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The human multidrug resistance-associated protein functionally complements the yeast cadmium resistance factor 1

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ABSTRACT **A *Saccharomyces cerevisiae* strain with a disrupted yeast cadmium resistance factor (*YCF1*) gene (DTY168) is hypersensitive to cadmium. YCF1 resembles the human multidrug resistance-associated protein MRP (63% amino acid similarity), which confers resistance to various cytotoxic drugs by lowering the intracellular drug concentration. Whereas the mechanism of action of YCF1 is not known, MRP was recently found to transport glutathione *S*-conjugates across membranes. Here we show that expression of the human *MRP* cDNA in yeast mutant DTY168 cells restores cadmium resistance to the wild-type level. Transport of *S*-(2,4-dinitrobenzene)-glutathione into isolated yeast microsomal vesicles is strongly reduced in the DTY168 mutant and this transport is restored to wild-type level in mutant cells expressing *MRP* cDNA. We find in cell fractionation experiments that YCF1 is mainly localized in the vacuolar membrane in yeast, whereas MRP is associated both with the vacuolar membrane and with other internal membranes in the transformed yeast cells. Our results indicate that yeast YCF1 is a glutathione *S*-conjugate pump, like MRP, and they raise the possibility that the cadmium resistance in yeast involves cotransport of cadmium with glutathione derivatives.**

Members of the adenosine 5′-triphosphate (ATP)-binding cassette family (ABC) of transporter proteins play a central role in cellular detoxification processes in animals (1), fungi (2, 3), and plants (4, 5). This is illustrated by multidrug resistant cancer cells, resistant against a range of drugs with different chemical structures and cellular targets following exposure to a single cytotoxic drug (1). At least two ABC transporters can contribute to multidrug resistance in human cancer cells: The *MDR1 P*-glycoprotein (for review see ref. 1) and the multidrug resistance-associated protein (MRP) (6). Both are plasma membrane glycoproteins that can lower the intracellular concentration of natural product drugs (1, 7, 8).

Another cellular detoxification mechanism with broad substrate specificity is the enzymatic (transferase-mediated) or nonenzymatic association of compounds with the thiol group of glutathione (GSH). The resulting GSH *S*-conjugates are eliminated from the cell by an ATP-dependent transport activity. This transport activity is known as the GS-X pump (see ref. 9), multispecific organic anion transporter (MOAT; see ref. 10), or the leukotriene C_4 (LTC_4) transporter (see ref. 11). These transporters have not only been found in many mammalian cell types (9), but also in yeast (3) and plant cells (4, 12). They have a relatively broad substrate specificity, but prefer substrates containing a large hydrophobic moiety and two negative charges (9, 10). GS-X pumps are also involved in the detoxification of heavy metals (13–15).

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Several recent studies indicate that the human MRP is one of these elusive GS-X pumps: (*i*) Overexpression of *MRP* in human cancer cells resulted in an increased ATP-dependent transport of GSH *S*-conjugates into isolated plasma membrane vesicles (16, 17). (*ii*) Transfection of an *MRP* cDNA in pig kidney epithelial cells increased the basolateral export of a GSH *S*-conjugate (18). (*iii*) The export of anti-cancer drugs by MRP requires intracellular GSH (19, 20). (*iv*) Overexpression of *MRP* increased the efflux of GSH from human lung cancer cells exposed to arsenite (20). The latter result supports the hypothesis that the GS-X pump transports arsenite as a complex with GSH (17, 20) and provides an explanation for the observed cross-resistance of some *MRP*-overexpressing cells against arsenite (8).

The yeast *Saccharomyces cerevisiae* contains a gene, denoted yeast cadmium resistance factor 1 (*YCF1*) that encodes a transporter with extensive homology (63% amino acid similarity) with human MRP (21). Disruption of the *YCF1* gene resulted in a yeast strain (DTY168) hypersensitive to cadmium (21). In this paper we demonstrate that heterologous expression of a human *MRP* cDNA can complement this deficiency. Furthermore, transport experiments with microsomal vesicles revealed that DTY168 cells had a strongly decreased GS-X transport activity, which was also complemented by human MRP. These data indicate that MRP and YCF1 are not only closely related in sequence but also in function.

MATERIALS AND METHODS

Chemicals. [^{14}C]-*S*-(2,4-dinitrobenzene)-glutathione ([^{14}C]DNB-GS) (10 Ci/mol; 1 Ci = 37 GBq) was synthesized as described (22) and purified using a C_{18} reversed phase column. Other chemicals were from Sigma, unless stated otherwise.

