Evidence for Altered Ion Transport in *Saccharomyces cerevisiae* Overexpressing Human MDR 1 Protein[†]

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ABSTRACT: Recently [Hoffman, M. M., and Roepe, P. D. (1997) *Biochemistry 36*, 11153−11168] we presented evidence for a novel Na^+- and Cl^--dependent H^+ transport process in LR73/hu MDR 1 CHO transfectants that likely explains pH_i, volume, and membrane potential changes in eukaryotic cells overexpressing the hu MDR 1 protein. To further explore this process, we have overexpressed human MDR 1 protein in yeast strain 9.3 following a combination of approaches used previously [Kuchler, K., and Thorner, J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 2302−2306; Ruetz, S., et al. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 11588−11592]. Thus, a truncated hu MDR 1 cDNA was cloned behind a tandem array of sterile 6 (Ste6) and alchohol dehydrogenase (Adh) promoters to create the yeast expression vector pFF1. Valinomycin resistance of intact cells and Western blot analysis with purified yeast plasma membranes confirmed the overexpression of full length, functional, and properly localized hu MDR 1 protein in independently isolated 9.3/pFF1 colonies. Interestingly, relative valinomycin resistance and growth of the 9.3/hu MDR 1 strains are found to strongly depend on the ionic composition of the growth medium. Atomic absorption reveals significant differences in intracellular K^+ for 9.3/hu MDR 1 versus control yeast. Transport assays using [^{3}H]tetraphenylphosphonium ([^{3}H]TPP^+) reveal perturbations in membrane potential for 9.3/hu MDR 1 yeast that are stimulated by KCl and alkaline pH_{ex}. ATPase activity of purified plasma membrane fractions from yeast strains and LR73/hu MDR 1 CHO transfectants constructed previously [Hoffman, M. M., et al. (1996) *J. Gen. Physiol. 108*, 295−313] was compared. MDR 1 ATPase activity exhibits a higher pH optimum and different salt dependencies, relative to yeast H^+ ATPase. Inside-out plasma membrane vesicles (ISOV) fabricated from 9.3/hu MDR 1 and control strains were analyzed for formation of H^+ gradients ± verapamil. Similar pharmacologic profiles are found for verapamil stimulation of MDR 1 ATPase activity and H^+ pumping in 9.3/hu MDR 1 ISOV. In sum, these experiments strongly support the notion that hu MDR 1 catalyzes H^+ transport in some fashion and lowers membrane potential in yeast when K^+ contributes strongly to that potential. In the accompanying paper [Santai, C. T., Fritz, F., and Roepe, P. D. (1999) *Biochemistry 38*, XXXX−XXXX] the effects of ion gradients on H^+ transport by hu MDR 1 are examined.

A molecular mechanism for how human MDR[1] 1 protein overexpression confers cellular resistance to a plethora of structurally and pharmacologically unrelated hydrophobic drugs (i.e., confers tumor multidrug resistance) remains elusive. Since MDR 1 protein overexpression may be important in some cases of tumor drug resistance in the cancer clinic (particularly for myeloma and other hematopoietic malignancies), since overexpression and/or mutation of homologues of hu MDR 1 protein have also been associated with pleiotropic drug resistance in microorganisms (*1*, *2*), since MDR protein plays a role in determining permeability of the blood−brain barrier (*3*), and since hu MDR 1 protein belongs to the ABC superfamily of membrane proteins (a family that also includes the highly important CFTR and SUR ion channels), elucidation of this mechanism is extremely important for many reasons. One popular model is that MDR proteins use the energy released from ATP hydrolysis to actively pump, "flip", or "vacuum" structurally divergent drugs and other hydrophobic compounds out of the cell or the cell membrane (*3*−*5*). This model has frequently been extended to suggest that other

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[1] Abbreviations: CHO, Chinese hamster ovary; MDR, multidrug resistance; ISOV, inside-out plasma membrane vesicles; RSO, right-side-out; ABC, ATP-binding cassette; CFTR, cystic fibrosis transmembrane conductance regulator; SUR, sulfonylurea receptor; pfMDR, *P. falciparum* multidrug resistance protein; pH_i, intracellular pH; $\Delta\Psi$, plasma membrane electrical potential; V_i, intracellular volume; ΔpH, transmembrane chemical gradient in protons; TPP^+, tetraphenylphosphonium; PMSF, phenylmethanesulfonyl fluoride; SC-ura, synthetic complete yeast growth medium lacking uracil; FCS, fetal calf serum; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); BSA, bovine serum albumin; KPi, potassium phosphate buffer; PEG, poly(ethylene glycol); Mes, 2-(*N*-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; SDS/PAGE, SDS/polyacrylamide gel electrophoresis; $[KCl]_{ex}$, extracellular KCl concentration; $[K^+]_i$, intracellular K^+; $\Delta\mu_{H^+}$, proton electrochemical potential.

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ABC transporters (like pfMDR from *Plasmodium falciparum* and others) also pump or "flip" drugs across cell membranes. However, the pump model contains a number of prominent difficulties: for example, it violates the law of enzyme specificity, appears to violate the coupling principle, and appears to be inconsistent with the kinetics of passive diffusion and the energetics of partitioning for many drugs (*6*, *7*).

Thus, other theories for MDR protein-mediated drug resistance that do not violate fundamental laws of biology or chemistry have also been entertained in recent years. For example, the altered partitioning model suggests that MDR protein does not *directly* pump drugs but *indirectly* alters their cellular accumulation and intracellular partitioning through some combination of intracellular pH (pH_i), plasma membrane electrical potential ($\Delta\Psi$),[2] and/or intracellular volume (V_i) perturbations (see ref *6* for a recent review). A variety of recent observations could conceivably help to expand on the molecular details of this model: for example, (1) MDR protein dramatically inhibits normal Cl^-/HCO_3^- exchange (*8*); (2) MDR protein has been hypothesized to be a potent regulator of cellular Cl^- conductance (*9*); (3) MDR protein has been associated with unusual Cl^--gradient-stimulated H^+ transport (*8*). One or more of these observations (and perhaps others, see refs *10* and *11*) are likely key to explaining alterations in pH_i, $\Delta\Psi$, and/or V_i due to overexpression of hu MDR 1 protein (*12*) and for thus providing a molecular mechanism for the altered partitioning model.

In previous work we have favored the altered partitioning model for several reasons. First, the model does not violate the law of enzyme specificity and other key principles as does the direct drug transport model. Second, a complex ion transport function for hu MDR 1 is perfectly consistent with the function of the homologues CFTR and SUR, as well as others, and is also consistent with a large amount of recent physical−chemical data (*6*−*8*, *12*, *13*). Third, detailed thermodynamic and kinetic evidence for the presence of an outward drug pump in membranes containing MDR proteins is sparse, and some data cited as support for the existence of a drug pump harbor a variety of contradictions (reviewed in refs *6*, *7*, and *13*). Outward chemotherapeutic drug transport against a concentration gradient with a rate constant faster than passive diffusion via hu MDR 1 protein has still not been directly measured; thus, outward drug pumping by hu MDR 1 remains a hypothesis.

Alternatively, other papers have argued against the altered partitioning model (e.g., see refs *14* and *15*). However, these arguments are complicated by the previous extensive use of chemotherapeutic drug-selected cell lines or chemotherapeutic drug-conditioned transfectants to study the function of MDR proteins. That is, since chemotherapeutic drugs are potent, complex compounds, they perturb cells in many ways, and it has therefore proved difficult (if not impossible) to isolate a "pure" (solely MDR 1 protein-mediated) phenotype in drug-conditioned cells (see refs *8* and *12*). In addition, the interaction of hydrophobic compounds with biological membranes induces a variety of physical−chemical effects that can influence binding and diffusion of drugs in vesicle or proteoliposomal systems. Most arguments against the altered partitioning model do not separate effects of MDR protein from other effects caused by chemotherapeutic drug exposure and/or the physical−chemical consequences of hydrophobic drug/membrane interactions. For example, it is sometimes argued that the altered partitioning model is problematic because it cannot account for 100-fold levels of drug resistance seen in model MDR tumor cell lines (e.g., *15*). In fact, MDR protein overexpression in and of itself likely does not confer resistance greater than 10−20-fold (*12*). Resistance higher than 10−20-fold in any drug-conditioned cell line is due to additional events caused by drug exposure, not to MDR protein overexpression per se; thus any model for MDR protein does not need to account for 100-fold resistance. Clearer separation of MDR protein effects from drug selection effects is also particularly relevant with regard to studying regulation of pH_i, $\Delta\Psi$, and/or V_i in MDR cells. Exposure to some chemotherapeutic drugs affects pH_i regulation via pathways independent of hu MDR 1 protein (*16*). Arguments against hu MDR 1 influencing cell pH_i regulation based on data from drug-conditioned cells (e.g., *14*) therefore do not necessarily contradict results from "true" transfectants (e.g., *12*). We have recently shown that the phenotype exhibited by "true" hu MDR 1 transfectants not previously exposed to chemotherapeutic drugs (and thus exhibiting a "pure" hu MDR 1-mediated phenotype) is quite different from that exhibited by cells that have been exposed to chemotherapeutic drug prior to analysis and that physical−chemical interactions between drugs and membranes are sufficient to explain the altered drug partitioning unequivocally due to MDR protein overexpression alone (*8*, *12*, *17*, *18*). A huge number of studies with drug-selected or drug-conditioned MDR cell lines have been published and the conclusions used to interpret subsequent experiments; however, it is important to realize that only a handful of studies have examined the "pure" hu MDR 1-mediated cellular phenotype.

