Effects of Ion Gradients on H^+ Transport Mediated by Human MDR 1 Protein[†]

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ABSTRACT: In the previous paper we presented a variety of data consistent with significant perturbations in 9.3 yeast plasma membrane ion transport upon overexpression of the hu MDR 1 protein. Thus, in this paper, we compare formation of ΔpH for inside-out yeast plasma membrane vesicles (ISOV) prepared from control 9.3/pVT versus 9.3/hu MDR 1 yeast. Since MDR 1 ATPase activity has a broader, more alkaline pH profile relative to endogenous yeast H^+ ATPase activity, we analyzed H^+ pumping at pH ≥ 8.0 in detail in order to selectively amplify hu MDR 1 contributions to H^+ movement over those of the endogenous yeast H^+ ATPase. We observed: (1) imposition of a Cl^- gradient oriented outside to in enhances acidification for 9.3/pVT ISOV (as expected), but decreases acidification for 9.3/hu MDR 1 ISOV; (2) imposition of a Cl^- gradient oriented inside to out decreases acidification for 9.3/pVT ISOV (as expected) but enhances acidification for 9.3/hu MDR 1 ISOV; (3) a Na^+ gradient oriented in the same direction as the Cl^- gradient amplifies the effects due to hu MDR 1 when both gradients are oriented inside to out, but not outside to in. The data are most easily explained by interesting Na^+, Cl^-, and ATP-dependent H^+ transport mediated by hu MDR 1 protein as previously suggested [Hoffman and Roepe (1997) *Biochemistry 36*, 11153−11168]. These data may help to resolve a variety of conflicting reports in the literature regarding ion transport mediated by hu MDR 1 and have implications for the physiology of a number of polarized epithelia in which hu MDR 1 is endogenously expressed.

Considerable evidence now suggests that hu MDR 1 protein, which is overexpressed in a number of multidrug-resistant tumor cell lines, catalyzes rather perplexing and novel ion transport in some fashion. The protein may do this either directly (i.e., as a pump, exchanger, cotransporter, or channel) or indirectly (i.e., as some type of ion transport regulator). However, the significance of this ion transport and whether it can fully explain the tumor drug resistance conferred by hu MDR 1 overexpression are still debated. One model is that hu MDR 1 protein directly translocates dozens of structurally divergent chemotherapeutic drugs and other hydrophobic compounds as some type of nonspecific hydrophobic drug "pump" or "vacuum cleaner" (*1*−*3*). Conversely, it has also been suggested that ion transport catalyzed by hu MDR 1 protein indirectly affects intracellular accumulation of drugs (*4*, *5*), by perturbing intracellular pH (pH_i) and membrane potential ($\Delta\Psi$) when the protein is overexpressed. $\Delta\Psi$ and pH_i perturbations, which have been measured in a variety of model MDR cell lines, have a number of important effects on the passive diffusion, cellular accumulation, and subcellular distribution of chemotherapeutic drugs and other compounds (*6*). These effects can easily and fully explain the drug resistance mediated by hu MDR 1 overexpression alone, when this resistance is not further amplified or affected by exposure of cell lines to chemotherapeutic drug(s) prior to analysis of chemotherapeutic drug resistance (*7*). Moreover, two close relatives of the hu MDR 1 protein, the cystic fibrosis transmembrane conductance regulator (CFTR) and the sulfonyl urea receptor (SUR), are complex ion transport proteins/ion transport regulators that when overexpressed or mutated are capable of significantly altering cellular cation, anion and H^+ transport and hence the regulation of $\Delta\Psi$ and ΔpH. Thus, an ion transport function for hu MDR 1 protein is reasonable and easily explains drug resistance and altered cellular drug retention in MDR cell lines mediated by hu MDR 1 (*7*) via a scenario that does not violate fundamental principles (e.g., the law of enzyme specificity) as do various drug pump models. However, there remains considerable controversy over the molecular details of ion transport catalyzed by hu MDR 1 protein (see refs *6* and *7* and references within).

In previous work (*7*) we constructed a series of "pure" hu MDR 1 transfectants that overexpressed hu MDR 1 protein but that were not cultured in the presence of chemotherapeutic drugs. This allowed us, for the first time, to rigorously define biophysical aspects of the cellular phenotype unequivocally mediated solely by overexpression of hu MDR 1 protein alone, without the complexities of other chemotherapeutic drug exposure effects superimposed upon this phenotype. Only a handful of studies have so far examined this "pure" phenotype (*7*−*12*). It is typically characterized by: (1) relatively low levels of chemotherapeutic drug resistance (e.g., 10−20-fold), (2) elevated pH_i and decreased plasma membrane $\Delta\Psi$, (3) slight negative effects on cell growth rate, (4) increased ATPase activity at the plasma

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membrane, and (5) lower apparent initial rates of drug accumulation without an increase in the rate constant for outward movement of drugs.

Recent single-cell photometry experiments with these pure transfectants under constant perfusion (*12*) suggested that hu MDR 1 protein may mediate (directly or indirectly) some type of Na^+- and ATP-dependent Cl^-/H^+ antiport. Although the process was found to be Na^+-dependent, we could not demonstrate any change in the efficiency of apparent Cl^-/H^+ antiport via imposition of a Na^+ gradient. This result was surprising since simplistically it predicts a twofold electrogenic ion transport process, and these are fairly uncommon. Regardless, the minimalistic model that was generated from these experiments with living cells was somewhat consistent with the endogenous physiological expression of the protein in the proximal tubule of the kidney, the liver bile cannaliculus, the blood−brain barrier, and elsewhere, since pH-dependent Cl^- transport is extremely important at these sites and perturbations in this transport can severely affect permeability of the epithelia to other substances. This is important, since MDR "knock-out" mice exhibit extremely interesting abnormalities in diffusion of drugs across the blood−brain barrier and elsewhere (*13*); thus, any model for hu MDR 1 protein must provide an explanation for these effects. An important role for hu MDR 1 in regulating net ion and water transport across the blood brain barrier and elsewhere is consistent with effects noted in knock-out mice; thus describing this transport in better molecular terms would have extremely important consequences for our understanding of the physiology of these tissues which extend beyond the more obvious importance of the data for the cancer clinic. Although single-cell photometry with living cells is an important approach, to more thoroughly examine Na^+ effects and transport stoichiometries requires the use of vesicle preparations and proteoliposomes.