Strains. Cloning in *Escherichia coli* was performed in strain DH5α. The isogenic yeast strains DTY7 (*Matα, ura 3–52, leu2–3,-112, his6*) and DTY168 (*Matα, ycf1Δ::hisG, ura 3–52, leu2–3,-112, his6*) were used for heterologous expression of the *MRP* cDNA.

Construction of the MRP Expression Vector. To excise the untranslated 5′ end of the cDNA encoding MRP (7), two primers were synthesized. The sense primer MRP1S [positions 196 to 212 as published (7)] (5′-ATAAGAATGCGGCCGCG-TAAAAGAAATGGCGCTCCGGGGCTT- 3′) contained a *Not*I site and the 5′ untranslated sequence of the *Arabidopsis thaliana* STP1 monosaccharide-H^+ symporter (underlined; ref. 23). The antisense primer MRP2AS corresponds to position 371 to 355 (5′-GGGAGAGATAGAGGAAG-3′). The PCR product was digested with *Not*I and *Dra*III and ligated with the MRP fragment excised from pJ3Ω-MRP after diges-

Abbreviations: DNB-GS, *S*-dinitrophenyl glutathione; MRP, multidrug resistance-associated protein; YCF1, yeast cadmium resistance factor 1; ATP, adenosine 5′-triphosphate; ABC, ATP-binding cassette; GSH, glutathione.

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tion with *Dra*III and *Kpn*I and pBluescript SK(+) prepared by digesting with *Not*I and *Kpn*I. The sequence replaced by the PCR fragment was verified by sequence analysis. A *Not*I fragment containing the *MRP* cDNA was isolated and cloned into the unique *Not*I site of the *E. coli/S. cerevisiae* shuttle vector NEV (24).

Cadmium Sensitivity. Cd^{2+} tolerance was investigated by inoculating 10 ml minimal medium (SD; 0.7% (wt/vol) yeast nitrogen base without amino acids (Difco), 0.07% (wt/vol) of complete supplement mixture supplemented with uracil as required (Bio 101), and 2% (wt/vol) glucose) to an OD_{600} of 0.01 with an overnight culture from DTY168, DTY7, DTY168(MRP), and DTY168 transfected with the empty vector, in the presence of 0, 10, 20, 40, 60, or 180 μM $CdCl_2$. The OD_{600} was measured every 12 h over a total period of 72 h.

Preparation of Vacuoles and Microsomes. Membrane fractions for Western blotting were isolated from yeast grown in SD medium (100 ml) to an OD_{600} of 0.5. Cells were harvested at 700 × *g* for 10 min, washed twice in H_20, and resuspended in 500 μl of TE (20 mM Tris·Cl, pH 7.5/1 mM EDTA/1 mM phenylmethylsulfonyl fluoride (PMSF)/1 ml leupeptin/1 ml aprotinin/1 ml pepstatin). Glass beads were added and after vortexing for 5 min, glass beads and unbroken cells were removed by centrifugation at 700 × *g* for 5 min. Subsequently, the supernatant was centrifuged at 100,000 × *g* for 30 min (in a Beckman TLA100 rotor) and the resulting pellet was dissolved in 50 μl of TE plus 0.2% SDS. The yeast microsomes for transport analysis were isolated by a modification of the procedure described by Kim *et al.* (25). Cells were grown overnight in 200 ml of YPD medium [1% (wt/vol) Bacto yeast extract (Difco)/2% (wt/vol) Bacto Peptone (Difco)/2% (wt/vol) glucose] at 30°C to an OD_{600} of approximately 10. The cells were sedimented at 1200 × *g* for 10 min, washed twice with water, and resuspended at an OD_{600} of 10 in 1.1 M sorbitol, 20 mM Tris·Cl (pH 7.6), 1 mM DTT containing 57 units of lyticase per ml. The suspension was incubated for 90 min at 30°C with gentle shaking, and spheroplasts were pelleted by centrifugation for 10 min at 1200 × *g* and resuspended in 25 ml of homogenization buffer [1.1 M glycerol/50 mM Tris-ascorbate, pH 7.4/5 mM EDTA/1 mM DTT/1.5% (wt/vol) polyvinylpyrrolidone/2 mg/ml bovine serum albumin (BSA)/1 mM PMSF/1 μg/ml leupeptin]. Experiments were done at 4°C. After disruption in a dounce homogenizer, unbroken cells and cell debris were removed by centrifugation at 4000 × *g* for 10 min. The sediment was resuspended in 10 ml of homogenization medium and homogenized and centrifuged as described above. The supernatants were pooled and centrifuged at 100,000 × *g* for 45 min (in a Beckman Ti70 rotor). The pellet was resuspended at an OD_{600} of 4 in suspension buffer (1.1 M glycerol/50 mM Tris·Mes, pH 7.4/1 mM EDTA/1 mM DTT/2 mg/ml BSA/1 mM PMSF/1 μg/ml leupeptin] and stored in liquid nitrogen. Yeast vacuoles were isolated according to Oshumi and Anraku (26). The purification of vacuoles was 4- to 5-fold with a final yield of 10–15%, as determined by measuring the α-mannosidase activity at different stages during the purification procedure. Comparison of α-mannosidase activity measured in microsomal vesicles and isolated vacuoles showed that the specific α-mannosidase activity was 2- to 3-fold higher in the vacuoles.