An additional strategy for probing unadulterated MDR protein function is to express it in yeast or bacteria using methods that do not require exposure of the cells to chemotherapeutic drugs and to isolate membrane fractions and vesicle preparations containing the protein. Several studies that follow this route have recently been published (*19*−*22*), but these studies have not investigated ion transport processes or the ion dependencies of MDR protein effects in these strains, which are key tests of the altered partitioning hypothesis for MDR protein function. These need to be investigated thoroughly before data with reconstituted systems can be accurately interpreted. In addition, with regard to MDR 1 protein ATPase activity, some disagreement over the stimulatory versus inhibitory effect of various hydrophobic drugs is found in the literature (*22*−*26*), although some of this may be related to differences between membrane-associated and solubilized enzyme. Additional data would be helpful in resolving these emerging controversies.

For these reasons, we have isolated and analyzed yeast strains expressing the hu MDR 1 protein. Strains were analyzed for growth rates on various media and in the

[2] Electrical membrane potential ($\Delta\Psi$) is formed when the permeability for one ion of a salt is greater than the other. The eukaryotic $\Delta\Psi$ that have previously been observed to be perturbed by hu MDR 1 overexpression are dominated by K^+ conductance under basal conditions. In contrast, a substantial contribution to $\Delta\Psi$ from K^+ conductance is only present for yeast plasma membrane under some conditions (i.e., higher pH) but not others (i.e., lower pH).

presence/absence of valinomycin and also used to prepare purified plasma membrane fractions and inside-out (ISO) membrane vesicles. Analysis of growth curve data, $\Delta\Psi$ and ΔpH of intact cells, intracellular K^+ levels, ATPase activity of plasma membrane fractions, ion dependencies of valinomycin resistance, and H^+ transport by the membrane vesicles confirms and extends predictions of the altered partitioning hypothesis for MDR protein.

MATERIALS AND METHODS

Materials. Zymolyase in powder form was from ICN Chemicals (Costa Mesa, CA); [^{3}H]TPP$^+$ (31 Ci/mmol), [^{14}C]-propionic acid (51 mCi/mmol), ^{86}RbCl (306 mCi/mmol), ^{36}Cl (as 1 M HCl; >0.2 mCi/mmol), [^{3}H]$_2$O (1 mCi/mL), and [^{14}C]methoxyinulin (7 mCi/g) were from DuPont/New England Nuclear (Wilmington, DE); phenylmethanesulfonyl fluoride (PMSF), pepstatin A, leupeptin, aprotinin, vanadate, MgATP, and amino acids were from Sigma (St. Louis, MO); components for YPD and SC yeast growth media (including Bacto yeast extract, Bacto peptone, and Bacto yeast nitrogen base extract without amino acids) were from Difco (Detroit, MI); acridine orange, nigericin, and valinomycin were from Molecular Probes (Eugene, OR). All other chemicals were reagent grade or better and used without additional purification.

Fibroblast Cell Lines and Purification of LR73 Plasma Membranes. The true hu MDR 1 transfectant cell line LR73/#27 and an appropriate control cell line LR73/neo have been described previously (*12*). These cells were grown in DME media/10% FCS with antibiotics as described (*12*), harvested by scrapping with a rubber policeman, and plasma membranes were purified by the following procedure.

To isolate purified plasma membranes from the LR73 cells, we lysed the cells by resuspending in homogenization buffer (10 mM Tris/0.25 M sucrose/0.2 mM $CaCl_2$/0.02% NaN_3) containing protease inhibitors (1 μg/mL each of aprotinin, PMSF, pepstatin A, and leupeptin), centrifuged at 1000*g* for 10 min to remove nuclei and most mitochondria, and then layered the supernatant on a 35% sucrose cushion. The mixture was centrifuged for 1 h at 95000*g* and the interface collected. The plasma membrane fraction was washed twice in 10 mM Tris/0.25 M sucrose (pH 7.3) plus protease inhibitors at 23 °C, and the final pellet was resuspended in the same buffer and stored at −70 °C.

Construction of Plasmid pFF1. A fragment harboring the Ste6 promoter and a truncated hu MDR 1 (missing the first 17 natural amino acid codons) was restricted from plasmid pYKM77 (kindly provided by Drs. K. Kuchler and J. Thorner) and subcloned behind the alcohol dehydrogenase (Adh) promoter of plasmid pVT102 (kindly provided by the Canadian Research Council). The plasmid was colony purified, and restriction analysis was used to confirm hu MDR 1 cDNA orientation relative to the tandem array of Ste6 and Adh promoters.

Yeast Strains and Culture Conditions. Saccharomyces cerevisiae w303-1A (Mat a ura3-1 leu2-3 leu2-112 ade2-1 trp1 his3) was a kind gift of Dr. Mary Ann Osley, Memorial Sloan-Kettering Cancer Center, New York, NY. *S. cerevisiae* 9.3 (Mat a leu2 ura3 trp1 ade2 trk1Δ trk2Δ ena1::His3::ena4) was kindly provided by Dr. Alonso Rodriguez-Navarro, Universidad Politechnica, Madrid, Spain.

Untransformed w303-1A was grown at 30 °C on standard YPD media. After transformation with the plasmid pVT102, pYKM77, or pFF1, cells were plated on agar made with selective minimal media (synthetic complete media lacking uracil, SC-ura). Propagation of single colonies isolated after transformation was on SC-ura. Transformed and untransformed strain 9.3 were grown under similar conditions but (with some exceptions, see Results) with additional 100 mM KCl in either the YPD or SC-ura media. Cultures grown on selective media were stored as frozen 15% glycerol suspensions and resuscitated as necessary. For growth experiments at high pH (8.0; see Results), SC-ura media was buffered with 100 mM Tris, and pH was constantly monitored and adjusted as necessary with NaOH to maintain pH near 8.0 (see Results).

Yeast Transformation. Yeast were transformed by the lithium acetate method of Ito et al. (*27*), with either 1 μg of plasmid pYKM77 (*21*), pFF1, or plasmid pVT102 (*28*) and approximately 10 μg of the carrier plasmid pEUKC (Invitrogen) to enhance transformation efficiency. Transformants were plated on selective SC-ura agar plates. Single colonies were picked, propagated in SC-ura medium to midlog to late log phase, used to prepare plasma membrane fractions or plasma membrane vesicles (see below), and screened by Western blot for hu MDR 1 expression as described (*29*). All subsequent cultures originated from frozen stock glycerol suspensions of these cultures.

Valinomycin Resistance Assays in Media of Various Salt Composition. Several groups (e.g., *21* and *22*) have made the interesting observation that yeast expressing MDR proteins are resistant to the K^+ ionophore valinomycin. The molecular basis for this resistance is unknown, but some have hypothesized that valinomycin is "pumped" from membranes by MDR proteins (*23*). We feel this explanation is highly unlikely because of the enormously high lipid:water partitioning coefficient for valinomycin and that it is more likely that the resistance is connected to altered ion transport or membrane bioenergetics in yeast expressing the protein. To test this, we performed valinomycin resistance assays for yeast plated on agar of various ionic composition (see Results). Valinomycin was added to the well-mixed liquid agar cooled to 50 °C and the solution stirred vigorously immediately before pouring plates. A defined number of exponentially growing yeast cells (counted with a hemacytometer) were then plated, and the yeast were allowed to grow 24−48 h at 30 °C. In each assay, data from three replicate plates were averaged. Results from three such assays (nine determinations in all) ± SD are reported (see Results).

Measurement of Intracellular K$^+$. Intracellular K^+ concentrations were determined by atomic absorption spectrophotometry using a Buck model 200A absorption spectrophotometer with a 766.5 nm lamp (7.5 mA, 2 nm slit). Standard K^+ solutions gave a linear response (see Results) in the 0.05−1.2 mM range (0.1−100 ppm); thus, 500 mL cultures of 9.3/pVT and 9.3/hu MDR 1 were grown overnight in SC-ura medium (typically containing 100 mM KCl; see Results) to an OD_{600} near 1, and K^+_i was extracted. Cells (5.0×10^{12} in total per sample) were collected by centrifugation, washed three times in 100 mM *N*-methylglucamine chloride (NMG-Cl) to remove extracellular K^+ under isotonic conditions, dried overnight in a vacuum oven (80 °C), and then hydrolyzed in 0.5 N HNO_3 for 12 h. The acid

hydrolysate containing intracellular K^+ was then filtered and aspirated into the Buck 200 A flame. Measurements were made in triplicate for each sample (see Results) and $[K^+]_i$ calculated using known intracellular volume (see below).

Preparation of Yeast Plasma Membrane Fraction. Overnight culture (2 L) of yeast cells grown in YPD medium to midlog phase (OD_{600} 1.4−2) was harvested at 2000*g* for 5 min at 25 °C. We note that MDR 1-expressing yeast strains can be passed once in nonselective YPD media without any loss of hu MDR 1 expression, as assayed by Western blot (data not shown). Thus "starter SC-ura cultures" were used to innoculate YPD media for growth of large-scale cultures. The cells were washed once in 10 mM Tris/10 mM DTT/5 mM EDTA, pH 7.5. The pellet was resuspended in 1.2 M sorbitol/2mM EDTA/20 mM HEPES, pH 7.0, at a ratio of 4 mL of buffer/g wet weight of cells; 5 mg of zymolyase 20T/g wet weight of cells was added to 1 mL of the same buffer, and DTT was added to the suspension to a final concentration of 10 mM. The mixture was incubated for 60 min with gentle shaking at 30° C.

All steps from this point on (except osmotic lysis) were carried out at 4 °C and in the presence of protease inhibitors (1 mM PMSF/2 μg/mL leupeptin/1 μg/mL pepstatin A/1 μg/mL aprotinin) which were added to the various buffers immediately before use.