Thus, we have constructed yeast strains expressing hu MDR 1 protein and have fabricated well-sealed inside-out (ISO) yeast plasma membrane vesicles that mediate well-defined ATP-dependent H^+ transport reactions. Initial observations with the intact yeast (*14*) indicated significant perturbations in yeast plasma membrane ion transport upon overexpression of hu MDR 1 and are consistent with, but do not directly show, the previously proposed Cl^-/H^+ antiport mechanism (*12*). With ISOV preparations, we are better able (relative to measurements with whole cells) to artificially manipulate ATP concentration, pH, and ion gradients and can conceivably "trigger" the function of hu MDR 1 protein more easily and study how ion transport mediated by this protein is influenced by a number of important biochemical parameters. Thus, in this paper, we present our initial data with ISOV preparations, which confirm a role for hu MDR 1 protein in mediating ATP-dependent H^+ transport that is strongly influenced by Na^+ and Cl^- gradients.

MATERIALS AND METHODS

Materials. All materials were reagent grade or better and used without additional purification as described in the previous paper (*14*). C219 is a monoclonal antibody directed to the ATP-binding site epitope of hu MDR 1. It is described in ref *1* and references within and was purchased from Signet Laboratories (Dedham, MA) and used without further purification.

Strains and Construction of ISO Vesicles. Transformation of yeast strain 9.3 with either empty control (pVT 102) or hu MDR 1-expressing (pFF1) vectors, growth of these strains, and fabrication of ISO vesicles from these strains are reported on in detail in the accompanying paper (*14*). In some cases (see Results) we fabricated vesicles in the presence of different salt concentrations (e.g., KCl or NaCl) or at different pH (7.5 vs 6.5). In these cases, the additional salt was added at the lysis step, wherein spheroplasts are converted to ISO vesicles, and all subsequent steps. To compensate for any additional salt and conserve osmolality, [sucrose] in these solutions was adjusted accordingly (*14*).

H^+-Pumping Assays. H^+ pumping into ISOV was assayed primarily via the acridine orange pumping assay as described in the preceding paper (*14*). Since some investigators might choose to interpret some data to indicate "pumping" of acridine via hu MDR 1, and not as passive redistribution of acridine in response to ΔpH as would conventionally be the case, in this study we do not emphasize comparisons between the amplitudes of acridine redistribution for control 9.3/pVT versus 9.3/hu MDR 1 ISO vesicle preparations. Instead, we scaled up production of our ISO vesicles and emphasized the relative effects of manipulation of ion concentrations/ion gradients on H^+ pumping for individual specific ISO preparations. That is, we do not emphasize conclusions that could possibly be biased by the nonratiometric response of acridine to ΔpH perturbations. In these initial studies we preferred acridine for analyzing pH relative to ratiometric probes (e.g., BCECF) because of the high signal-to-noise at relatively acid pH, ease of use, and expense of incorporating BCECF during formation of ISOV (acridine is less expensive and is passively loaded during the assay, see Results).

As an additional caution, possible effects of all reagents on the basal fluorescence of acridine were studied in detail. To form ion gradients, we (for example) typically used either gluconate or glutamate salts to balance tonicity of the transport medium yet to create a chemical gradient in Cl^-. Since different salt concentrations could conceivably affect acridine fluorescence independent of effects due to formation of ΔpH, we assayed acridine fluorescence versus a number of variables (different [NaCl], [KCl], [Na gluconate], [K gluconate], vanadate, ATP, pH, etc.). From these data, similar to previous observations (*15*), it is obvious that the effects we are measuring could not be due to changes in salt composition.

In many experiments, we wished to examine the influence of a particular ion gradient (e.g., Cl^- gradient oriented inside to out) on the relative H^+-pumping efficiency of a given ISO preparation. In these instances, all additions to the transport medium (of ATP, DTT, etc.; see ref *14*) were made prior to addition of vesicles, and then the ISOV were injected into a rapidly stirred cuvette exhibiting a stable acridine fluorescence baseline. Unless otherwise noted, baseline was verified to be stable before and after all reagent additions, as shown in Figure 4 in the previous paper. Careful comparisons between assays wherein the order of addition of ATP and ISOV was reversed revealed that this practice did not affect the baseline or the ΔpH-dependent response of acridine (not shown, but see the previous paper in this issue).

Curve-Fitting Analysis. Fluorescence data were initially stored as Ascii files, transferred to a Macintosh computer, and analyzed with SigmaPlot software using the Marquardt Levenberg algorithm. Rate constants for acridine fluorescence quenching were computed by the iterative least-squares method, with convergence typically satisifed in <25 iterations (tolerance < 0.00001). In some cases, determination of this rate constant, knowledge of intial pH_i in the ISO preparations (see the previous paper in this issue), and quantitative estimate of the pH_i in the ISO after achieving steady state in ΔpH (see Results) allowed us to quantitatively estimate rates of H^+ movement into the ISO (see Results).