Uptake Experiments. Uptake of [^{14}C]DNB-GS was measured by the rapid filtration technique using nitrocellulose filters (0.45-μm pore size; Schleicher & Schuell). One part of microsomes was mixed with six parts of transport solution (0.4 M glycerol/100 mM KCl/20 mM Tris·Mes, pH 7.4/1 mM DTT), and then incubated at 25°C in the presence of 5 mM My-ATP and 40 μM of [^{14}C]DNB-GS, unless stated otherwise. Samples (100 μl) were taken at the indicated time points. Experiments with cadmium were done in the absence of DTT and BSA, which had no affect on DNB-GS uptake. The calculation of the K_m for DNB-GS was done using the Enzfitter program (version 1.05; Elsevier Biosoft, Cambridge, U.K.).

Subcellular Fractionation of Yeast Extracts. To prepare crude yeast extracts, cells growing exponentially in SD medium without uracil (200 ml cultures; OD_{600} = 0.5–0.7) were used. Subsequent steps were according to the fractionation protocol described by Kölling and Hóllenberg (27) with the modification that DTT was omitted in all steps as this made the NADPH dependent cytochrome *c* reductase assay unreliable. Briefly, lysis of the cells was achieved by vortexing in STED10 [10% (wt/vol) sucrose/10 mM Tris·Cl, pH 7.6/10 mM EDTA/1 mM PMSF/1 μl leupeptin/1 μl pepstatin A/1 μl aprotinin] in the presence of glass beads for 3 min. The cell extract was cleared (700 g, 10 min), and loaded onto a sucrose gradient prepared as follows. STED53, STED35, and STED20 [53, 35, and 20% (wt/vol) sucrose, respectively, 10 mM Tris·Cl (pH 7.6), 1 mM EDTA] were layered in this order in a centrifuge tube (4 ml each; Beckman SW41 rotor). After loading with extract (0.5 ml), gradients were spun for 17 h at 30,000 rpm at 4°C. Fractions (700 μl) were collected from the top of the gradient. Marker proteins were detected by Western blotting and incubation with rabbit polyclonal antibodies against the yeast plasma membrane ATPase (PMATPase), the Golgi protein dipeptidyl aminopeptidase A (DPAPA), and a monoclonal antibody against MRP (MoAb MRPr1; ref. 28). The activity of the endoplasmic reticulum marker NADPH-dependent cytochrome *c* reductase was measured in a reaction mixture consisting of 40 mM KP_i (pH 7.8), 0.4 mM NADPH, 0.1% (vol/vol) Triton X-100, 1 μM Rotenone, 70 μM cytochrome *c*, complemented with 10–20 μl of the gradient fractions. The increase in absorbance at 550 nm was followed for 7.5 min in a Cobas analyzer (Roche Clinical Laboratories, Burlington, NC). The reaction was linear over this time period. Activity of the vacuolar marker α-mannosidase was measured as described (29), with the modification that 4-methylumbelliferyl-α-D-mannopyranoside was used as the substrate.

