$47.4 \pm 1.7$ (61.0)	$6.4 \pm 0.3$ (41.8)
Gramicidin D	$45.8 \pm 1.4$ (58.9)	$5.8 \pm 0.1$ (37.7)
$\text{NH}_4\text{Cl}$	$69.1 \pm 2.9$ (88.9)	$14.9 \pm 0.7$ (96.8)
$\text{NH}_4\text{Cl}$ + gramicidin D	$42.6 \pm 1.8$ (54.8)	$4.1 \pm 0.2$ (26.6)
Bafilomycin $\text{A}_1$	$43.2 \pm 1.0$ (55.6)	$4.3 \pm 0.3$ (27.9)
Bafilomycin $\text{A}_1$ + gramicidin D	$39.2 \pm 2.6$ (50.5)	$3.8 \pm 0.1$ (24.7)

the inability of uncouplers to markedly increase the inhibitions caused by V-ATPase inhibitors, alone, and the resistance of 50–60% of total uptake to inhibition by any one of these compounds (Table I), DNP-GS uptake by vacuolar membranes from wild type cells is concluded to proceed via two parallel mechanisms: a V-ATPase inhibitor- and uncoupler-insensitive pathway that is directly energized by MgATP, and a  $\Delta\bar{\mu}_{\text{H}^+}$ -dependent, V-ATPase inhibitor- and uncoupler-sensitive pathway that is primarily driven by the inside-positive  $\Delta\psi$  established by the V-ATPase.

Of these two pathways, the  $\Delta\psi$ -dependent pathway predominated in membranes from DTY167 cells (Table I). FCCP, gramicidin D, and bafilomycin  $\text{A}_1$  diminished net DNP-GS uptake by DTY167 vacuolar membranes from  $15.4 \pm 0.4$  nmol/mg/10 min to between  $4.3 \pm 0.3$  and  $6.4 \pm 0.3$  nmol/mg/10 min. Moreover, although the effects of FCCP or gramicidin D and V-ATPase inhibitors in combination were slightly greater than those seen when these agents were added individually, the transport remaining was only about 10% of that seen with wild type membranes and only 2–4-fold stimulated by MgATP. In conjunction with the negligible inhibitions seen with  $\text{NH}_4\text{Cl}$ , alone, indicating that  $\Delta\psi$ , not  $\Delta\text{pH}$ , is the principal driving force for the transport activity remaining in their vacuolar membranes, DTY167 cells are inferred to be preferentially impaired in MgATP-energized,  $\Delta\bar{\mu}_{\text{H}^+}$ -independent DNP-GS transport.

The nonhydrolyzable ATP analog, AMP-PNP, did not promote DNP-GS uptake by vacuolar membrane vesicles from either DTY165 or DTY167 cells (Table I), indicating a requirement for hydrolysis of the  $\gamma$ -phosphate of ATP regardless of whether uptake was via the YCF1- or  $\Delta\psi$ -dependent pathway.

**Abolition of High Affinity, Uncoupler-insensitive Uptake**—Examination of the concentration dependence of [ $^3\text{H}$ ]DNP-GS uptake revealed a near total abolition of high affinity, MgATP-dependent, uncoupler-insensitive transport by vacuolar membrane vesicles from the *ycf1* $\Delta$  mutant strain (Fig. 3). When measured in the presence of uncoupler (gramicidin D), the rate of DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 cells increased as a simple hyperbolic function of MgATP (Fig. 3A) and DNP-GS concentration (Fig. 3B) to yield  $K_m$  values of  $86.5 \pm 29.5$   $\mu\text{M}$  (MgATP) and  $14.1 \pm 7.4$   $\mu\text{M}$  (DNP-GS) and a  $V_{\text{max}}$  of  $51.0 \pm 6.3$  nmol/mg/10 min (DNP-GS). By contrast, uncoupler-insensitive uptake by the corresponding