RESULTS

First, we wished to determine whether unusual ATP-dependent H^+ movement previously attributed to hu MDR 1 protein in LR73 CHO transfectants (*12*) and consistent with our previous observations with 9.3/hu MDR 1 yeast (*14*) could be measured in yeast inside-out (ISO) vesicles containing the protein. However, normal yeast contain an endogenous ATP-driven H^+ pump whose function will be superimposed upon any ATP-driven H^+ movement that may be catalyzed by hu MDR 1 protein, thus potentially complicating interpretation. Therefore, our initial experiments were designed to identify biochemical conditions that would enhance the contribution of hu MDR 1 protein to overall H^+ movement in ISOV preparations over the relative contribution of the endogenous H^+ ATPase. Since the endogenous ATPase has a pH optimum lower than that of hu MDR 1 (see the preceding paper in this issue) and since it is not affected by the presence of Cl^- gradients (as is predicted for hu MDR 1, see ref *12*) our approach was fairly straightforward.

Figure 1A,B shows representative ATP-dependent H^+-pumping data for control 9.3/pVT (A) and 9.3/hu MDR 1 (B) ISO vesicles versus bulk pH of the transport medium, measured as described in the accompanying paper (*14*). Traces shown in each panel are for a single vesicle preparation, but all conclusions in this paper have been tested with multiple, independently prepared 9.3/pVT and 9.3/hu MDR 1 ISO vesicle preparations (e.g., see Figures 3 and 5). In this assay, as described in the previous paper, inward movement of H^+ into the ISOV creates an acid inside pH gradient, and the weak base acridine then passively partitions inward in response to this gradient (see ref *6* and references within for theory behind weak base partitioning). This inward partitioning leads to quenching of acridine fluorescence as described (see the previous paper in this issue), and the magnitude of quenching is directly proportional to the magnitude of the ΔpH formed (see below).

However, even though the amplitudes for the 9.3/hu MDR 1 ISO vesicle traces obtained at each pH (Figure 1B) consistently appear larger than those for 9.3/pVT ISO vesicles (Figure 1A), it could in theory be misleading to draw quantitative conclusions regarding the relative magnitude of ΔpH from the amplitudes of acridine traces obtained for different vesicle preparations. This is because average ISOV internal volume (which can also affect the magnitude of the acridine response along with generation of ΔpH upon addition of ATP) can conceivably vary preparation to preparation. However, it is reasonable to compare the amplitudes of traces for experiments with a specific individual vesicle preparation in order to examine *trends* induced by altering a specific variable (e.g., pH, $[Cl^-]$, etc.), as long as it is known that the variable does not independently affect acridine fluorescence (see Materials and Methods).

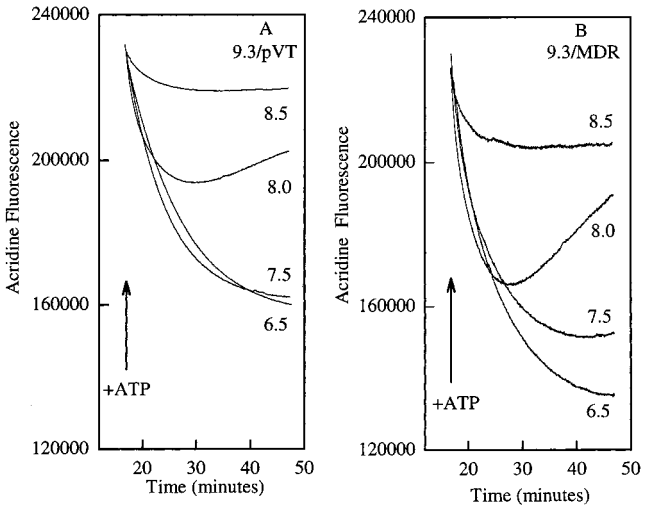

FIGURE 1: Comparison of the pH_{ex} dependency of H^+ pumping [measured by acridine distribution as described previously (*14*)] for control 9.3/pVT (A) and 9.3/hu MDR 1 (B) ISO preparations. In each case, H^+ pumping was assayed at external pH 6.50 (bottom trace), 7.50 (second trace from bottom), 8.00 (third trace from bottom), and 8.50 (top trace in each panel) for a given specific ISO preparation. In all experiments, stock additions of acridine, ATP, and DTT [see Materials and Methods (*14*)] were identical, and initial baseline of the acridine fluorescence (prior to addition of the ISO, not shown) was virtually identical (±2%). Standard transport buffer composition is as described in the previous paper but buffered to different pH values (with HEPES) as shown. Comparing the relative trends in activity versus pH in a given panel is more important than comparing the amplitudes between panels A and B. The arrow indicates initiation of H^+ pumping via the addition of ATP (*14*); in these experiments, vesicles were incubated with transport buffer for 60 s before addition of ATP; thus pH_i is likely close to pH_{ex} at the time ATP is added. Although comparisons between only two vesicle preparations are shown, similar trends in different pH_{ex} sensitivities are found in two other comparisons between two other independent 9.3/pVT and 9.3/hu MDR 1 ISO preparations (not shown).