Spheroplasts were washed twice at 2500*g* for 10 min in the same buffer without zymolyase but supplemented with protease inhibitors. They were resuspended in an equal volume of 10 mM $MgCl_2$/2 mM $CaCl_2$/2 mM $MnSO_4$/250 mM mannitol/50 mM Tris, pH 7.50, supplemented with 1.2 mg of concanavalin A/g wet weight of cells. The suspension was incubated on ice for 20 min and then layered over an equal volume of 500 mM mannitol/10 mM $MgCl_2$/50 mM Tris, pH 7.50. The spheroplasts were then centrifuged at 300*g* for 10 min and resuspended in a minimal volume of 1.2 M sorbitol/2 mM EDTA/20 mM HEPES, pH 7.0. The suspension was fast diluted into 200−300-fold volumetric excess of 10 mM Tris/5 mM $MgSO_4$/50 μg/mL RNase/10 μg/mL DNase, pH 7.50, prewarmed to 37 °C, which was stirred vigorously. Protease inhibitors were added immediately before osmotic lysis. Unlysed spheroplasts and cellular debris were collected by twice centrifuging at 400*g* for 5 min and twice at 1000*g* for 5 min, and the resultant supernatant was centrifuged again at 13500*g* for 30 min. The pellet was resuspended in 10 mM Tris/5 mM $MgSO_4$/50% glycerol (v/v), pH 7.5, and the membranes were stored in aliquots at −70 °C.

Alternatively, cells grown as above were washed twice in harvest buffer (100 mM glucose/50 mM imidazole/5 mM DTT, pH 7.5) and lysed by rapid mixing in the presence of glass beads (1.00−1.05 mm diameter; B. Braun Biotech, Allentown, PA). The platform of a conventional vortex (Fischer Scientific) was modified to accommodate 50 mL conical tubes attached to the base of the platform. A suspension of cells [10 g wet weight of cells, 20 mL of breaking buffer (50 mM imidazole/0.25 M sucrose/0.1 M glucose/1.0 mM $MgCl_2$, pH 7.50), 20 mL of glass beads] was vortexed at 3000 rpm for 20 min at 4 °C. The suspension was decanted away from the glass beads and centrifuged twice at 1000*g* and once at 3000*g* for 5 min each, to remove unlysed cells and cellular debris. The supernatant was collected and spun again at 100000*g* to collect the crude membrane fragment. These fragments were then acid-precipitated via the procedure of Goffeau and Dufour (*30*) to remove any mitochondrial membrane contamination. Resultant purified plasma membranes were stored in suspension medium (10 mM imidazole/1 mM $MgCl_2$) and analyzed by Western blot and ATPase assays as described below.

Preparation of ISO Plasma Membrane Vesicles. For preparation of inside-out membrane vesicles we followed the procedure described in Menendez et al. (*31*) with some modifications. As starting material we generally used 10 g wet weight of yeast cells, obtained from a 1−2 L overnight culture grown to midlog phase. Cells were harvested at 2000*g* for 3 min and washed once in 700 mM sorbitol/10 mM Tris, pH 7.50. The pellet was diluted to a final volume of 5 mL/g wet weight of cells, and zymolyase (1 mg/mL final concentration) and DTT (10 mM final concentration) were added. After a 1 h incubation with occasional shaking at 30 °C, the resulting spheroplasts were washed twice with 500 mM sorbitol/100 mM glucose/15 mM MES (adjusted to pH 6.50 with Tris base). Spheroplasts were resuspended in 15 mL per 10 g wet weight cells, using the same buffer and incubated for 10 min at 30 °C. The suspension was diluted into twice the volume of ice-cold 25 mM MES/5 mM EDTA/0.2% (w/v) BSA/0.2% (w/v)/casein hydrolysate/1 mM DTT, pH 6.50, containing protease inhibitors as described above. The suspension was lysed in ice-cold buffer using a Potter homogenizer and centrifuged for 3 min at 300*g*. The resulting supernatant was centrifuged for 15 min at 35000*g*. The pellet was resuspended to a final weight of 4 g using 5 mM potassium phosphate (KPi)/330 mM sucrose/1 mM DTT, pH 7.80. This suspension was added to 12 g of dextran/PEG 3350/sucrose/EDTA/DTT/KPi buffer, pH 7.80, such that the final composition was 5.7% (w/w) Dextran/5.7% (w/w) PEG 3350/5 mM KPi/330 mM sucrose/1 mM EDTA/1 mM DTT, pH 7.80. The resulting suspension was mixed thoroughly and allowed to separate into two phases on ice for 30 min until a sharp interface appeared. The upper phase was discarded, and the interphase and the lower phase, containing the ISO plasma membrane vesicles (see ref *31*), were diluted in 9 volumes of 15 mM MES/330 mM sucrose/1 mM DTT, pH 6.50, and centrifuged for 30 min at 60000*g*. The pellet was resuspended in 1.5 mL of the same buffer and an aliquot removed for an amido black protein asssay. The vesicles were aliquoted and stored under liquid nitrogen. Repeated freeze−thaw cycles revealed loss of H^+ ATPase activity (not shown), so frozen aliquots were thawed once and used immediately and any leftover was discarded. Western blot analysis revealed the protease cocktail employed was sufficient to prevent degradation of expressed MDR protein in the vesicles.

ATPase Assays. ATPase activity was measured for purified plasma membranes as the vanadate-inhibitable release of inorganic phosphate, essentially following the procedure of Chifflet et al. (*32*), with some modifications. In general, 10 μg of purified plasma membrane protein was suspended in 200 μL of 2X assay buffer (300 mM NH_4Cl/10 mM $MgCl_2$/100 mM Tris, pH 7.5). Subsequent additions of ATP (to initiate the reaction) and various modifiers (various drugs, etc.) were added along with H_2O to bring the reaction volume to 400 μL. All additions were made to tubes on ice, with ATP (to 2.5 mM final concentration) being the final addition (as an isolated drop on the side of the tube). The reaction was then initiated by mixing and incubating the tubes at

37 °C for exactly 1 min. Reactions were continued at 25 °C for various time (see Results), and the reaction was quenched by addition of 200 μL of "stop" solution (12% SDS/1% ammonium molybdate/0.5 M HCl). Immediately after stop solution was added, 200 μL of a 6% ascorbate solution (stored wrapped in foil at 4 °C but prewarmed to room temperature before use) was added, and the developing reaction was timed for exactly 10 min. Finally, 400 μL of "stabilizer" solution (2% sodium arsenate/2% sodium citrate/2% glacial acetic acid) was added and the absorbance at 700 nm measured exactly 1 h later. A standard curve was generated using stock K_2HPO_4 solutions and used to calculate mol of phosphate released/mg of membrane protein (see Results).

Whole Cell Transport Assays and Measurement of Membrane Potential. Transport assays with whole yeast cells essentially followed the procedures described in De La Pena et al. (*33*) with some modifications (see also refs *34* and *35*). Cultures were prepared by inoculating 200 mL of the appropriate media, and 12 h later the cells were harvested at midlog phase (OD_{600} 0.5−1.5). The cells were counted with a hemacytometer, centrifuged as above, washed once in the appropriate transport buffer, resuspended to 2×10^9 cells/mL, and incubated for 1 h at room temperature. All transport buffers contained 0.15 M glucose as described (*33*). The cells were centrifuged again and resuspended to the same concentration in the same buffer. For uptake experiments 50 μL of the cell suspension was added to 150 μL of the transport buffer (*33*) containing 1 μL of either 0.5 mM [^{3}H]-TPP^+ (136 mCi/ml), 1 mM [^{14}C]propionic acid (52 mCi/mL), or 2 M ^{86}RbCl (0.04 mCi/mL). At specific times, the reactions were stopped by addition of 3 mL of ice-cold transport buffer containing 20 mM $HgCl_2$ and cells trapped by filtering through Whatman GF/C filters. After the filters were washed twice with the same cold transport buffer and dried, trapped radioactivity was quantified by liquid scintillation spectrometry. Uptake at 0 time was determined by adding cells to ice-cold transport media and ice-cold wash buffer simultaneously. For experiments at pH 4.5 we used 100 mM aminocaproic-HCl as the buffer, and for experiments at pH 8.5, 100 mM Tris-Cl was the buffer. $\Delta\Psi$ and ΔpH were calculated using these uptake data, following the procedures outlined by Rottenberg (*36*).

Measurement of Intracellular and Intravesicular Water Volume. Internal water volume was determined as described in (*33*, *36*); 2×10^9 cells/mL were incubated for 30 min at room temperature in 100 mM ϵ-aminocaproic-HCl at pH 4.5 or 100 mM Tris at pH 8.5, both buffers containing 330 mM glucose. After the incubation, 1 μL of [^{3}H]$_2$O (1 mCi/mL) and 10 μL of [^{14}C]methoxyinulin (0.1 mCi/mL) were added to 0.5 mL of the cell suspension and 0.5 mL of the same buffer. The mixture was incubated at room temperature for 10 min. Two aliquots of 400 μL were removed and centrifuged for 5 min at 14 000 rpm in an Eppendorff microfuge; 20 μL of the supernatant and of the cell pellet were separated, incubated overnight in 1 mL of 1% SDS, and then counted in a scintillation counter. The internal volume was calculated as described in (*33*, *36*).

Transport Measurements with ISO Vesicles. An acridine orange-based assay for following ATP-driven acidification of ISO yeast vesicles is described in detail *31*. We used similar methods with some modifications. Acridine orange quenching upon redistribution due to formation of a H^+ gradient can be followed by monitoring changes in either the visible absorbance or fluorescence of the probe. We monitored fluorescence emission at 535 nm (465 nm excitation) with a Photon Technologies Inc. alphascan fluorometer. Vesicles were continuously mixed using a magnetic stirrer situated beneath the cuvette, and samples were rapidly injected through a custom port above the sample chamber. Our standard H^+ pumping assay used a reaction volume of 3 mL containing 10 mM MES−Tris buffer/140 mM KCl/0.1 mM Na EDTA/20 μM acridine orange/1 mM DTT/330 mM sucrose/1 mM NaN_3/0.1 mM ammonium molybdate/50 mM KNO_3/4 mM $MgCl_2$ and 75 μg of vesicle membrane protein at 37 °C. After the mixture was equilibrated for 1−2 min (see Results), ATP was added to initiate H^+ ATPase activity and formation of a H^+ gradient. In some experiments dependent upon the formation of an ion gradient (see the following paper in this issue), the order of addition of ATP and ISO vesicles was reversed. Control experiments revealed that reversing the order of ATP and vesicle additions did not affect the value of ΔpH formed.