RESULTS

Expression of Human MRP in Yeast Cells Lacking YCF1. To investigate whether human *MRP* cDNA could complement *S. cerevisiae* lacking the cadmium resistance factor (YCF1), we transformed yeast cells without a functional *YCF1* gene (strain DTY168; ref. 21) with a yeast expression vector containing human *MRP* cDNA. Protein blot analysis of a crude membrane fraction from transformed cells [DTY168(MRP)] showed that these cells contained a substantial amount of a 170-kDa protein reacting with an anti-MRP MoAb (MRPr1; ref. 28). This protein was not detected in untransformed DTY168 cells (Fig. 1, lanes 1 and 2). The molecular mass of human MRP made in the pig kidney epithelial cell line LLC-PK1 transfected with *MRP* cDNA is approximately 190 kDa (Fig. 1, lane 3; ref. 18). The difference in size of MRP present in yeast and mammalian cells is probably caused by reduced or absent *N*-glycosylation in yeast cells. This has also been observed for *P*-glycoprotein expressed in yeast (30). Glycosylation of MRP is not required for its function in multidrug resistance, as inhibitors of glycosylation did not affect drug resistance of *MRP*-overexpressing cells (for review see ref. 37).

To investigate whether MRP could complement the Cd^{2+} sensitivity of DTY168 cells, cultures of DTY168, DTY168(MRP), and DTY7 wild-type cells were exposed to various concentrations of $CdCl_2$ and growth was measured for 72 h. The IC_{50} values for the different strains are shown in Table 1. Expression of *MRP* restores cadmium tolerance in the cadmium sensitive DTY168 cells to the wild-type level. The presence of the empty vector did not affect cadmium tolerance.

Uptake of DNB-GS into Yeast Microsomal Vesicles. It has been shown that overexpression of human *MRP* cDNA in tumor cells results in an increased ATP-dependent transport

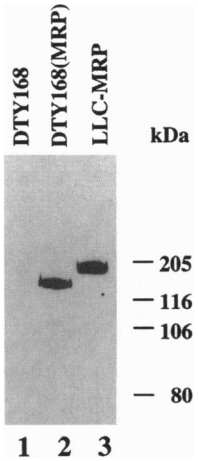

FIG. 1. Detection of MRP in crude membrane fractions from DTY168, DTY168(MRP), and LLC-MRP cells. Protein (5 μg) was size fractionated in a 7.5% polyacrylamide gel containing 0.1% (wt/vol) SDS. After electroblotting, MRP was visualized with MoAb MRPr1 (28). Protein antibody interaction was detected using the enhanced chemiluminescence technique. The source of the membrane fractions is indicated over the lanes.

activity of the GSH conjugates DNB-GS and LTC_4 into isolated plasma membrane vesicles prepared from such cells (17, 32). ATP-dependent transport of DNB-GS was also shown in membrane vesicles isolated from an *S. cerevisiae* secretory mutant (3), indicating that these cells also contain a GS-X pump. YCF1 could be responsible for this activity, based on its homology with MRP. To verify this, microsomal vesicles (a mixture of vesicles derived from internal membranes and the plasma membrane) were prepared from DTY168, DTY168(MRP), and DTY7 cells and assayed for ATP-dependent DNB-GS uptake. Microsomal vesicles from DTY168(MRP) and DTY7 showed a time-dependent accumulation of DNB-GS. This accumulation continued for at least 12 min for DTY168(MRP) and DTY7 (Fig. 2). The uptake activity of the DTY168(MRP) vesicles over 12 min was roughly 1.5-fold higher than that of the wild-type DTY7 vesicles, whereas microsomes isolated from DTY168 cells accumulated DNB-GS only at a very low rate. The ATP-dependent DNB-GS uptake in DTY168(MRP) vesicles is a saturable process (Fig. 3). The apparent K_m value was calculated to be 23 ± 3 μM. Uptake of DNB-GS was not stimulated by the nonhydrolyzable ATP analogue AMP-PNP and was inhibited by 1 mM vanadate (Table 2). In contrast, NH_4^+, which dissipates the pH gradient build-up by proton pumps (33) and thus secondarily affects transport processes dependent on activated proton gradients, did not inhibit the uptake of DNB-GS. Decyl-GS inhibited the uptake of DNB-GS almost completely, oxidized GSH (GSSG) was less effective, whereas GSH had no effect. Probenecid and sulfinpyrazone, which effectively inhibits MRP mediated export from mammalian cells (18, 34), also had an inhibitory effect.

Table 1. Relative resistance of yeast strains to cadmium

Strain	IC_{50}, μM
DTY7	46.7 ± 7.3
DTY168	8.1 ± 0.6
DTY168(MRP)	47.7 ± 7.4
DTY168(NEV)	9.5 ± 3.0

IC_{50} values for DTY7, DTY168, DTY168(MRP), and DTY168(NEV) (DTY168 containing the empty expression vector) were determined. The IC_{50} values are the $CdCl_2$ concentrations where the growth rate was 50% of the growth rate in the absence of $CdCl_2$. Values are the means of three independent experiments.