Thus, when H^+ pumping for control 9.3/pVT and 9.3/hu MDR 1 ISOV is compared at a range of pH_{ex} (Figure 1A vs 1B), it is clear that the pH profiles differ. Control 9.3/pVT have a pH optimum for ΔpH formation consistent with previous studies of endogenous yeast H^+ ATPase H^+ pumping (*15*) and ATPase (*16*) activities. In contrast, the 9.3/hu MDR 1 ISO vesicles have a broader pH profile for H^+ pumping, with proportionally more activity at higher pH ($\geq$8.0) relative to control (e.g., compare pH_{ex} 8.5 trace to pH_{ex} 7.5 trace in Figure 1A vs 1B). Interestingly, this different pH profile for 9.3/hu MDR 1 H^+ pumping parallels the different pH profile for hu MDR 1 ATPase activity (*14*). Formation of ΔpH at $pH_{ex} = 8.0$ appears more "leaky" for 9.3/hu MDR 1 ISO vesicles since the baseline indicating the steady-state value of ΔpH between 30 and 45 min has a steeper positive slope. This "leaky" character is not due to premature depletion of external ATP under these conditions (not shown) or to some defect in the ISO vesicle preparation (the same vesicles are used in the pH 6.5−7.5 experiments). At pH_o 8.5, the baseline is stable once again, because a ΔpH of lower magnitude has been formed and there is thus a

lowered driving force for H^+ to leak back out of the ISO down the acid inside pH gradient established upon addition of ATP. That is, since the same vesicle preparation does not appear particularly "leaky" at pH_o 6.50, 7.50, or 8.50, we suggest a significantly higher ΔpH for 9.3/hu MDR 1 ISO vesicles must be formed initially at pH 8.0 (9−10 min after addition of ATP), relative to 9.3/pVT ISO. This is consistent with the trend noted when comparing H^+-pumping data at pH 7.5 to data obtained at pH 8.0 for the two preparations (the size of the initial ΔpH formed at pH 8.0 is about one-half that at pH 7.5 for 9.3/pVT but is nearly the same as that for 9.3/hu MDR 1). Thus, consistent with relative ATPase activity versus pH profiles (*14*), from these data we conclude that 9.3/hu MDR 1 ISO vesicles are capable of generating a proportionally higher ATP-dependent ΔpH at $pH_o \geq 8.0$ relative to 9.3/pVT ISO vesicles.

As an alternative interpretation, we note that some investigators propose that MDR proteins are capable of physically translocating dozens of structurally divergent compounds with high lipid/saline partitioning coefficients (*1*). Thus, it could conceivably be argued that differences between control and MDR ISO are due to "pumping" of acridine orange by the hu MDR 1 protein. Since there are a number of points that are inconsistent with an acridine-pumping interpretation as described below and elsewhere (*6*), we favor the more conventional interpretation that differences between the traces likely reflect different H^+ movement. Further distinction between acridine-pumping and H^+-transport interpretations of these data would be assisted by calibration of the ΔpH-dependent response of acridine partitioning. Historically, it has proven difficult to use acridine data to quantify the magnitude of a generated ΔpH, since acridine is a nonratiometric probe. Thus, possible differences in internal volume of multiple ISOV preparations (which could further affect the acridine quenching caused by formation of ΔpH) in theory could limit the ability to quantitatively compare H^+ flux for multiple ISO vesicle preparations. However, as shown in Figure 2A the intravesicular acridine orange signal can be titrated by simultaneous addition of the potonophore nigericin (to collapse ΔpH) and aliquots of HCl (to alter pH_{ex} to different values). Moreover, if total internal ISO vesicle volume in a continuously mixed acridine/vesicle suspension is purposefully manipulated by addition of different amounts of ISO vesicles, plots of the acridine fluorescence recovered upon collapse of ΔpH (e.g., Figure 2A) versus the size of the ΔpH at collapse (manipulated by addition of HCl along with nigericin, see Figure 2 caption) intersect at a common point on the *x* axis (Figure 2B). This point must correspond to a value that is close to the internal ISOV pH (ISO pH_i) upon achieving steady state in the H^+-pumping assay, since this steady-state value will be independent of the number of vesicles present and independent of the total ISOV internal volume. Quantitatively estimating steady-state pH_i (via multiple titrations such as those shown in Figure 2A and summarized in Figure 2B) and knowing the initial ISO pH_i (set during preparation of the ISO vesicles, see Materials and Methods) allow for a quantitative estimate of the rate of H^+ movement. From analyses such as that shown in Figure 2, we estimate the magnitude of the ΔpH that is formed by the 9.3/hu MDR 1 ISOV at pH 8.50 is at least 0.4 unit larger than the ΔpH formed by 9.3/pVT ISOV (see Figure 2 caption).