Uptake experiments using vesicles and radioisotopes were performed similar to the whole cell transport experiments: 10 μg of vesicle membrane protein was added to 300 μL of the appropriate transport buffer as described above; 1 μL of either 1 mM [^{36}Cl] (0.5 μCi/mL) or 1 mM [^{14}C]propionic acid (52 mCi/mL) was added and the mixture incubated for 5 min at room temperature. ATPase activity was initiated by adding MgATP to a final concentration of 2 mM. The reaction was quenched by adding 3 mL of ice-cold transport buffer and immediatly filtering through Whatman nitrocellulose filters (45 μm). Filters were washed twice with 3 mL of the same buffer and radioactivity determined by liquid scintillation spectrometry.

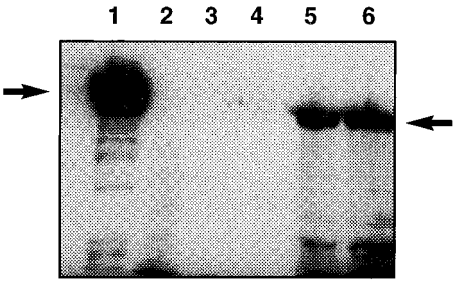

FIGURE 1: Western blot analysis of hu MDR 1 expression in yeast 9.3, using the monoclonal antibody C219. Lane 1 is 10 μg of purified plasma membrane protein for LR73/hu MDR 1 clone #27 (*12*) used as a positive control, lane 2 is a negative control for LR73/# 27 [control LR73/neo transfectant plasma membranes (*12*)], lanes 3 and 4 are 10 μg of purified plasma membrane protein for control 9.3/pVT yeast transformants, and lanes 5 and 6 are 10 μg of purified plasma membrane protein for 9.3/hu MDR 1 yeast transformants. MDR protein in the yeast is fully functional (see below) but is unglycosylated, leading to migration closer to 140 kDa (right-hand arrow), compared to 170 kDa (left-hand arrow) for the protein expressed in LR73 cells. The minor bands between 70 and 150 kDa for LR73/#27 membranes and near 30 kDa for the yeast membranes likely represent proteolytic product(s) produced during cell fractionation, since levels of protease inhibitors appear to influence their abundance (not shown).

RESULTS

Protein Expression. Figure 1 presents Western blot analysis of hu MDR 1 expression in the plasma membrane

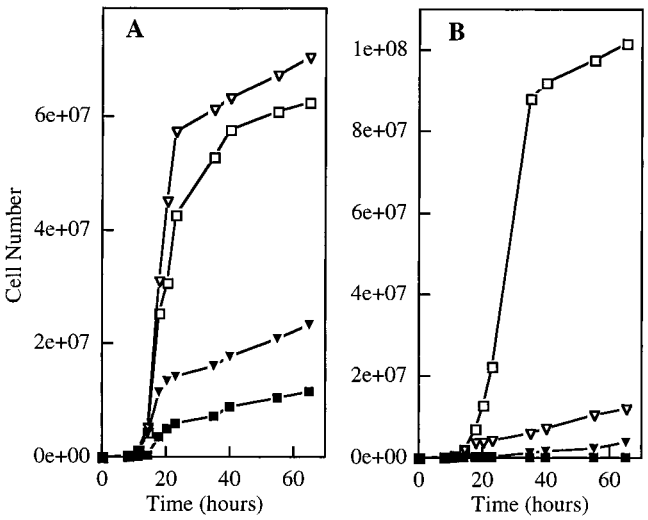

FIGURE 2: Growth of control 9.3/pVT yeast (open symbols) versus 9.3/hu MDR 1 (closed symbols) on minimal SC-ura media at either pH < 5.0 (A) or pH = 8.0 (B) containing either 5 mM (squares) or 100 mM (triangles) KCl. There is no growth for 9.3/hu MDR 1 at pH 8.0 and 5 mM KCl, but trace growth in 100 mM KCl (see text).

fraction of yeast strains we have isolated and compares this to expression for true hu MDR 1 Chinese hamster ovary (CHO) fibroblast transfectants (*12*). Levels of hu MDR 1 in our yeast strains are comparable to levels found for LR73/hu MDR 1 clone #27, a high-level-expressing CHO fibroblast hu MDR 1 transfectant clone (*12*). Thus, there appears to be no barrier to expressing hu MDR 1 protein in yeast to reasonably high levels. Moreover, similar to results from other recent studies (*22*) the MDR protein is well-localized to the plasma membrane and on SDS/PAGE gels migrates at the predicted molecular mass (140−145 kDa) for the unglycosylated species (20−30 kDa of the mass of hu MDR 1 protein expressed in higher eukaryotic cells is due to carbohydrate that is not necessarily attached in yeast nor essential for function; see also ref *22*). Thus the expressed protein is full length and properly localized.

Growth Rates. Figure 2 presents growth data for 9.3/pVT (control; open symbols) versus 9.3/hu MDR 1 yeast (closed symbols) in media at pH ≤ 5.0 (panel A) or pH 8.0 (panel B) and either 5 mM KCl (squares) or 100 mM KCl (triangles). Similar to previous results (*22*), growth of yeast expressing hu MDR 1 is slower than growth of the control strain. This is observed in either rich media (e.g., YPD, not shown) or minimal media (e.g., SC-ura, cf. Figure 2). We expressed hu MDR 1 in strain 9.3 in part because of the somewhat decreased ability of the strain to accumulate potassium (see Discussion). Therefore, although a requirement for high $[K^+]_{ex}$ is not absolute, growth of the control 9.3/pVT strain is somewhat dependent on the concentration of supplemental KCl added to minimal medium. Under standard conditions (i.e., pH_{ex} < 5.0, cf. Figure 2A) 9.3/pVT growth is slowed at low [KCl] (e.g., 5 mM KCl, open squares, Figure 2A) relative to high KCl (e.g., 100 mM, open triangles, Figure 2A). 9.3/hu MDR 1 cells are more dramatically slowed in growth (approximately 5-fold more, relative to control) when suspended in pH < 5.0 minimal media with 5 mM KCl (closed squares, Figure 2A) compared to 100 mM KCl (closed triangles, Figure 2A). That is, growth of 9.3/hu MDR 1 yeast (at pH < 5.0) is more sensitive to changes in [KCl] relative to the control strain. We wondered if this was a general ionic strength effect or an effect specific to either K^+ or Cl^-; 100 mM NaCl does not substitute for 100 mM KCl (not shown), and relative 9.3/hu MDR 1 growth increases further in high K^+ when Cl^- is also removed (i.e., in SC-ura + 100 mM KHEPES) (not shown), arguing for the latter.

We also tested growth at higher pH (pH = 8.0, Figure 2B). Control 9.3/pVT yeast grow somewhat better at pH 8.0 relative to pH < 5.0 when the KCl concentration is low (5 mM, compare open squares in Figure 2B and 2A and note different axes) but grow poorly at pH 8.0 and 100 mM KCl (open triangles, Figure 2B). That is, the effect of high KCl (growth stimulatory at low pH) is reversed for 9.3/pVT at high-pH growth conditions. This is not surprising, since high $[KCl]_{ex}$ has a potent depolarizing effect (i.e., lowers $\Delta\Psi$) for yeast (see below), and relative growth rates should depend on the magnitude of plasma membrane electrochemical potential, $\Delta\mu_H^+$ (simplistically, $\Delta\mu_H^+ = \Delta\Psi + \Delta pH$). At low external pH (Figure 2A) there is low $\Delta\Psi$/high ΔpH, and $\Delta\mu_H^+$ is thus dominated by the chemical gradient in H^+; at high pH (Figure 2B) substantial $\Delta\Psi$ exists to compensate for greatly reduced ΔpH.

However, interestingly, 9.3/hu MDR 1 cells do not grow at all at higher pH and low KCl (conditions where $\Delta\Psi$ would normally be maximal). There is trace but measurable growth (OD_{600} = 0.3 at 60 h) in media at pH 8.0 and 100 mM KCl. Also, in contrast to results at low pH (Figure 2A) high NaCl *does* substitute for high KCl in promoting growth but KHEPES does not (not shown), arguing that the effect is more Cl^- specific than K^+ specific. That is, high KCl inhibits growth for 9.3/pVT at high pH as expected (likely by lowering $\Delta\Psi$) but is paradoxically essential for very modest growth of 9.3/hu MDR 1, which is further found to depend on high $[Cl^-]_{ex}$ more than high $[K^+]_{ex}$. Also, curiously, in and of itself high pH is extremely toxic to 9.3/hu MDR 1 yeast, whereas it actually stimulates control 9.3/pVT growth in media of low $[KCl]_{ex}$.