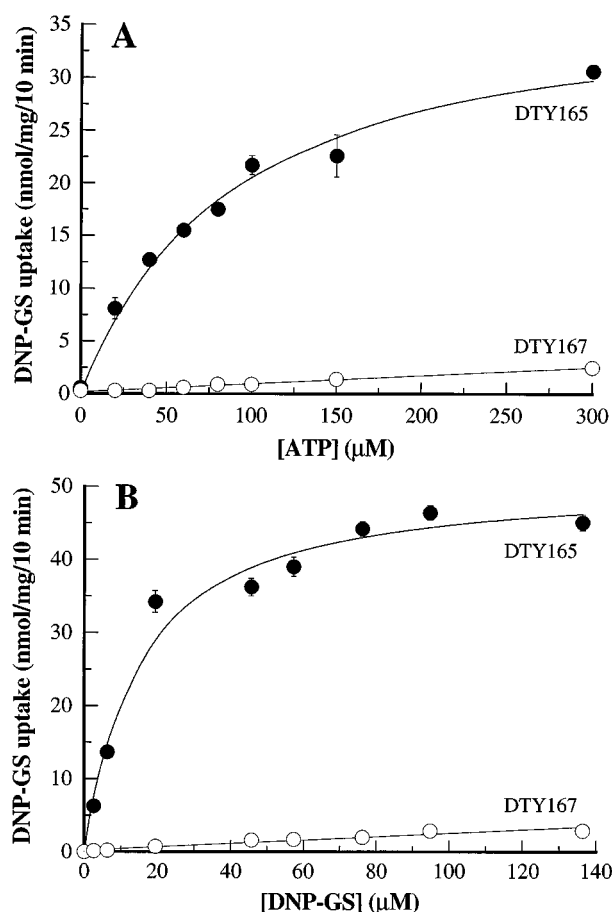


FIG. 3. Kinetics of uncoupler-insensitive [ $^3\text{H}$ ]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells. A, MgATP concentration-dependence of uncoupler-insensitive uptake. B, DNP-GS concentration-dependence of MgATP-dependent, uncoupler-insensitive uptake. The MgATP concentration-dependence of uptake was measured with  $66.2 \mu\text{M}$  [ $^3\text{H}$ ]DNP-GS. The DNP-GS concentration-dependence of uptake was measured with  $3 \text{ mM}$  MgATP. Uptake was allowed to proceed for 10 min in standard uptake medium containing  $5 \mu\text{M}$  gramicidin D. The kinetic parameters for vacuolar membrane vesicles purified from DTY165 cells were  $K_m(\text{MgATP})$   $86.5 \pm 29.5 \mu\text{M}$ ,  $K_m(\text{DNP-GS})$   $14.1 \pm 7.4 \mu\text{M}$ ,  $V_{\text{max}}(\text{MgATP})$   $38.4 \pm 5.6 \text{ nmol/mg/10 min}$ ,  $V_{\text{max}}(\text{DNP-GS})$   $51.0 \pm 6.3 \text{ nmol/mg/10 min}$ . The lines of best fit and kinetic parameters were computed by nonlinear least squares analysis (Marquardt, 1963). Values shown are means  $\pm$  S.E. ( $n = 3$ ).

membrane fraction from DTY167 cells was more than 15-fold slower over the entire concentration range, showed no evidence of saturation and increased as a linear function of both DNP-GS and MgATP concentration (Fig. 3).

**Selective Inhibitors of YCF1-mediated Transport**—MgATP-dependent, uncoupler-insensitive DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 cells was sensitive to inhibition by vanadate, vinblastine, verapamil, GSSG and glutathione S-conjugates other than DNP-GS (Tables II and III). One hundred  $\mu\text{M}$  concentrations of metolachlor-GS, azidophenacyl-GS, and monochlorobimane-GS and 1 mM GSSG inhibited uptake by about 50% (Table II), while vanadate, vinblastine, and verapamil exerted 50% inhibitions at concentrations of 179, 89, and 203  $\mu\text{M}$ , respectively (Table III). None of these agents significantly inhibited residual MgATP-dependent, uncoupler-insensitive DNP-GS uptake by vacuolar membrane vesicles from DTY167 cells (Tables II and III).