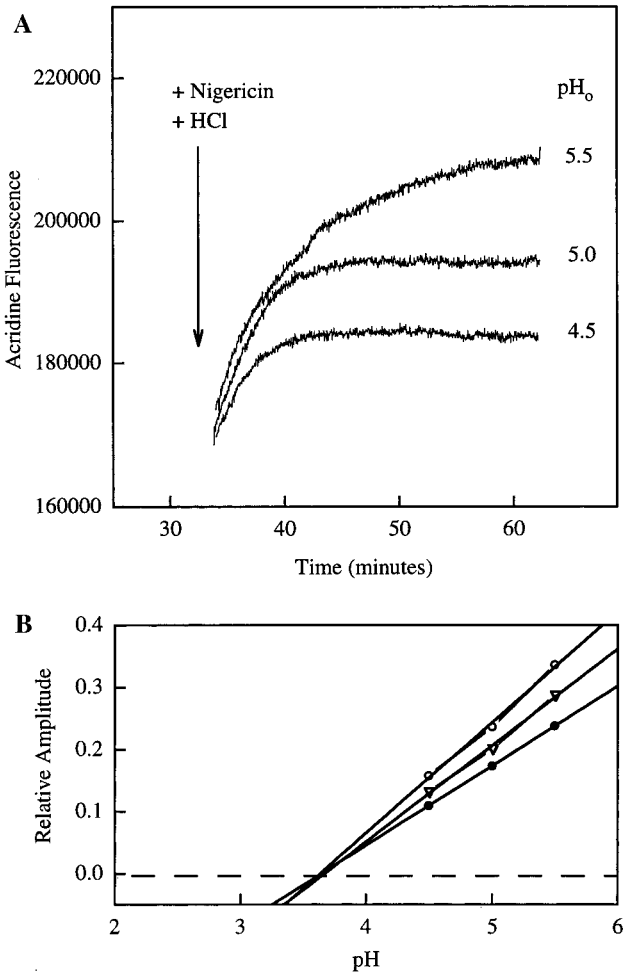

FIGURE 2: Null point titration calibration technique for acridine redistribution upon formation of an ISO vesicle ΔpH. The total change in acridine fluorescence in a given experiment after addition of ATP is dependent upon both the magnitude of the ΔpH that is formed [which causes pH-dependent accumulation/aggregation of the weakly basic acridine via the weak base effect] and the internal volume of the ISO vesicles. As shown in panel A, when the protonophore nigericin is added after achieving steady state in formation of ΔpH, the pH gradient collapses and acridine redistributes (fluorescence intensity is recovered). If we collapse the ΔpH by addition of nigericin and simultaneously titrate the transport buffer to successively more acidic pH by addition of aliquots of HCl (from the top, transport buffer titrated to pH 5.50, 5.00, and 4.50, respectively) the net increase in acridine fluorescence (due to collapse of ΔpH) becomes less dramatic as a smaller ΔpH is collapsed. If we plot this "recovery of acridine fluorescence upon collapse of ΔpH" (i.e., relative amplitude) versus the pH of the transport medium at addition of nigericin (panel B, open circles, top curve) a straight line is obtained (via linear regression analysis) that in theory should intersect the *x* axis at the pH equal to ISO vesicle pH_i. That is, in theory, no "recovery" of acridine fluorescence should be obtained if addition of nigericin does not result in collapse of ΔpH (e.g., when ISO vesicle pH_i equals the titrated pH_{ex} upon addition of nigericin). However, the absolute magnitude of acridine quenching also depends on ISO vesicle internal volume, so to test whether this calibration scheme would be unduly affected by slightly different internal volume for different ISO vesicle preparations, we purposefully added one-fourth less vesicles (open triangles, panel B) and one-half less vesicles (closed circles, panel B) and repeated the nigericin calibration (as shown in panel A). Plots of the "recovery of acridine fluorescence amplitude" versus "pH of the transport medium upon addition of nigericin" for these experiments intersect the *x* axis at the same point as the initial plot (Figure 2B). Thus, the *x* intercept is a reasonable quantitative estimate of the ISO vesicle pH_i at steady state in the H^+-pumping assay.

We also analyzed H^+ pumping by these vesicles versus various concentrations of ATP and vanadate. Similar to others (*17*) we previously found that inhibition of yeast H^+ ATPase and hu MDR 1 ATPase activity by vanadate is similar (*14*). Thus, it was not surprising to find that vanadate inhibited formation of ΔpH in both ISO vesicle systems with similar relative K_i (not shown). In addition, at bulk pH 6.50, titration of $[ATP]_{ex}$ versus the relative magnitude of the ΔpH that is formed revealed that 2.0 mM ATP was well above K_m in both systems (not shown) and sufficient to maintain ΔpH steady state for at least 40 min in both systems (not shown, see ref *14*). This is consistent with previous estimates of the K_m for ATP for fungal plasma membrane H^+ ATPases (*16*) as well as for hu MDR 1 (see ref *14* and references within). Titration of ATP revealed that ΔpH formation in both ISO vesicle preparations has a similar K_m for ATP (not shown). Therefore, we were unable to further "isolate" hu MDR 1 effects via manipulation of [ATP] or [vanadate].

However, we also analyzed the effects of Cl^- gradients, since imposition of a Cl^- gradient was earlier found to affect movement of H^+ for LR73/hu MDR 1 CHO transfectants in a manner clearly very different than control (*12*). Figure 3A,B shows that imposition of a Cl^- gradient oriented outside to in ($\Delta Cl^{o/i}$) enhances net inward H^+ movement in 9.3/pVT ISO vesicles (as expected; compare top dashed trace to top solid trace, Figure 3A). In contrast, surprisingly, an inward directed Cl^- gradient (bottom solid trace, Figure 3A) slows acidification for 9.3/hu MDR 1 relative to acidification measured in the absence of the gradient (bottom dashed trace, Figure 3A). Thus, when H^+ pumping is analyzed for vesicles diluted into KCl ($+\Delta Cl^{o/i}$) versus K gluconate (no $\Delta Cl^{o/i}$) and traces from the latter experiments are subtracted from traces for the former (see Figure 3B), a difference trace with negative slope [reflecting enhanced inward movement of H^+ due to the Cl^- counterion passive influx effect (*18*)] is found for 9.3/pVT (dashed traces each comparison, Figure 3B), but a positive hyperbolic curve (reflecting net elimination of H^+ from the ISO vesicles; solid trace each comparison Figure 3B) is found for 9.3/hu MDR 1.