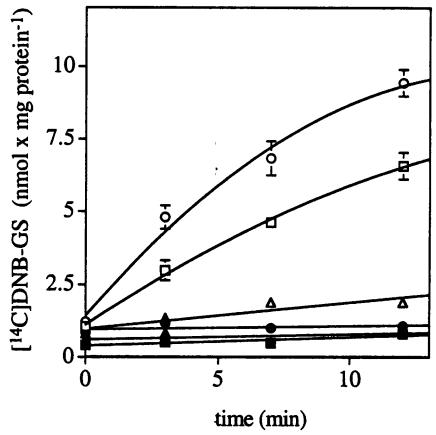

FIG. 2. Time dependent [^{14}C]DNB-GS uptake in microsomal vesicles from yeast cells. Yeast microsomal vesicles from DTY7 (squares), DTY168(MRP) (circles), and DTY168 with vector alone (triangles) were incubated in the presence of 40 μM of [^{14}C]DNB-GS. Samples were taken at t = 0, 3, 7, and 12 min. Open and solid symbols represent uptake in the presence and absence of Mg-ATP, respectively. Experiments were carried out in triplicate. Bars indicate SD.

Subcellular Localization of MRP in DTY168(MRP). Despite several attempts with various anti-MRP antibodies we were not able to reliably immunolocalize MRP in DTY168(MRP) cells using either fluorescence or electron microscopy. To determine the subcellular localization of MRP in DTY168(MRP) cells we therefore fractionated cell extracts on a sucrose gradient. Fractions collected from the gradient were assayed by Western blot analysis for the presence of MRP, plasma membrane ATPase (PMATPase), and the Golgi protein dipeptidyl aminopeptidase A (DPAPA). Endoplasmic reticulum membranes were identified by measuring NADPH-dependent cytochrome *c* reductase (cyt. red.) activity and vacuolar membranes by determining the α-mannosidase activity. Fig. 4 shows the data from a typical fractionation experiment. Cytochrome *c* reductase activity was enriched in fractions 2–8 (Fig. 4*D*), whereas most of the PMATPase was detected in fractions 10–16 (Fig. 4*A*). The bulk of MRP was found in fractions 6–12 (Fig. 4*B*), the same fractions where the DPAPA marker protein was found (Fig. 4*C*). α-Mannosidase activity partially overlapped with the presence of MRP and was detected in fractions 6–10 (Fig. 4*D*). These data indicate that

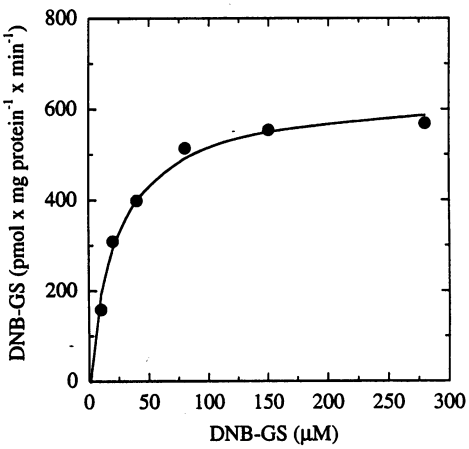

FIG. 3. Concentration dependence of [^{14}C]DNB-GS uptake into microsomal vesicles isolated from DTY168(MRP) cells. Vesicles were incubated in the presence of various concentrations of [^{14}C]DNB-GS in the absence and presence of Mg-ATP. Uptake rates after 7 min were calculated by subtracting the radioactivity measured in the absence of Mg-ATP from the radioactivity measured in the presence of Mg-ATP. The plot was made with the Enzfitter program.

Table 2. Effect of inhibitors and AMP–PNP on the uptake of DNB-GS into microsomes isolated from DTY168(MRP)

Treatment	% of control
+MgATP (control)	100
+MgATP + 1 mM vanadate	9 ± 6
+MgATP + 10 mM probenicide	42 ± 12
+MgATP + 4 mM sulfinpyrazone	14 ± 8
+MgATP + 5 mM NH_4Cl	92 ± 7
+MgATP + 1 mM GSH	99 ± 8
+MgATP + 1 mM GSSG	48 ± 12
+MgATP + 0.1 mM Decyl-GS	13 ± 7
+MgAMP–PNP	2 ± 2

Yeast microsomal vesicles were incubated in the presence of 40 μM of [^{14}C]DNB-GS, 3 mM of ATP or AMP–PNP, 5 mM $MgSO_4$, and the compounds listed in the table. Values are means of at least three independent experiments ± SD, each performed in triplicate. Uptake rates were calculated by subtracting the values determined in the absence of ATP from those in the presence of ATP.

the majority of the MRP protein is associated with intracellular membranes, but not primarily with endoplasmic reticulum or plasma membrane.