**Vacuolar Membrane Localization**—The capacity for MgATP-dependent, uncoupler-insensitive [ $^3\text{H}$ ]DNP-GS uptake strictly copurified with the vacuolar membrane fraction (Table IV). By comparison with crude microsomes (total membranes) pre-

TABLE II  
Effects of GSH, GSSG, and glutathione S-conjugates other than DNP-GS on MgATP-dependent, uncoupler-insensitive [ $^3\text{H}$ ]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells

Uptake was measured as described for Table I except that  $5 \mu\text{M}$  gramicidin D was included in all of the uptake media. Values outside parentheses are means  $\pm$  SE ( $n = 3-6$ ); values inside parentheses are rates of uptake expressed as percentage of control.

Compound	DNP-GS uptake	
	DTY165	DTY167
	nmol/mg/10 min	
Control	47.9 $\pm$ 2.5 (100)	6.5 $\pm$ 0.8 (100)
GSH (1 mM)	50.6 $\pm$ 2.3 (105.6)	4.6 $\pm$ 1.1 (70.8)
GSSG (1 mM)	26.0 $\pm$ 0.9 (54.3)	4.4 $\pm$ 0.4 (67.7)
Metolachlor-GS (100 $\mu\text{M}$ )	27.6 $\pm$ 0.9 (57.7)	4.8 $\pm$ 0.7 (73.8)
Azidophenacyl-GS (100 $\mu\text{M}$ )	16.0 $\pm$ 1.4 (33.5)	5.2 $\pm$ 0.3 (80.0)
Monochlorobimane-GS (100 $\mu\text{M}$ )	25.2 $\pm$ 1.1 (52.6)	4.5 $\pm$ 0.4 (69.2)

TABLE III  
Sensitivity of MgATP-dependent, uncoupler-insensitive [ $^3\text{H}$ ]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells to inhibition by vanadate, vinblastine, and verapamil

Uptake was measured as described in Table I except that  $5 \mu\text{M}$  gramicidin D was included in all of the uptake media. The concentrations of the compounds causing 50% inhibition of uptake ( $I_{50}$  values) were estimated by nonlinear least squares analysis after fitting the data to a single negative exponential (Marquardt, 1963).

	DTY165	$I_{50}$	DTY167
		$\mu\text{M}$	
Vanadate	179.1		Insensitive
Vinblastine	88.8		>500
Verapamil	202.6		Insensitive

pared from whole spheroplast homogenates of DTY165 cells, vacuolar membrane vesicles derived from vacuoles purified by the Ficoll flotation technique (see "Materials and Methods") were coordinately enriched for DNP-GS uptake and both of the vacuolar membrane markers assayed,  $\alpha$ -mannosidase and bafilomycin  $A_1$ -sensitive ATPase (V-ATPase) activity. The respective enrichments of MgATP-dependent, uncoupler-insensitive DNP-GS uptake,  $\alpha$ -mannosidase and bafilomycin  $A_1$ -sensitive ATPase activity were 28-, 53- and 22-fold. By contrast, the vacuolar membrane fraction was 4.5-, 6.3-, 11.1- and 4.3-fold depleted of NADPH cytochrome *c* reductase (endoplasmic reticulum), latent GDPase (Golgi), vanadate-sensitive ATPase (plasma membrane), and azide-sensitive ATPase activity (mitochondrial inner membrane), respectively. Accordingly, when vacuolar membrane vesicles derived from Ficoll-fractionated vacuoles were subjected to further fractionation on linear 10–40% (w/v) sucrose density gradients, MgATP-dependent, uncoupler-insensitive [ $^3\text{H}$ ]DNP-GS uptake,  $\alpha$ -mannosidase and bafilomycin  $A_1$ -sensitive ATPase activity were found to comigrate and exhibit identical density profiles (Fig. 4).