Figure 4 summarizes the effects upon reversing the direction of the Cl^- gradient. In striking contrast to the effects noted for control (Figure 4, top two dashed traces), imposition of a Cl^- gradient oriented inside to out ($\Delta Cl^{i/o}$) accelerates the inward movement of H^+ for the 9.3/hu MDR 1 ISO (seen as a negative inflection upon subtracting H^+-pumping traces for KCl-loaded ISO vesicles diluted into K gluconate from those diluted into KCl; second solid trace from bottom). That is, outward movement of Cl^- promoted by establishing $\Delta Cl^{i/o}$ (see Materials and Methods and Figure 4 caption) slows the rate of net inward H^+ movement for 9.3/pVT ISO vesicles (again, as expected based on the counterion passive diffusion effect) but, in contrast, enhances apparent inward movement of H^+ for 9.3/hu MDR 1 ISO vesicles. These results could be interpreted in terms of ATP-dependent H^+ movement by hu MDR 1 protein that is enhanced by the presence of a Cl^- gradient in the opposite orientation (e.g., ATP-dependent Cl^-/H^+ antiport), which is identical to the conclusion recently reached by a completely different approach, namely, rapid ion-substitution experiments of living cells under constant perfusion monitored by single-cell photometry (*12*).

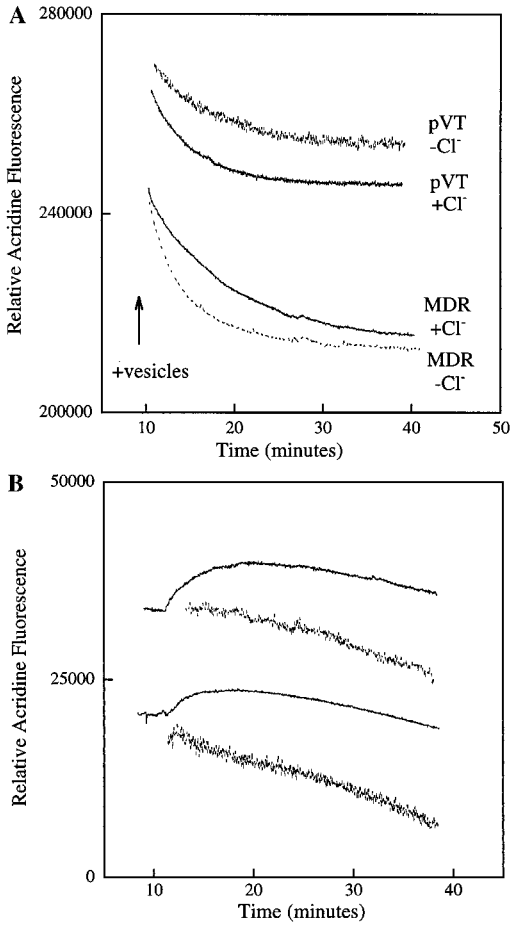

FIGURE 3: Effect of an inward directed Cl^- gradient ($\Delta Cl^{o/i}$) on the rate of ISO vesicle acidification for 9.3/pVT and 9.3/hu MDR 1. (A) Acidification for ISO diluted into either KCl (solid traces labeled "$+Cl^-$") or K gluconate (dashed traces labeled "$-Cl^-$") is compared for 9.3/pVT (top two traces) or 9.3/hu MDR 1 (bottom two traces; osmolality maintained by adjusting sucrose concentration, see Materials and Methods). Cl^- composition of the standard transport buffer (see caption to Figure 1) was changed to essentially zero by substituting equimolar K gluconate for KCl and $MgSO_4$ for $MgCl_2$ and adjusting pH with H_2SO_4 instead of HCl, etc. Rate constants for the two 9.3/pVT curves are similar (analysis not shown), but an increase in amplitude of the ΔpH formed is found upon imposition of $\Delta Cl^{o/i}$, consistent with additional inward passive diffusion of H^+ with the counteranion (*18*). When these two traces are subtracted, the result (top dashed trace, Figure 3B) reveals the kinetics of this additional inward H^+ movement (see below). In contrast, rate constants for the two 9.3/hu MDR 1 traces differ with (unexpectedly) a higher rate in the absence of $\Delta Cl^{o/i}$ (bottom dashed trace, Figure 3A). In the top comparison (B) we present difference traces that illustrate the results obtained upon subtracting the dashed traces in panel A (no $\Delta Cl^{o/i}$) from the solid traces in panel A ($+\Delta Cl^{o/i}$); that is, we illustrate the net effect promoted by inducing an inward Cl^- gradient. These are addition of a linear component to acidification for 9.3/pVT ISO vesicles that corresponds to passive diffusion of Cl^- inward (top dashed trace) and a positive hyperbolic curve that reflects effective elimination of H^+ from 9.3/hu MDR 1 ISO vesicles (top solid trace). The bottom comparison in Figure 3B is an analogous set of subtractions for four different experiments involving different 9.3/pVT and 9.3/hu MDR 1 ISO vesicle preparations to illustrate reproducibility. The two 9.3/pVT and two 9.3/hu MDR 1 ISO preparations were fabricated from different yeast cultures more than 6 months apart by two different students (C.T.S. and F.F.) in two different laboratories (Georgetown and Sloan-Kettering, respectively). The H^+-pumping assays for the two sets of ISO vesicles were performed by two different students in the two laboratories (C.T.S. and Mary M. Hoffman, respectively) approximately 6 months apart.