Valinomycin Resistance. A previously described assay for the presence of functional hu MDR 1 protein in yeast is resistance to valinomycin (*21*, *22*). As described in Materials and Methods, we wondered if the basis for valinomycin resistance is at all related to the K^+ ionophore function of valinomycin, and not necessarily to valinomycin being "pumped" out of the yeast plasma membrane by MDR protein as has been suggested by others (*23*). This idea is all the more appealing because of the interesting effects of pH and $[KCl]_{ex}$ on growth of the 9.3/hu MDR 1 strains (Figure 2). Thus, 9.3/hu MDR 1 and control 9.3/pVT were plated on SC-ura agar at various [KCl] and ± 50 μM valinomycin (Table 1; note growth assays using solid agar are performed at low pH, see caption). As described (*21*, *22*) at relatively low $[KCl]_{ex}$ hu MDR 1 expression confers partial resistance to the toxic effects of valinomycin (Table 1). However, at progressively higher $[KCl]_{ex}$ 9.3/hu MDR 1 yeast appear even more resistant to valinomycin [i.e., 73% survival at 40 mM KCl (98/134) compared to 17% at 10 mM (21/122)]. Also, control 9.3/pVT yeast are much less sensitive to the toxic effects of valinomycin at $[KCl]_{ex}$ > 50 mM, and the protective effect of hu MDR 1 protein expression thus becomes less evident (Table 1). That is, >50 mM $[KCl]_{ex}$ (at external pH < 5.0) mimics the protective

Table 1: Resistance to Valinomycin versus $[K^+]_{ex}$[a]

	9.3/pVT control		9.3/hu MDR 1	
$[K^+]$, mM	−val	+val	−val	+val
10	126	0	122	21
15	151	0	119	64
40	155	0	134	98
60	136	65	134	91
110	144	114	121	98

[a] Colony formation assays for control 9.3/pVT and 9.3/hu MDR 1 yeast in the presence (+val) and absence (−val) of 50 μM valinomycin versus concentration of exogenous K^+ added to the agar. See Materials and Methods for description of the assay; in each case, the same number of yeast is plated per well of a 6-well dish, and resultant colonies were counted by eye 2 days later. Results shown are the average of three assays, with growth on each concentration of K^+ determined in triplicate in each assay (nine determinations in all, SE ≤ 7%). Valinomycin is clearly more toxic to control yeast, but toxicity appears to depend on [KCl]. The pH of the semisolid growth medium is ≤ 5.0 due to metabolism of the growing yeast; we were unable to uniformly buffer pH at high values in these experiments; thus the effect of KCl should be analogous to what is observed in Figure 2A. However, note also that colony formation assays are not analogous to growth assays performed in liquid media; the former measure survival, whereas the latter measure relative growth rates. Colonies for 9.3/hu MDR 1 were in general smaller than 9.3/pVT in these plating assays; thus, low KCl is more growth inhibitory for 9.3/hu MDR 1, but not necessarily more toxic.

Table 2: Intracellular K^+ Concentrations[a]

	$[K^+]_i$ (mM)	
conditions	9.3/pVT	9.3/hu MDR 1
pH 5.0/100 mM KCl	197	313
pH 5.0/5 mM KCl	296	427
pH 8.0/100 mM KCl	307	331
pH 8.0/5 mM KCl	190	216

[a] Intracellular concentration of K^+ determined by atomic absorption as described in Materials and Methods. Results are the average of three determinations, each determination performed in duplicate (six determinations in all) with a linear standard curve (see Materials and Methods) obtained each time. After determination of ppm K^+ for samples from a known number of yeast cells, the data were converted to concentration using determined intracellular volumes (see Materials and Methods) which were not significantly different for the two strains.

function of hu MDR 1 expression in these yeast. At high external concentrations of KCl the plasma membrane chemical gradient in K^+ obviously decreases, plasma membrane $\Delta\Psi$ decreases, and plasma membrane ΔpH increases (*33*, *37*). Thus, the toxic action of valinomycin could be due to loss of internal K^+ as it dissipates down its chemical gradient at low external K^+ (internal K^+ is nearer 200−300 mM, see below) and/or an intracellular acidification secondary to loss of $[K^+]_i$ (i.e., outward movement of K^+ promotes inward movement of H^+ to conserve electrical neutrality). A decrease in the K^+ chemical gradient would be slowed by any process that either (1) encouraged net inward translocation of K^+ or (2) hyperpolarized the membrane (raised the magnitude of $\Delta\Psi$, thereby producing a higher electrical barrier for downhill K^+ efflux) under these conditions.

Intracellular K^+. Thus, we measured intracellular K^+ concentrations ($[K^+]_i$) for these cells by atomic absorption (Table 2). When control 9.3/pVT yeast are grown at pH 5.0 in SC-ura medium plus 5 or 100 mM KCl, $[K^+]_i$ was determined to be 296 and 197 mM, respectively. Lower $[K^+]_i$ at higher external K^+ in the medium might appear paradoxical but is logical based upon effects of high external KCl on $\Delta\Psi$ (see below). 9.3/hu MDR 1 yeast grown at pH 5.0 and either 5 or 100 mM KCl harbor 427 and 313 mM $[K^+]_i$, respectively. This elevated $[K^+]_i$ under conditions that mimic the valinomycin colony formation assay (Table 1) argues that possibility 1 (increased net inward movement of K^+) is the explanation for valinomycin resistance in 9.3/hu MDR 1 yeast.

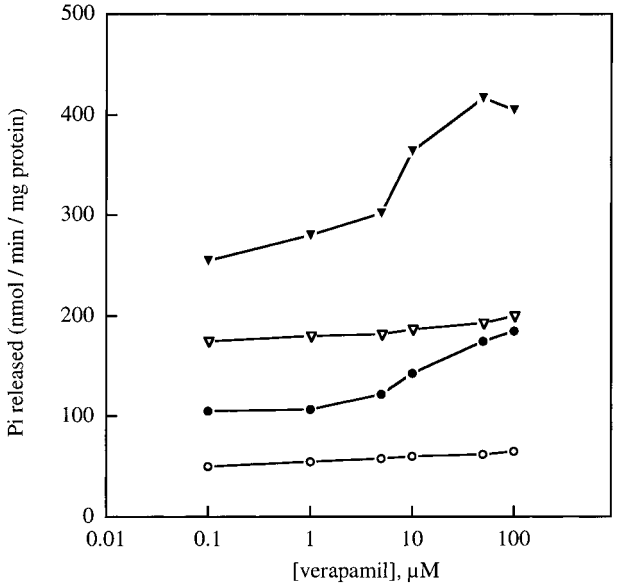

FIGURE 3: Steady-state ATPase activity (measured as vanadate-inhibitable release of inorganic phosphate, see Materials and Methods) for purified plasma membrane fractions of CHO transfectants described previously (*12*) that either do (LR73/hu MDR #27; closed circles) or do not (LR73/neo; open circles) express hu MDR 1 protein as well as purified yeast plasma membranes from either control 9.3/pVT (open triangles) or 9.3/hu MDR 1 (closed triangles) yeast. As described by others (*23*, *24*) the measured ATPase activity is time-dependent (not shown); thus a time near the plateau of phosphate release (20 min) was chosen to analyze the effects of drugs on the ATPase activity (e.g., this presented stimulation versus verapamil; see also Table 3 for effects of other drugs).

Since 9.3/hu MDR 1 yeast do not grow at high pH and low KCl (Figure 2) and grow very poorly at pH 8.0/100 mM KCl, we could not compare $[K^+]_i$ for the two strains grown under these conditions. Thus, the yeast were grown at pH 5.0 and 100 mM KCl and then resuspended at pH 8.0 and either 100 mM NMG^+Cl^- (0 KCl) or 100 mM KCl (0 NMG^+Cl^-) for 5 h before atomic absorption. $[K^+]_i$ for the two strains was found to be similar under high pH conditions. Thus, poor growth for 9.3/hu MDR 1 at high pH is likely not due to a significant change in the K^+ chemical gradient.

ATPase Activity. Many studies have shown that the calcium channel blocker verapamil resensitizes MDR tumor cells to the toxic effects of chemotherapeutic drugs, and although a mechanism has not been elucidated, via the drug pump hypothesis one explanation is that it must therefore inhibit MDR protein function. The drug has been reported to stimulate MDR protein ATPase activity under some conditions (see ref *38* for a review). Figure 3 shows that at low concentrations of verapamil, ATPase activity is approximately 2.1-fold higher for purified LR73/#27 plasma membranes, relative to control LR73/neo, and approximately 1.5-fold higher for purified 9.3/hu MDR 1 plasma membranes relative to control 9.3/pVT. Basal ATPase activity is

Table 3: Relative ATPase Activity versus Drugs[a]

[drug], μM	9.3/pVT control	9.3/hu MDR 1
verapamil		
1	102	159
10	110	220
100	114	263
vinblastine		
1	107	170
10	110	185
100	91	132
doxorubicin		
1	106	147
10	98	135
100	101	141
colchicine		
1	109	158
10	113	178
100	118	188
vanadate		
1	60	112
10	8	28
100	0	0

[a] Relative ATPase activity (measured as release of inorganic phosphate) for purified plasma membrane fractions of 9.3/pVT and 9.3/hu MDR 1 yeast. Activity for control 9.3/pVT membranes in the absence of modifier and under standard assay conditions (see Materials and Methods) is defined as 100% [thus, activity for 9.3/hu MDR 1 membranes under standard assay conditions is 152% (Figure 3), and a number less than 152 for 9.3/hu MDR 1 signifies inhibition; for example, 1 μM vanadate inhibits approximately 40% of the ATPase activity of both 9.3/pVT and 9.3/hu MDR 1]. Results shown are the average of 5−6 assays, each assay involving duplicate determinations (10−12 determinations in all, SE <6%).

higher for yeast plasma membranes relative to CHO fibroblast membranes, likely due to higher yeast H^+ ATPase versus eukaryotic Na^+/K^+ ATPase activity under these conditions.