**Plasmid-encoded YCF1 Mediates Vacuolar DNP-GS Transport and CDNB Resistance**—Immunoblots of vacuolar membranes from pYCF1-HA-transformed DTY165 or DTY167 cells, probed with mouse anti-HA monoclonal antibody, demonstrated incorporation of YCF1-HA polypeptide into the vacuolar membrane fraction (Fig. 5B). Immunoreaction with the 12CA5 epitope was not detectable in lanes loaded with membranes from pRS424-transformed cells but the same quantities of membranes prepared from pYCF1-HA-transformed cells yielded a single intensely immunoreactive band with an electrophoretic mobility ( $M_r = 156,200$ ) commensurate with a computed mass of 172 kDa for the fusion protein encoded by YCF1-HA (Fig. 5B).

TABLE IV

Direct participation of the plasmid-borne *YCF1-HA* gene product in DNP-GS transport and CDNB detoxification was verified by the finding that vacuolar membrane vesicles purified from pYCF1-HA-transformed DTY167 cells exhibited a 6-fold enhancement of MgATP-dependent, uncoupler-insensitive [<sup>3</sup>H]DNP-GS uptake (Fig. 5A) which was accompanied by a decrease in the susceptibility of such transformants to growth retardation by exogenous CDNB (Fig. 6). Whereas pYCF1-HA-transformed DTY167 cells exhibited a similar resistance to growth retardation by CDNB as untransformed DTY165 cells (compare Fig. 6B with Fig. 1A), the same mutant strain showed neither increased vacuolar DNP-GS transport *in vitro* nor decreased susceptibility to CDNB *in vivo* after transformation with parental plasmid pRS424, lacking the YCF1-HA insert (Fig. 6B).

*Vacuolar Accumulation of Monochlorobimane-GS in Vivo*—Monochlorobimane, a membrane-permeant, nonfluorescent compound, is specifically conjugated with GSH by cytosolic glutathione *S*-transferases (GSTs) to generate the intensely fluorescent, membrane-impermeant *S*-conjugate, monochlorobimane-GS (Shrieve *et al.*, 1988; Oude Elferink *et al.*, 1993; Ishikawa *et al.*, 1994). Given that the GS-*X* pumps of both animal and plant cells exhibit activity toward a broad range of *S*-conjugates, including monochlorobimane-GS (Ishikawa *et al.*, 1994; Martinoia *et al.*, 1993), and DNP-GS uptake by the yeast enzyme was reversibly inhibited by this compound (Table II), suggesting competition between monochlorobimane-GS

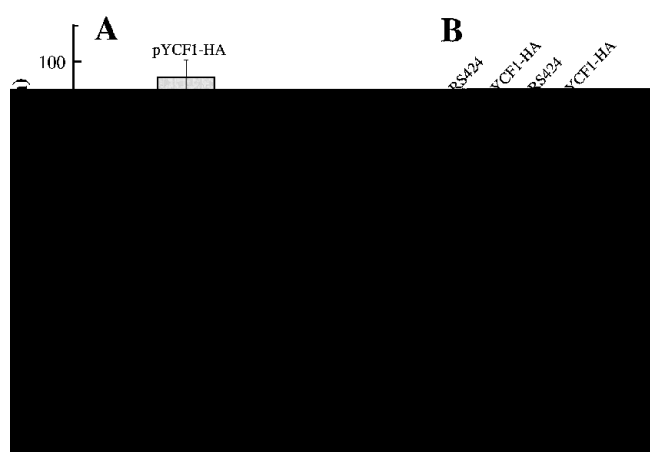


FIG. 5. A, Effect of transformation with pYCF1-HA or pRS424 on MgATP-dependent, uncoupler-insensitive [<sup>3</sup>H]DNP-GS uptake by vacuolar membranes purified from DTY165 and DTY167 cells. Uptake was measured in standard uptake medium containing 66.2  $\mu$ M [<sup>3</sup>H]DNP-GS and 5  $\mu$ M gramicidin D. B, immunoreaction of vacuolar membrane proteins prepared from pYCF1-HA-transformed and pRS424-transformed DTY165 and DTY167 cells with mouse monoclonal antibody raised against the 12CA5 epitope of human influenza hemagglutinin. All lanes were loaded with 25  $\mu$ g of delipidated membrane protein and subjected to SDS-polyacrylamide gel electrophoresis and Western analysis as described under "Materials and Methods." The *M<sub>r</sub>* of YCF1-HA (*boldface type*) and the positions of the *M<sub>r</sub>* standards are indicated.