We have previously shown that plants contain a GS-X pump in the vacuolar membrane and not in the plasma membrane (4). As our fractionation experiments showed an overlap between the α-mannosidase activity and the presence of MRP we investigated whether vacuolar membranes contain functionally active MRP. Vacuoles were isolated from DTY7, DTY168(MRP), and DTY168 cells using a flotation gradient. The vacuolar fractions had a 2- to 3-fold higher specific α-mannosidase activity than the microsomal fractions, indicating that they were enriched for vacuolar membranes. Fig. 5 shows that the vacuoles isolated from DTY7 cells accumulated DNB-GS in a time and ATP-dependent manner, whereas this accumulation was 2- and 5-fold lower in vacuoles isolated from DTY168(MRP) and DTY168 cells, respectively. Comparison of the uptake rates in vacuoles and microsomes as a function of α-mannosidase activity after an incubation of 8 min indicated that in the DTY7 cells most activity was associated with vacuolar membranes (data not shown). In contrast, in the vacuolar fraction from the DTY168(MRP) cells, the specific transport activity per microgram of protein was lower than in the microsomal vesicles (compare Figs. 2 and 5). Furthermore, the ratio of DNB-GS transport activity to α-mannosidase activity was lower in the vacuolar fraction of DTY168(MRP) than in the microsomal vesicles (data not shown). We conclude that most of YCF1 is localized in the vacuolar membrane. In contrast, as already suggested by the fractionation experiment, MRP is associated both with the vacuole and with other internal membranes. The fractionation in Fig. 4 suggests that there is no significant amount of MRP in the yeast plasma membrane. This conclusion is supported by observations with monobromobimane, a compound that reacts readily with GSH. After incubation of DTY7, DTY168(MRP), or DTY168 cells with monobromobimane the resulting product, GS-bimane, was only detected intracellularly and not in the medium (data not shown).

DISCUSSION

In this report we demonstrate that a human *MRP* cDNA can restore both GSH *S*-conjugate transport activity and cadmium tolerance in yeast cells lacking the *YCF1* gene. This suggests that yeast YCF1 and human MRP are functional homologues. The properties of MRP in yeast resemble those of MRP produced in mammalian cells (17): (*i*) DNB-GS uptake is strongly ATP dependent, and the nonhydrolysable ATP analogue AMP–PNP cannot drive DNB-GS uptake in microsomal vesicles; (*ii*) uptake is strongly inhibited by decyl-GS, weakly by GSSG, and not at all by GSH; (*iii*) the apparent K_m value of MRP in yeast for DNB-GS (23 μM) is nearly the same as that