The effects of a variety of hydrophobic drugs on the ATPase activity of MDR protein have been heavily studied (see ref *38* for a review). Some hydrophobic drugs appear to inhibit, some to stimulate, and others promote "biphasic" effects, where stimulation at comparatively lower levels of a drug is followed by return to basal levels of activity or even inhibition at higher levels of the drug. Figure 3 shows that the elevated ATPase activity attributed to hu MDR 1 protein expressed in strain 9.3/hu MDR 1 exhibits a characteristic verapamil stimulation profile, similar to the profile observed for LR73/#27. These data are similar to effects previously noted by others (*38*). We also note a milder (10−15% increase at 50−100 μM) effect of verapamil on yeast H^+ ATPase activity (in control 9.3/pVT yeast transformants) and hamster Na^+/K^+ ATPase (in control LR73/neo transfectants).

Table 3 summarizes these effects of verapamil as well as effects of several other drugs on the ATPase activity attributed to hu MDR 1 expressed in 9.3/hu MDR 1 yeast. In general, although they differ in absolute magnitude to some extent, the pattern of stimulatory and inhibitory effects of various hydrophobic drugs is similar to what others have reported in other membrane preparations (*38*). However, it is important to note that if these effects are expressed as a percent of basal activity in the absence of drug, some of the inhibitory or stimulatory effects for membranes harboring hu MDR 1 are not significantly different from those observed for control. For example, at 100 μM colchicine there is 124% of the activity at 0 colchicine for 9.3/hu MDR 1 membranes (188/152), and at 100 μM colchicine there is 118% of the activity at 0 colchicine for 9.3/pVT. That is, although the absolute increase in ATPase activity is greater for 9.3/hu MDR 1, when expressed as a percent of basal activity, colchicine stimulates the ATPase activity of both membrane preparations to essentially the same extent. Similarly, net effects of vinblastine, doxorubicin, and vanadate are similar. Of the compounds tested, only verapamil appears to stimulate the ATPase activity of membranes from 9.3/hu MDR 1 to a significantly greater extent.

Table 4: Relative ATPase Activity (% control) versus Ionic Perturbations[a]

	9.3/pVT control	9.3/hu MDR 1
pH		
5.0	12	29
6.0	62	110
7.0	98	162
8.0	41	159
9.0	7	142
[Mg^{2+}], μM		
1	2	4
10	7	3
100	5	11
1000	62	142
3000	100	165
[Ca^{2+}], μM		
1	5	3
10	2	6
100	6	7
1000	9	5
3000	12	8
[KCl], mM		
5	103	165
25	125	190
50	122	185
100	130	187
[NaCl], mM		
5	101	152
25	99	194
50	93	253
100	86	310

[a] Relative ATPase activity for purified 9.3/pVT control and 9.3/hu MDR 1 plasma membranes under a variety of ionic conditions. Activity for control 9.3/pVT membranes under standard assay conditions is defined as 100%. The effect of Ca^{2+} was measured in the absence of Mg^{2+}; the effect of pH and other salts was measured in the presence of 10 mM Mg^{2+}. Results shown are the average of 4−6 assays, each assay involving duplicate determinations (10−12 determinations in all, SE <6%).

Since we have isolated clear perturbations in Cl^--dependent H^+ transport for hu MDR 1 transfectants (*8*), we also investigated the effects of pH and various ions on MDR and yeast H^+ ATPase activities (Table 4). Ionic strength effects on MDR protein ATPase activity (or even *S. cerevisiae* H^+ ATPase) have not previously been reported to our knowledge. However, Bowman and Slayman (*39*) previously reported ionic strength effects on the plasma membrane H^+ ATPase of *N. crassa*, and effects on the *S. cerevisiae* H^+ ATPase are expected to be similar. Effects of Mg^{2+} versus Ca^{2+} on MDR protein ATPase activity have previously been examined by several groups (*38*), and effects of pH have previously been measured for plasma membrane H^+ ATPases from *N. crassa* (*39*), *S. cerevisiae* (*40*, *41*), and *C. albicans* (*42*) and optima found at slightly acidic pH values (6.0−7.0).

Table 5: ΔΨ and ΔpH versus pH_o[a]

		9.3/pVT control		9.3/hu MDR 1	
pH_o	[KCl], mM	ΔΨ	ΔpH	ΔΨ	ΔpH
4.5	0	−23	−94	−14	−101
7.5	0	−27	−73	−20	−82
8.5	0	−63	+2	−58	+5
8.5	25	−48	+10	−30	+9
8.5	50	−30	+22	−13	+21
8.5	100	−28	+20	−11	+24

[a] Membrane potential and ΔpH measured for intact yeast via TPP^+ and propionic acid distribution, respectively (see refs *33* and *36* and Materials and Methods). Results shown are the average of three assays, each assay performed in triplicate (SE < 9%). The ΔpH (in mV) is equal to $-nRT \ln([H^+]_i/[H^+]_o)$, where *T* is temperature and *R* is the gas constant (*nRT* at room temperature in this case is 25.693 mV).

As reported previously (*38*), we find the MDR ATPase activity to be highly Mg^{2+}-dependent similar to endogenous yeast H^+ ATPase and that equivalent $[Ca^{2+}]$ cannot substitute for Mg^{2+} (Table 4). In addition, we now find that increasing NaCl concentrations, which previously were reported to decrease *N. crassa* Mg^{2+}-dependent H^+ ATPase activity relative to equivalent concentrations of KCl (*39*) and which appear to have a similar effect for *S. cerevisiae* H^+ ATPase (Table 4), appear to stimulate membrane-associated MDR protein ATPase activity *more* than equivalent KCl concentrations. Neither equivalent K gluconate or Na gluconate substitute for KCl or NaCl in producing these effects (not shown). These results are intriguing and suggest that there may be ion-specific effects for hu MDR 1 ATPase activity superimposed upon general ionic strength effects. Moreover, these may be at least as significant as many drug stimulatory effects, at least for membrane-associated hu MDR 1.

Previously determined pH profiles for MDR protein ATPase activity vary somewhat, with Senior and colleagues reporting a rather broad bell-shaped curve with a maximum near pH 7.5−8.0 (*24*), Sharom and colleagues reporting a similar profile but with less activity near pH 6.0 (*26*), and Hamada and Tsuruo (*25*) reporting a rather flat profile between 6.5 < pH < 8.0 with more activity at pH 6.0 relative to that reported by Senior and colleagues. Regardless, in all of these studies, as well as in ours (Table 4), it is clear that MDR protein ATPase activity has a broader, more alkaline pH profile compared to that of the endogenous yeast H^+ ATPase.

Membrane Potential and pH. We also examined membrane potential in these yeast. Under proper conditions (*33*) uptake of $[^3H]TPP^+$ and [^{14}C]propionic acid, followed by determination of equilibrium distribution of the probes and intracellular water volume (*36*), can be used to quantitatively estimate the magnitude of ΔΨ and ΔpH, respectively. As pH_{ex} increases plasma membrane ΔΨ increases for yeast via the activation of K^+ channels (*43*), because as ΔpH (normally alkaline inside) begins to dissipate yeast strive to maintain electrochemical energy (*33*). Therefore, ΔΨ and ΔpH are more easily quantified at higher and lower pH_{ex}, respectively. Table 5 summarizes results from ΔΨ and ΔpH measurements. Interestingly, even though hu MDR 1 overexpression clearly alkalinizes the cytosol of higher eukaryotic cells (*12*), effects of hu MDR 1 expression on the pH_i of 9.3 yeast at their normal physiologic pH_{ex} (i.e., ≤7.0) are not as significant. Thus, any movement of H^+ by hu MDR 1 protein

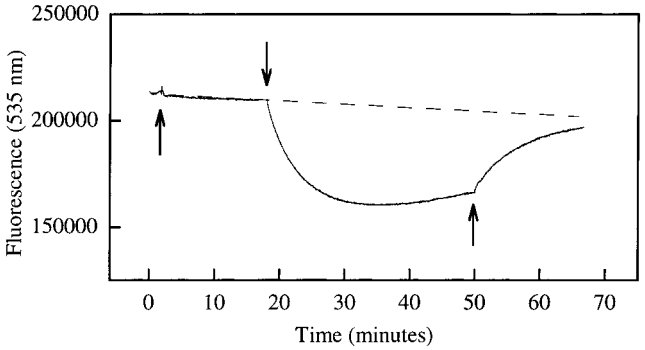

FIGURE 4: Results of a standard H^+-pumping assay for ISO vesicles fabricated from control 9.3./pVT yeast. In this assay, [acridine] is 20 μM, [ATP] is 2.5 mM, pH_o is 6.50, and 75 μg of vesicle protein is added (at the first arrow). Interaction between acridine and vesicles in the absence of ATP (time between 2 and 20 min) does not result in appreciable changes in acridine fluorescence; however, addition of ATP to activate the endogenous H^+ ATPase (second arrow) results in rapid quenching of acridine fluorescence as the probe redistributes in response to the rapidly induced acid inside ΔpH. Addition of nigericin or vanadate with ATP (dashed line) yields a flat line. After ΔpH is achieved, addition of vanadate (third arrow) collapses ΔpH due to inhibition of the ATPase. The addition of ATP (second arrow) induces an instantaneous mild increase in acridine fluorescence, but this "blip" is removed for clarity of presentation.

(*8*) does not have the kinetic capacity to strongly compete with endogenous yeast pH_i regulation at pH_{ex} 7.0. However, similar to depolarizing effects noted previously for higher eukaryotic cells (*6*, *7*) 9.3/hu MDR 1 yeast clearly exhibit decreased membrane potential at high pH_{ex} relative to control transformants (Table 5). Also, this effect (similar to effects on growth and valinomycin resistance) is dependent upon KCl.

H^+ Transport Measurements with ISO Vesicles. We also fabricated ISO membrane vesicles from the 9.3/hu MDR 1 and 9.3/pVT strains and analyzed their ATP-driven H^+ transport activity. In the ISO preparations, endogenous H^+ ATPase (as well as hu MDR 1 ATPase) sites are extrafacially disposed. Thus, addition of ATP stimulates rapid intravesicular acidification for the ISO preparations.