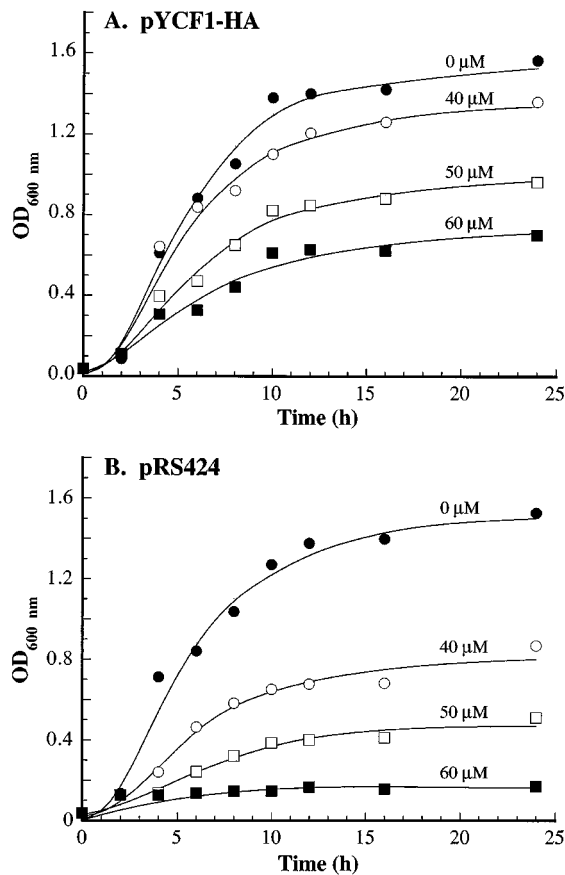


FIG. 6. Effect of transformation with pYCF1-HA (A) or pRS424 (B) on sensitivity of DTY167 cells to growth retardation by CDNB. Cells were grown at 30 °C for 24 h to an  $OD_{600\text{ nm}}$  of approximately 1.4 in AHC medium before inoculation of aliquots into 15-ml volumes of the same medium containing 0–60  $\mu\text{M}$  CDNB.  $OD_{600\text{ nm}}$  was measured at the times indicated.

and DNP-GS for a common uptake mechanism, exogenous monochlorobimane satisfies the minimum requirements of a sensitive probe for monitoring the intracellular transport and localization of its *S*-conjugate.

Fluorescence microscopy of DTY165 and DTY167 cells after incubation in growth medium containing monochlorobimane provided direct evidence that YCF1 contributes to the vacuolar accumulation of its glutathione *S*-conjugate by intact cells (Fig. 7). DTY165 cells exhibited an intense punctate fluorescence, corresponding to the vacuole as determined by Nomarski microscopy, after 6 h of incubation with monochlorobimane (Fig. 7, A and C). The fluorescence associated with vacuolar monochlorobimane-GS was by comparison severely attenuated in most, and completely absent from many, DTY167 cells (Fig. 7, B and D).

#### DISCUSSION

The experiments described demonstrate that uptake of the model glutathione *S*-conjugate, DNP-GS, by vacuolar membrane vesicles isolated from *S. cerevisiae* proceeds by two parallel pathways: one that is directly energized by MgATP and another that is driven by the inside-positive  $\Delta\psi$  established by the V-ATPase (Fig. 8). Of these two pathways, only the former is catalyzed by YCF1. Vacuolar membrane vesicles purified from the wild type strain, DTY165, are competent in both MgATP-dependent, uncoupler-insensitive uptake and uncoupler-sensitive uptake but membranes from the *ycf1* $\Delta$  strain, DTY167, are more than 90% deficient in MgATP-dependent, uncoupler-insensitive uptake.