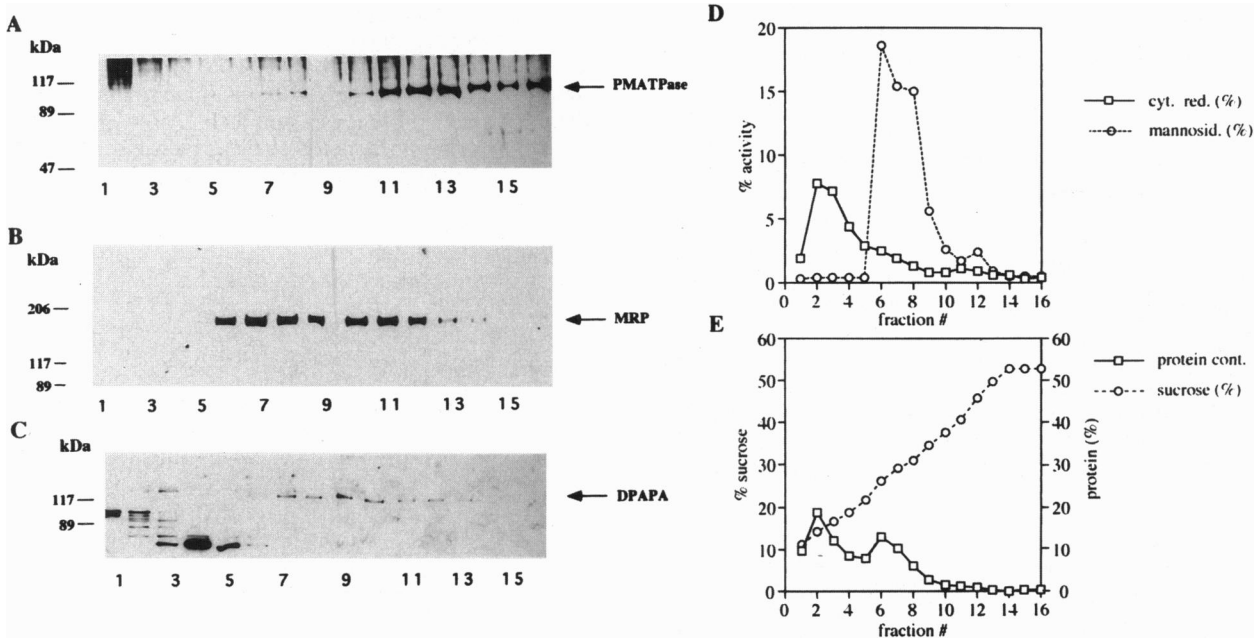

FIG. 4. Fractionation of an MRP168(MRP) homogenate on sucrose gradients. A whole-cell lysate from DTY168(MRP) cells was fractionated on a sucrose gradient. Aliquots of the gradient fractions were size-fractionated as in Fig. 1. Blots were incubated with anti-PMATPase antibodies (*A*), MoAb MRPr1 (*B*), and anti-DPAPA antibodies (*C*). The strong bands detected in lanes 1–5 of *C* with the anti-DPAPA antibodies are due to the crossreactivity of this serum with other yeast proteins. Fraction numbers are indicated below the panels. The activities measured in the NADPH-dependent cytochrome *c* reductase (cyt. red.) and α-mannosidase assay are plotted against the fraction number (*D*). Activity is expressed as the percentage of total activity recovered from the input lysate, which was loaded onto the gradient. Total recovery of the enzymatic activities were 40% and 80%, respectively. The sucrose percentage (wt/vol) and protein concentration (expressed as the percentage of total protein measured in the input lysate) are plotted against the fraction number (*E*).

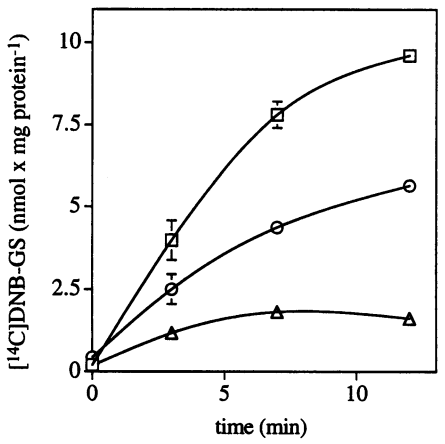

FIG. 5. Time dependent [^{14}C]DNB-GS uptake into isolated yeast vacuoles. Vacuoles isolated from DTY7 (□), DTY168(MRP) (○), and DTY168 (Δ) were incubated in the presence of 40 μM [^{14}C]DNB-GS. Samples were taken after 0, 3, 8, and 12 min. Uptake rates were calculated by subtracting the radioactivity measured in the absence of Mg-ATP (ca. 0.5 nmol DNB-GS/mg of protein during the experiment) from the values measured in the presence of Mg-ATP. Experiments were carried out in triplicate. Bars indicate SD.

of MRP present in drug resistant human lung cancer cells (30 μM; ref. 17). We conclude that heterologous expression of human *MRP* in yeast may be used as a tool for a structure/function analysis of MRP.

Our fractionation and transport experiments suggest that the majority of functional MRP is present in internal membranes and not in the plasma membrane. This is remarkably similar to what has been shown for the yeast ABC-transporter Ste6, a protein required for the secretion of the yeast pheromone **a**-factor (27). These authors speculate that Ste6 may secrete **a**-factor into vesicles that are subsequently delivered to the plasma membrane. This is reminiscent of what has been observed for the cellular localization of MRP in normal mammalian tissue cells. Although MRP is predominantly present in the plasma membrane in resistant tumor cells (7), MRP in normal lung epithelial cells is only detected in the membranes of intracellular vesicles (35). Localization of MRP in the plasma membrane of mammalian cells may therefore be due to overexpression of *MRP*, resulting in an overflow of protein toward the plasma membrane. This type of behavior is typical of proteins that reside in the trans-Golgi network (36).