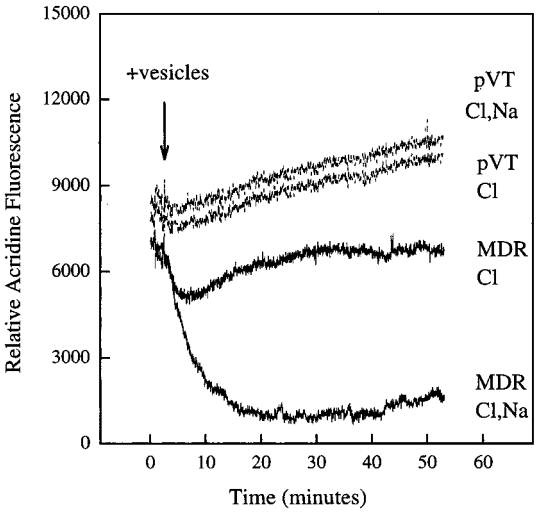

FIGURE 4: Difference traces computed to show the net effect of $\Delta Cl^{i/o} \pm \Delta Na^{i/o}$. The top two dashed traces are for 9.3/pVT ISO vesicles fabricated in the presence of either NaCl (top trace) or KCl (second trace from top); that is, the internal ISOV ionic composition was adjusted by replacing K^+ salts with equimolar Na^+ salts in the ISOV fabrication buffers (see the previous paper). The vesicles containing these salts were then diluted into either KCl or K gluconate (transport buffer composition as described above in Figure 3 caption), and the trace from the former experiment was subtracted from the trace for the latter to illustrate the effect of imposing $\Delta Cl^{i/o}$ (e.g., a decrease in the rate of ISOV acidification, apparent as a line with positive slope, second trace from the top) or the effect of imposing both $\Delta Cl^{i/o}$ and $\Delta Na^{i/o}$ (a similar decrease in the rate of acidification, top trace). The bottom two solid traces illustrate an analogous set of experiments and subtractions for 9.3/hu MDR 1 ISO vesicles that illustrate the net effects of imposing $\Delta Cl^{i/o}$ and $\Delta Na^{i/o}$ (bottom trace) or $\Delta Cl^{i/o}$ (second trace from bottom). In this case, an outward directed Cl^- gradient promotes 9.3/hu MDR 1 ISO vesicle acidification, and addition of $\Delta Na^{i/o}$ enhances the effect.

In previous work (*12*) we also showed that apparent Cl^-/H^+ antiport was dependent upon the presence of Na^+, but we were unable to demonstrate an effect of ΔNa^+, most likely a consequence of the fact that this parameter is very difficult to artificially manipulate for living cells. In Figure 4 we show the effects of adding a $\Delta Na^{i/o}$ to the $\Delta Cl^{i/o}$ with regard to H^+ movement by control 9.3/pVT and 9.3/hu MDR 1 ISO vesicles. We isolate the effects due to the sum of $\Delta Cl^{i/o}$ and $\Delta Na^{i/o}$ more clearly via subtracting traces recorded for a given vesicle preparation in the absence of a $\Delta Cl^{i/o}$ (but otherwise identical conditions) from a trace recorded for the exact same preparation in the presence of a $\Delta Cl^{i/o}$ (see caption). Thus, the difference traces (top dashed, 9.3 pVT; bottom solid, 9.3/hu MDR 1) represent ISO vesicle acidification promoted by addition of ATP, $\Delta Cl^{i/o}$, and $\Delta Na^{i/o}$. From this difference analysis, it is clearly apparent that the additional presence of a $\Delta Na^{i/o}$ has no strong effect on acidification of the control ISO vesicles but very strongly enhances ATP- and $\Delta Cl^{i/o}$-dependent acidification of 9.3/hu MDR ISO vesicles.

However, interestingly, we found that imposition of $\Delta Na^{o/i}$ (Na^+ gradient in the opposite direction) did not appreciably enhance or decrease the amplitude of the effect of $\Delta Cl^{o/i}$ for the 9.3/hu MDR 1 ISO vesicle preparations as described in Figure 3. Thus the ΔNa^+ effect, unlike the ΔCl^- effect, does not appear to be symmetrical. That is, Cl^- gradients oriented in either direction clearly appear to significantly affect H^+ movement for 9.3/hu MDR 1, but a Na^+ gradient effect is much more easily identified when oriented inside to out for the ISO vesicles (e.g., the orientation corresponding to the physiologic orientation of the Na^+ gradient for epithelial cells that express hu MDR 1 endogenously).

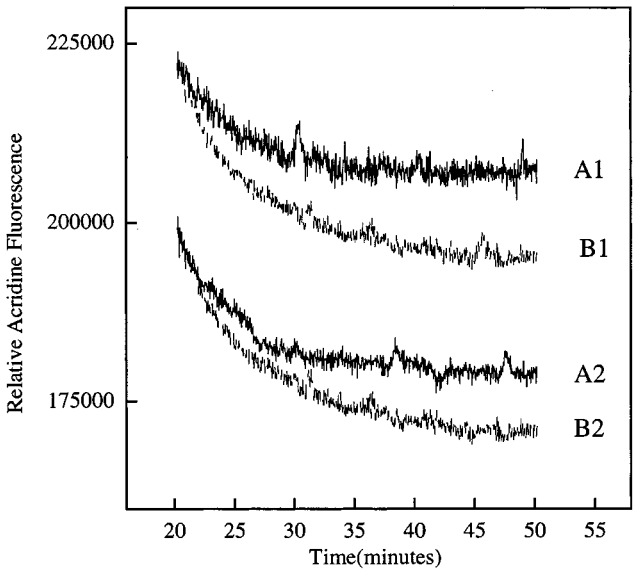

FIGURE 5: Effect of C219 pretreatment on H^+ transport in 9.3/hu MDR 1 ISO vesicles. ISO vesicles were preincubated in either the presence (dashed lines) or absence (solid lines) of 20 μg/mL C219 antibody and then diluted into standard transport buffer containing Cl^- but at pH 8.50. To demonstrate reproducibility, the results from two sets of experiments using different independent ISO vesicle preparations are shown (top and bottom comparisons). In constrast, there is no effect of C219 on the H^+ pumping by 9.3/pVT ISO vesicles (not shown).