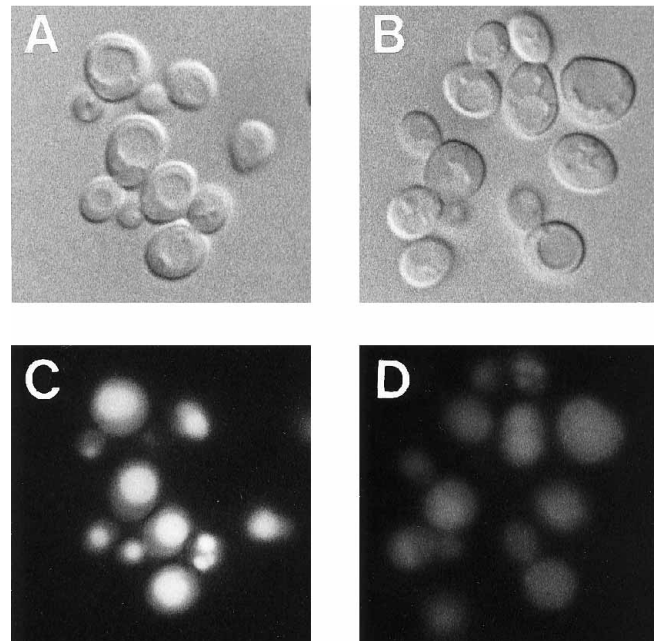


FIG. 7. Photomicrographs of DTY165 (A and C) and DTY167 cells (B and D) after incubation with monochlorobimane. Cells were grown in YPD medium for 24 h at 30 °C and 100- $\mu\text{l}$  aliquots of the suspensions were transferred into 15-ml volumes of fresh YPD medium containing 100  $\mu\text{M}$  monochlorobimane. After incubation for 6 h, the cells were washed and examined in fluorescence (C and D) or Nomarski mode (A and B) as described under "Materials and Methods."

Three primary findings implicate MgATP, rather than a preformed electrochemical gradient, as the immediate energy source for YCF1-mediated DNP-GS transport. (i) Abrogation of the development of an inside-acid, inside-positive  $\Delta\bar{\mu}_{\text{H}^+}$  across the vacuolar membrane by uncouplers (gramicidin D and FCCP) exerts little or no effect on the rate or extent of YCF1-mediated uptake. (ii) Total abolition of the capacity of the V-ATPase, coresident on the vacuolar membrane, for  $\text{H}^+$ -translocation by treatment with bafilomycin  $\text{A}_1$ , does not inhibit YCF1-catalyzed uptake. (iii) Nonhydrolyzable ATP analogs, such as AMP-PNP, do not support YCF1-catalyzed uptake. Findings (i) and (ii) unequivocally exclude energization by  $\Delta\bar{\mu}_{\text{H}^+}$ . Finding (iii), in conjunction with the sensitivity of YCF1-mediated transport to inhibition by the phosphoryl transition state analog, vanadate, and the results of previous investigations demonstrating that the integrity of Phe<sup>713</sup> in the first NBF of YCF1 is essential for cadmium detoxification (Szczyepka *et al.*, 1994), implies a requirement for hydrolysis of the  $\gamma$ -phosphate of ATP, possibly with formation of a phosphoenzyme intermediate, rather than nucleoside phosphate-elicited gating of an otherwise exergonic pathway.

YCF1-independent uptake, by contrast, has the characteristics of a DNP-GS (anion) uniport driven by the inside-positive  $\Delta\psi$  established as a result of electrogenic  $\text{H}^+$ -translocation by the V-ATPase. Much of the DNP-GS transport activity remaining in the vacuolar membrane fraction from *ycf1* $\Delta$  mutants is abolished by V-ATPase inhibitors and electrogenic uncouplers (FCCP and gramicidin D) but unaffected by vanadate, verapamil, vinblastine, and electroneutral uncouplers ( $\text{NH}_4\text{Cl}$ ) (Fig. 8).