Figure 4 illustrates typical results from our H^+-pumping assay for 9.3/pVT yeast at $pH_{ex} = 6.50$. We analyzed formation of ISO vesicle H^+ gradients by continuously monitoring fluorescence of the weak base probe acridine (e.g., ref *31* and references within). After achieving a stable baseline, ISO vesicles are rapidly injected into a well-stirred cuvette (first arrow, solid line, Figure 4). A stable baseline (e.g., Figure 4, between 2 and 20 min) is verified before proceeding. Addition of ATP (second arrow) stimulates inward H^+ pumping (solid trace) and hence inward partitioning of the weak base acridine. Partitioning leads to quenching of acridine fluorescence because of the formation of aggregates as the intravesicular acridine concentration becomes >20 μM (*44*). Control experiments performed in the presence of 100 μM vanadate (to inhibit induction of ATPase activity upon addition of ATP) or 20 μM nigericin (to immediately collapse any generated ΔpH that is formed by the ATPase) verify the assay is measuring ATP-driven H^+ pumping (dashed line, Figure 4). The mild negative slope to the dashed trace is likely due to acridine photobleaching. Additional control experiments (not shown) verify that extravesicular ATP is not depleted for at least 40 min under these assay

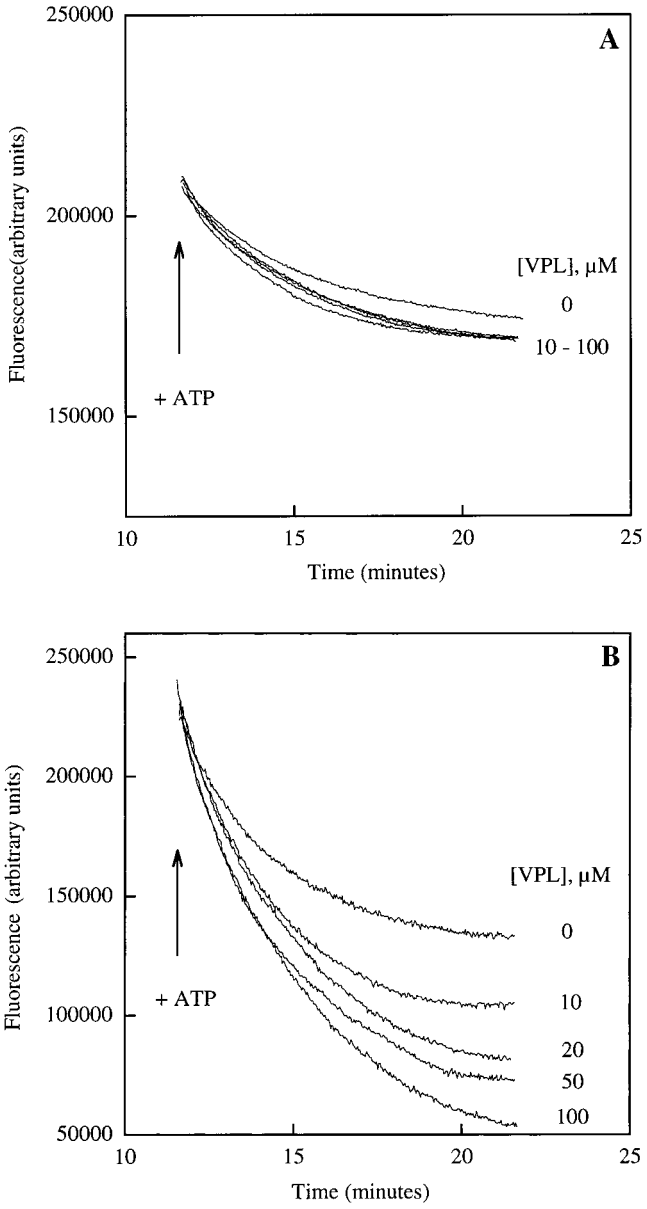

FIGURE 5: Effect of increasing concentrations of verapamil on the apparent H^+-pumping activity of ISO vesicles fabricated from control 9.3/pVT (A) or 9.3/hu MDR 1 (B) yeast. From top, each panel: 0, 10, 20, 50, 100 μM verapamil. Verapamil alone has no appreciable effect on the fluorescence of acridine orange (not shown). Similar to the trace shown in Figure 4, baseline AO fluorescence is stable prior to addition of ATP in each experiment (not shown).

conditions. After H^+ pumping plateaus, the ΔpH that is formed can be collapsed by addition of vanadate (third arrow), a potent inhibitor of the yeast H^+ ATPase, or by the protonophore nigericin (not shown).

Since the putative ion transport activity of hu MDR 1 protein appears to be ATP-dependent (*8*) and since the ATPase activity of hu MDR 1 protein is more stimulated by verapamil than the ATPase activity of the endogenous yeast H^+ ATPase (Figure 3, Table 3), we compared H^+ pumping for control 9.3/pVT and 9.3/hu MDR 1 ISOV versus [verapamil] (Figure 5A,B). Addition of 10−100 μM verapamil has only a small effect on the apparent magnitude of ΔpH formed for 9.3/pVT ISOV (Figure 5A). However, parallel to the more potent effects of verapamil on hu MDR 1 ATPase activity (Figure 3), H^+ pumping for 9.3/hu MDR 1 ISOV is increased significantly by verapamil (Figure 5B) and in a concentration-dependent fashion with an estimated K_m that parallels the K_m for effects on hu MDR 1 ATPase activity. One obvious straightforward interpretation of these data is that the ATPase activity of hu MDR 1 protein results in an additional movement of H^+ that is directly or indirectly mediated by hu MDR 1. Alternatively, via the "drug pump" model, these data could be envisioned to indicate heightened inward "pumping" of acridine by hu MDR 1 (that is, if MDR 1 protein is hypothesized to pump dozens of structurally divergent hydrophobic compounds, why not acridine as well). However, the drug pump model proposes that verapamil *competes* for pumping of drugs via hu MDR 1 protein via occupying the putative "drug-binding" site. Thus, if the assay was revealing pumping of acridine by MDR 1 protein, elevated [verapamil] would be expected to *lower* the extent of acridine quenching (reflecting inward transport), not *increase* it. Therefore, considering all the data together and including previous results that also showed anomalous ATP-dependent movement of H^+ due to hu MDR 1 in CHO transfectants (*8*), it is much more logical to us to interpret these data in terms of increased inward movement of H^+ due to ATP-dependent activation of hu MDR 1. In the accompanying paper, this anomalous H^+ movement is analyzed versus pH, ΔNa^+, and ΔCl^-.

DISCUSSION

First, our data confirm a pH profile for hu MDR 1 ATPase activity that is distinctly different from that of endogenous yeast H^+ ATPase. This profile should be useful in further diagramming ion transport mediated by hu MDR 1 protein (see the following paper in this issue). We also confirm mild stimulation of hu MDR 1 ATPase activity by some hydrophobic drugs, but the magnitude of some effects is not essentially any greater than effects of other nonspecific membrane perturbants (e.g., drugs, hormones, detergents, etc.) on the activities of several other membrane-associated ATPases, or any greater than the effects of some salts on the ATPase activity of hu MDR 1. These salt effects may also be useful in further elucidating the function of hu MDR 1 protein. It is also interesting to find an apparent increase in ΔpH formation for 9.3/hu MDR 1 ISOV in the presence of increasing [verapamil] that parallels the verapamil concentration dependence in stimulation of hu MDR 1 ATPase activity. Thus, the two phenomena exhibit similar "pharmacologic profiles" and are likely connected by a common underlying mechanism.

Several additional lines of independent evidence presented in this paper support the conclusion that ion transport is significantly perturbed in yeast 9.3 expressing hu MDR 1 protein. First, growth of 9.3/hu MDR 1 yeast appears to be much more sensitive to $[KCl]_{ex}$ and pH_{ex} than growth of the control strain. High NaCl is unable to substitute for lack of high KCl in 9.3/hu MDR 1 cultures at low pH (not shown), suggesting high K^+ is particularly important for optimal growth of the 9.3/hu MDR 1 strain at low pH. Relative growth of 9.3/hu MDR 1 at low pH in SC-ura medium + 100 mM KHEPES is higher than in 100 mM KCl (not shown), suggesting that growth in high K^+ is even better when $[Cl^-]_{ex}$ is also low at low pH. Thus, higher $[K^+]_{ex}$ is apparently helpful in balancing an effect of hu MDR 1 protein function (perhaps dependent upon $[Cl^-]_{ex}$; see ref *8*)

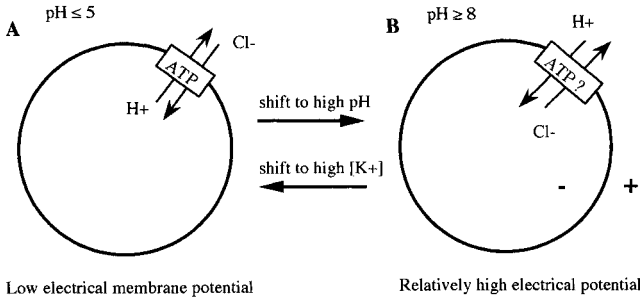

FIGURE 6: Cartoon illustrating one possible interpretation of all the different data in this paper. We propose that under conditions of low $\Delta\Psi$ (as occurs in yeast at low pH_{ex} where the chemical gradient in protons dominates) inward Cl^- and outward H^+ transport by hu MDR 1 is favored and driven by ATP hydrolysis. At relatively high $\Delta\Psi$ (as occurs at high pH_{ex} when yeast K^+ channels are activated) the direction of Cl^- and H^+ transport is reversed because of the increased electrical barrier to transport in the opposite direction. This scheme is consistent with the fully reversible characteristics of net Cl^-/H^+ transport mediated by hu MDR 1 protein in CHO transfectants described earlier (*8*) in single-cell photometry experiments.

that is deleterious for growth at low pH. Our previous suggestion that hu MDR 1 may catalyze net electrogenic Cl^-/H^+ transport in some fashion (*8*) is consistent with this conclusion, since conditions that favored net inward movement of K^+ (e.g., high $[K^+]_{ex}$) would balance effects of the proposed electrogenic Cl^-/H^+ transport (*8*) (Figure 6A). However, interestingly, we essentially find the converse at high pH; high KCl is essential for any growth of 9.3/hu MDR 1 at pH 8.0, but in this case NaCl substitutes for KCl and KHEPES does not, arguing that the growth-promoting effect is due to high $[Cl^-]_{ex}$. The explanation for this effect may be related to the previously observed (*8*) symmetrical reversibility of Cl^-/H^+ transport mediated by hu MDR 1 (see below).