Sulfinpyrazone and probenecid, known inhibitors of multispecific organic anion transport in living cells (37) are specific inhibitors of DNB-GS and cytotoxic drug export from cells overexpressing *MRP* (18, 34). We now show that both compounds are also directly inhibiting MRP *in vitro* in microsomes isolated from yeast. Probenecid and sulfinpyrazone inhibited DNB-GS uptake only by 10–20% and 40%, respectively, in microsomes isolated from wild-type DTY7 cells, suggesting that these compounds can discriminate between YCF1 and MRP (data not shown).

It has been shown that MRP can confer arsenite resistance to human cells (8), and arsenite increased the rate of GSH efflux from *MRP* overexpressing cells (20). Since it is known that arsenite can react with GSH to form an $As(SG)_3$ complex (38), it is most likely that this complex is transported by MRP (20). There is also indirect evidence for transport of a cisplatin–GSH complex by MRP (15). It is therefore tempting to speculate that MRP can also transport a cadmium–GSH complex. Such a complex is indeed known to exist (for review see ref. 39), but its structure is not known. A contribution of GSH to cadmium detoxification in mammalian cells is suggested by experiments in which LLC-PK1 kidney cells depleted of GSH showed an increased cadmium sensitivity (40). However, no resistance against cadmium has been observed in *MRP* overexpressing cells (ref. 8; G.J.R.Z., unpublished observation). Moreover, the cadmium detoxification mechanism thus far characterized in humans is binding to the carrier protein metallothionein. This complex accumulates mainly in the kidney where it has a half-life of 12–35 years (41).

How does MRP confer Cd^{2+}-resistance to yeast cells? Various mechanisms are involved in cadmium detoxification in yeast. (*i*) It has been shown that overexpression of the transcription factors CAD1 and yAP1 leads to pleiotropic drug resistance and cadmium resistance (42). However, only a mutant in which the yAP1 gene was disrupted showed cadmium hypersensitivity. (*ii*) Heavy metal complexing proteins such as metallothioneins have been described in animals, fungi, and plants. Expression of the *Arabidopsis thaliana* metallothionein genes in DTY168 cells rescues the cadmium sensitivity of these cells (unpublished results). (*iii*) Phytochelatins, GSH-derived peptides able to complex heavy metals, are present in fungi and plants. (*iv*) Finally, recent results suggest that members of the ABC transporter family can also play an important role in the detoxification of cadmium. In *Schizosaccharomyces pombe* HMT1, a "single half" ABC-transporter localized in the vacuolar membrane, is associated with heavy metal tolerance. Detoxification occurs by transport of a complex of phytochelatins and cadmium across the vacuolar membrane. Transport of GSH and cadmium could not be detected (43, 44).

The fact that MRP confers cadmium resistance to DTY168 cells in combination with the strong decrease in GS-X pump activity in the DTY168 mutant is compatible with the idea that MRP is directly involved in the transport of a cadmium–GSH complex. However, as yet, there is no experimental support for this idea. We were unable to detect ATP-dependent uptake of cadmium in the presence of GSH in our microsomal uptake system under various conditions. Furthermore, inhibition of DNB-GS transport by GSH (1 mM) and Cd^{2+} (100–300 μM) was also weak (data not shown). It is therefore possible that the role of MRP in cadmium detoxification is an indirect one. In plants it has been shown that a Cd^{2+}/H^+ antiporter exhibiting a high affinity for cadmium is localized in the vacuolar membrane (45), and that phytochelatins are transported into the vacuole by a transport system that may correspond to a GSH-conjugate ATPase (ref. 46; R.T., E. Grill, and E.M., unpublished data). Cd^{2+} transport due to a proton antiporter was also detected in *S. pombe* (44). Therefore, an alternative role of MRP in yeast cadmium tolerance could be that cadmium is detoxified by a H^+/Cd^{2+} antiporter and subsequently complexed with a cadmium binding molecule transported by MRP. Further studies on the substrate specificities of MRP are needed to settle this matter.

Note Added in Proof. A recent paper by Li *et al.* (47) shows that the yeast YCF1 protein is localized in the vacuolar membrane and that it is a glutathione *S*-conjugate transporter, in full agreement with our findings.

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