The functional resemblance between the YCF1-encoded DNP-GS transporter of *S. cerevisiae* and the GS-*X* pumps of animal and plant cells is striking. Like YCF1-mediated transport, DNP-GS uptake by membrane vesicles derived from rat liver canalculus (Akerboom *et al.*, 1991; Kobayashi *et al.*, 1990), MRP1-transfected human cells (Leier *et al.*, 1994;

Muller *et al.*, 1994) and plant vacuoles (Martinoia *et al.*, 1993; Li *et al.*, 1995) is sensitive to inhibition by vanadate and GSSG but not GSH. Moreover, in strict correspondence with the functional characteristics of MRP1 (Muller *et al.*, 1994) and the plant vacuolar GS-*X* pump (Li *et al.*, 1995), YCF1-catalyzed DNP-GS transport is also subject to inhibition by *S*-conjugates of herbicides (*e.g.* metolachlor-GS) and the alkaloids, vinblastine and verapamil. These similarities, in combination with the congruence of their kinetic parameters, notably  $K_{m(\text{DNP-GS})}$  and  $K_{m(\text{MgATP})}$  (see Table IV in Li *et al.*, 1995), suggest that the glutathione-conjugate transporters of animals, plants, and yeast are catalytically equivalent. Two important corollaries therefore follow. First, YCF1 is not only a structural homolog of MRP1 but also a functional homolog. By implication, the yeast *YCF1* gene and *ycf1* $\Delta$  mutants, respectively, fulfill the requirements of probes for and null backgrounds against which the corresponding genes from molecularly less well characterized systems, such as plants, may be identified. Second, it is probable that MRP1 and YCF1 serve similar roles *in vivo*. Since the glutathione *S*-conjugation reaction mediated by cytosolic GSTs is known to be instrumental in the detoxification of lipophilic electrophiles derived from exogenous or endogenous sources, it has been proposed that through the concerted actions of GSTs and the GS-*X* pump, mammalian and plant cells can confer a common structural determinant on and increase the water solubility of the toxins in question and thereby eliminate them from the cytosol by MgATP-dependent transport (Ishikawa *et al.*, 1991; Martinoia *et al.*, 1993; Li *et al.*, 1995). The existence of ostensibly the same transport function in the vacuolar mem-

will also directly impinge on xenobiotic detoxification in plants and bioremediation in general.