Along with these interesting salt effects on growth, we observe perturbations in membrane potential for 9.3/hu MDR 1 yeast that are relatively easy to isolate at higher pH_{ex} in the presence of KCl. Since it has been proposed that TPP^+ is "pumped" by hu MDR 1 protein (*45*), it could be argued that reduced TPP^+ accumulation in these assays is not due to perturbations in $\Delta\Psi$ but to increased "pumping" of the probe. However, considering all the data together (see also the following paper in this issue), we feel it is much more logical to propose the altered TPP^+ distribution is due to a perturbation in $\Delta\Psi$, because the perturbation is particularly dramatic at higher pH and is dependent upon the presence of KCl. These are variables that are certainly known to affect $\Delta\Psi$ regulation in yeast but that have never been suggested to affect "drug pumping". However, if, as described above, we explain salt effects on growth at lower pH via Cl^-/H^+ transport as previously proposed (*8*; Figure 6A), we are left with a paradox when attempting to rationalize lowered $\Delta\Psi$ at high pH_{ex}; inward Cl^- and/or outward H^+ transport via hu MDR 1 would be predicted to *hyperpolarize* the yeast under these conditions, not depolarize as is measured (Table 5). Thus, we speculate that as $\Delta\Psi$ is increased in the yeast via activation of K^+ conductance (as the magnitude of ΔpH decreases upon elevating pH_{ex}, see ref *33*), the regulation of ion movement catalyzed by hu MDR 1 (see ref *8*) becomes more responsive to that increased $\Delta\Psi$, such that the direction of Cl^- and H^+ movement is reversed, leading to depolarization (Figure 6B). Complete reversibility of the Cl^-/H^+ transport mediated by hu MDR 1 upon reversing the direction of the Cl^- chemical gradient was observed earlier (*8*) and is examined further in the accompanying paper. On the basis of this scenario, high external Cl^- would then be predicted to inhibit outward movement of Cl^- at high pH_{ex} (high $\Delta\Psi$), thereby *protecting* the cells from the toxic action of hu MDR 1. Indeed, 100 mM KCl is absolutely essential for any growth of 9.3/hu MDR 1 at pH 8.0, and again, unlike the high KCl effect at low pH, NaCl *does* substitute for KCl and KHEPES *does not* (not shown), arguing the effect is due to high $[Cl^-]_{ex}$.

With regard to valinomycin resistance, presumably, control yeast are killed by valinomycin at relatively low $[K^+]_{ex}$ because internal K^+ (at low pH_{ex} and hence low $\Delta\Psi$) is lost as it moves down its concentration gradient via the K^+ ionophore. At high $[K]_{ex}$ this is inhibited, leading to enhanced survival (Table 1). Alternatively, it is important to note a high chemical gradient in H^+ (alkaline inside) can be maintained by yeast at pH < 5.0 because inward K^+ transport typically electrically balances the H^+ movement; disruption of this vectorial transport with a K^+ ionophore could disrupt maintenance of ΔpH. Thus, hu MDR 1 protein ion transport activity must allow the yeast to at least partially compensate for one or both of these deleterious effects, by essentially acting to retain higher $[K^+]_i$ via increasing the net inward K^+ permeability and/or the net outward movement of H^+. Inward movement of Cl^- by hu MDR 1 (which would promote passive influx of K^+) and/or outward movement of H^+ as proposed previously (*8*; also following paper in this issue) are thus both obvious suggestions (Figure 6A). As $[K^+]_{ex}$ is elevated, control 9.3/pVT yeast maintain higher $[K^+]_i$ by passive effects; thus they do not require hu MDR 1 expression to survive in the presence of 50 μM valinomycin.

As another possibility, in theory, valinomycin toxicity could also be due to ionophoric actions at the mitochondrial membrane (*46*). However, it is likely that the protective function of hu MDR 1 expression is via some effect at the plasma membrane because: (1) MDR 1 protein is well-localized to the plasma membrane and (2) high external KCl (the consequences of which are lower plasma membrane $\Delta\Psi$ and/or higher plasma membrane ΔpH) mimics the protective effect of hu MDR 1 expression.

Regardless of our specific hypothesis for ion transport by hu MDR 1 (Figure 6; *8*), considering all these data together, it is obvious that hu MDR 1 overexpression perturbs regulation of $\Delta\mu_{H^+}$ in these yeast. Normally, at relatively low pH_{ex} and in the absence of ionophores, pumping of H^+ (via the endogenous yeast plasma membrane H^+ ATPase) is adequate to maintain $\Delta\mu_{H^+}$ for yeast, which is negative and alkaline inside under these optimal growth conditions and dominated by a high ΔpH. If ΔpH dissipates (as happens when pH_{ex} is raised), K^+ transport becomes essential to the survival of yeast, since K^+ channel activity is used to restore electrochemical energy in the form of $\Delta\mu_{K^+}$. That is, when $\Delta\mu_{H^+}$ of yeast (usually dominated by ΔpH) is compromised, they respond by generating $\Delta\Psi$ via activating K^+ conductance. Any movement of ions that interferes with this regulation of electrochemical energy would have the effects measured in this study. The most obvious suggestion to us is movement of Cl^- and H^+ by hu MDR 1 protein since previous data suggested this (*8*; see also the following paper

in this issue). The larger depolarizing effects of hu MDR 1 expression at higher pH (where $\Delta\mu_{K^+}$ is dominant), the KCl dependency of these effects, the lack of growth of 9.3/hu MDR 1 yeast at high pH without high $[Cl^-]_{ex}$, the K^+ dependency of the valinomycin resistance effect, and the linkage between verapamil effects on hu MDR 1 ATPase activity and anomolous H^+ movement in 9.3/hu MDR 1 ISOV all support this notion.

Finally, we note that several other studies (*19*−*22*, *47*) have reported expression of either murine or human MDR proteins in various *S. cerevisiae* strains. With one exception (*47*) these studies have not investigated $\Delta pH/\Delta\Psi$ values or other key predictions of the altered partitioning hypothesis for MDR 1 protein function (*6*, *13*). In contrast to our data, one previous study (*47*) did not find appreciable differences in $\Delta\Psi$ or ΔpH for secretory vesicle preparations expressing MDR protein, versus control yeast secretory vesicles. However, these measurements were apparently made at pH 7.5 and in the absence of Cl^-. As further elaborated upon in the accompanying paper, our results are not necessarily incompatible with these previous data, since the effects that we measured with 9.3/hu MDR 1 strains appear to be Cl^--dependent and are enhanced at slightly alkaline pH. It may be difficult to measure full ion transport activity of hu MDR 1 in vesicle preparations if the measurements are performed in the absence of Cl^- and at pH < 8.0. Alternatively, the decreased ability of yeast 9.3 to accumulate K^+ might make the effects we measured easier to isolate.

In conclusion, considering all these data together, one could logically propose that the ATPase activity of hu MDR 1 protein is linked to a process or processes that would have a depolarizing effect (e.g., increased transport of Cl^- or inhibition of proper K^+ transport, particularly at higher pH) as well as a process that would affect formation of a H^+ gradient for yeast ISOV in the absence of CO_2/HCO_3^- (e.g., movement of H^+ or OH^-). Such a model has also recently been proposed based on independent results from Cl^- substitution experiments for CHO transfectants monitored by single-cell photometry (*8*) and is further elaborated upon in the accompanying paper. Independent of this specific hypothesis however, a deluge of data from this laboratory and others over the past few years (much reviewed in ref *6*) now leave little doubt that expression of hu MDR 1 protein perturbs transmembranous ion transport in quite interesting ways. One permutation of this observation that has been suggested is that hu MDR protein must therefore be *both* some type of ion transport regulator *and* a multisubstrate hydrophobic drug pump that actively translocates many different chemotherapeutic drugs and other compounds (ref *9* and references within). As argued previously (*6*, *12*, *13*, *48*, *49*) we feel it is much more logical to suggest that the biophysical perturbations caused by hu MDR 1 overexpression (due to this abnormal ion transport) are fully responsible for the altered drug partitioning (*12*), drug resistance (*12*), and signal transduction (*18*) unequivocally attributed to the expression of hu MDR 1 protein alone and that it is not necessary to invoke a direct active drug-pumping process that violates the law of enzyme specificity, the coupling principle, and other important concepts in order to explain the phenotype mediated solely by hu MDR 1 overexpression. With regard to possible direct chemotherapeutic drug pumping via hu MDR 1 protein, we cannot as yet find any evidence for an increased rate constant in transport of chemotherapeutic drugs across 9.3/hu MDR 1 yeast plasma membranes (data not shown). However, although these results are reproducible with multiple vesicle preparations, they are negative data and as such are not definitive. Further examination of altered passive drug translocation in these yeast vesicle systems and further analysis of the possible contributions of biophysical perturbations to this altered translocation (*6*) will prove informative.

In the accompanying paper, molecular details of the altered ion transport due to hu MDR 1 protein are analyzed further.

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