## REFERENCES

- Akerboom, T. P. M., Narayanaswami, V., Kunst, M., and Sies, H. (1991) *J. Biol. Chem.* **266**, 13147–13152
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. O., Siedman, J. G., Smith, J. A., and Struhl, K. (1987) *Current Protocols in Molecular Biology*, Wiley, New York
- Balzi, E., and Goffeau, A. (1994) *J. Bioenerg. Biomembr.* **27**, 71–76
- Cole, S. P., Bhardwaj, G., Gerlach, J. H., Mackie, J. E., Grant, C. E., Almquist, K. C., Stewart, A. J., Kurz, E. U., Duncan, A. M. V., and Deeley, R. G. (1992) *Science* **258**, 1650–1654
- Higgins, C. F. (1995) *Cell* **82**, 693–696
- Hyde, S. C., Elmsley, P., Hartshorn, M. J., Mimmack, M. M., Gileadi, U., Pearce, S. R., Gallagher, M. P., Gill, D. R., Hubbard, R. E., and Higgins, C. F. (1990) *Nature* **346**, 362–365
- Ishikawa, T. (1992) *Trends Biochem. Sci.* **17**, 463–468
- Ishikawa, T., and Akimura, K. (1995) in *International ISSI-Workshop on Glutathione-S-Transferases* (Vermeulen, N. P. E., Mulder, G. J., Niewenhuyse, H., Peters, W. H. M., van Bladeren, P. J., eds), Taylor & Francis, London, in press
- Ishikawa, T., Wright, C. D., and Ishizuka, H. (1994) *J. Biol. Chem.* **269**, 29085–29093
- Kim, E. J., Zhen, R.-G., and Rea, P. A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 6128–6132
- Kim, E. J., Zhen, R.-G., and Rea, P. A. (1995) *J. Biol. Chem.* **270**, 2630–2635
- Kobayashi, K., Sogame, Y., Hayashi, K., Nocotera, P., and Orrenius, S. (1990) *FEBS Lett.* **240**, 55–58
- Kosower, E. E., and Pazhenchevsky, B. (1980) *J. Am. Chem. Soc.* **102**, 4983–4993
- Kubota, S., Yoshida, Y., Kumaoka, H., and Furumichi, J. (1977) *J. Biol. Chem.* **81**, 197–201
- Kunst, M., Sies, H., and Akerboom, T. P. M. (1989) *Biochim. Biophys. Acta* **983**, 123–125
- Leier, I., Jedlischky, G., Buchholz, U., Cole, S. P., Deeley, R. G., and Keppler, D. (1994) *J. Biol. Chem.* **269**, 27807–27810
- Li, Z.-S., Zhao, Y., and Rea, P. A. (1995) *Plant Physiol.* **107**, 1257–1268
- Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math.* **11**, 431–441
- Martinoia, E., Grill, E., Tommasini, R., Kreuz, K., and Amrhein, N. (1993) *Nature* **364**, 247–249
- Muller, M., Meijer, C., Zaman, G. J. R., Borst, P., Scheper, R. J., Mulder, N. H., de Vries, E. G. E., and Jansen, P. L. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 13033–13037
- Opheim, G. W. (1978) *Biochim. Biophys. Acta* **524**, 121–125
- Oude Elferink, R., Bakker, C. K. K., Roelofsen, H., Middelkoop, E., Ottenhoff, R., Heijn, M., and Jansen, P. L. M. (1993) *Hepatology* **17**, 343–444
- Parry, R. V., Turner, J. C., and Rea, P. A. (1989) *J. Biol. Chem.* **264**, 20025–20032
- Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346–356
- Rea, P. A., and Turner, J. C. (1990) *Methods Plant Biochem.* **3**, 385–405
- Rea, P. A., Britten, C. J., and Sarafian, V. (1992) *Plant Physiol.* **100**, 723–732
- Ruetz, S., and Gros, P. (1994) *Cell* **77**, 1071–1081
- Roberts, C. J., Raymond, C. K., Yamashiro, C. T., and Stevens, T. H. (1991) *Methods Enzymol.* **194**, 644–661
- Sherman, F., Fink, G. R., and Hicks, J. B. (1983) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Shrieve, D. C., Bumps, E. A., and Rice, G. C. (1988) *J. Biol. Chem.* **263**, 14107–14114
- Singhal, R. K., Anderson, M. E., and Meister, A. (1987) *FASEB J.* **1**, 220–223
- St-Pierre, M. V., Ruetz, S., Epstein, L. F., Gros, P., and Arias, I. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 9476–9479
- Szczyzka, M., Wemmie, J. A., Moye-Rowley, W. S., and Thiele, D. J. (1994) *J. Biol. Chem.* **269**, 22853–22857
- Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) *EMBO J.* **1**, 945–951
- Wemmie, J. A., Szczyzka, M. S., Thiele, D. J., and Moye-Rowley, W. S. (1994) *J. Biol. Chem.* **269**, 32592–32597
- Wu, A., and Moye-Rowley, W. S. (1994) *Mol. Cell. Biol.* **14**, 5832–5839
- Yanagisawa, K., Resnick, D., Abeijon, C., Robbins, P. W., and Hirschberg, C. B. (1990) *J. Biol. Chem.* **265**, 19351–19355
- Zaman, G. J. R., Lankelma, J., Telling, O. V., Beijnen, J., Dekker, H., Paulusma, C., Oude Elferink, R. P. J., Baas, F., and Borst, P. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7690–7694