To further test whether this anomalous H^+ movement is due to hu MDR 1 ATPase activity, we analyzed the effects of the antibody C219, which binds to the ATP hydrolysis sites of hu MDR 1 protein and has been reported to partially inhibit hu MDR 1 protein activity. We wished to design experiments in this regard that might also further help distinguish between acridine-pumping and H^+-transport interpretations for hu MDR 1 function in these ISO vesicles. Thus, as shown in Figure 5, we analyzed H^+ pumping at high pH for 9.3/hu MDR 1 ISO vesicles in the presence of an inward directed Cl^- gradient in either the absence (solid lines, each comparison, labeled A1, A2; the numbers refer to two independent ISO vesicle preparations) or presence (dashed lines, each comparison, labeled B1, B2) of C219 antibody. Since an inward Cl^- gradient *slows* 9.3/hu MDR 1 ISO vesicle acidification (cf. Figure 3A) by promoting H^+ movement in the opposite direction (cf. Figure 3B), then inhibition of hu MDR 1 by C219 in the presence of an inward Cl^- gradient would be predicted to *increase* acidification for 9.3/hu MDR 1 ISO vesicles. This is indeed observed, as seen by an increase in acridine quenching (B1, B2 vs A1, A2, Figure 5). If we analyze these data in the context of a drug pump hypothesis for hu MDR 1, C219 should have *decreased* acridine quenching, since it would be predicted to *inhibit* inward "pumping" of acridine. Taken together, these data strongly support a role for hu MDR 1 protein in ATP-, Na^+-, and Cl^--dependent H^+ transport.

DISCUSSION

In previous work (*12*) we isolated unusual Cl^- gradient effects on plasma membrane H^+ transport for living LR73/

hu MDR 1 CHO transfectants under constant perfusion. These were unusual because the relative orientation of the Cl^- gradient that appeared to enhance inward H^+ movement mediated by hu MDR 1 in these cells would normally be predicted to decrease inward H^+ movement. This is also the case for the present data: predicted outward movement of Cl^- upon imposing $\Delta Cl^{i/o}$ should (simplistically) generate an interior positive electrical potential, which should inhibit net inward movement of H^+ (as is indeed seen for control 9.3/pVT ISO vesicles, cf. Figure 4). However, $\Delta Cl^{i/o}$ actually enhances net inward H^+ movement for 9.3/hu MDR 1 ISO vesicles. The transport mediated by hu MDR 1 also requires ATP, and this presumably supplies sufficient energy for this predicted active transport; we do not find that $\Delta Cl^{i/o}$ alone is capable of acidifying 9.3/hu MDR 1 ISOV. In addition, along with Cl^- gradient effects, there were several other interesting features to the Cl^-/H^+ transport described previously (*12*), including a strong dependency on Na^+. Due to the high intrinsic permeability of eukaryotic plasma membrane to Na^+, we were unable to determine whether the presence of a Na^+ gradient further affected the apparent Cl^-/H^+ antiport. Nonetheless, we suspected that some other ion (e.g., Na^+) might be cotransported in the measured reaction, since a Cl^-/H^+-exchange reaction would predict a two fold electrogenic process, and these are rare. By overexpressing hu MDR 1 to high levels in yeast strain 9.3 and fabricating ISO vesicles, we are now better able to test the effects of artificial Na^+ gradients on the Cl^-/H^+ transport putatively catalyzed by hu MDR 1 protein. Although we do not directly measure Na^+ transport via hu MDR 1, we find that a Na^+ gradient oriented in the same direction as the Cl^- gradient strongly enhances H^+ transport in the opposite direction for 9.3/hu MDR 1 ISO, but preferentially when that gradient is oriented inside to out for the ISOV. By the most straightforward objective analysis we can envision, this suggests that hu MDR 1 protein (directly or indirectly) catalyzes Na^+:Cl^-:OH^- cotransport, preferentially inside to out for these ISOV (corresponding to outside to in for typical epithelial cells in which hu MDR 1 is endogenously expressed), but that a different reaction, not dependent on the Na^+ gradient, occurs in the opposite direction (corresponding to inside to out for epithelial cells). Depending on the stoichiometry of the cotransported ions, the Na^+:Cl^-:OH^- process could still be electrogenic but could also (at 2:1:1 stoichiometry) be electroneutral. That is, we propose that these dramatic (in some cases counterintuitive) effects of Cl^- and Na^+ gradients on H^+ transport argue that Cl^- and Na^+ may be antiported with H^+. However, note we have not directly measured Na^+ or Cl^- transport in this study; although H^+ transport clearly appears to be affected by hu MDR 1, firm proof that hu MDR 1 protein directly catalyzes Na^+:Cl^-:OH^- cotransport (or only some component of this overall reaction scheme) will await detailed quantitative studies with purified reconstituted protein.

An alternative ion-transport interpretation is also possible, but more speculative. We could envision that Cl^- and/or Na^+ gradient effects in this study modulate an appropriate ion channel activity of hu MDR 1 via effects on plasma membrane potential and that the resultant novel H^+ movement attributed to hu MDR 1 is passive and an indirect result of this channel activity. However, this would presumably entail ATP-dependent generation of an interior negative $\Delta\Psi$ in the 9.3/hu MDR 1 ISO vesicles upon imposition of $\Delta Cl^{i/o}$ and generation of an interior positive $\Delta\Psi$ upon imposition of $\Delta Cl^{o/i}$. It would also necessitate an unusually high passive H^+ permeability for these ISO vesicles; thus we favor the former interpretation.

Regardless, in previous work (*12*) we suggested that the unusual ion transport due to the overexpression of hu MDR 1 in LR73 CHO fibroblasts could be either the result of direct ion transport by hu MDR 1 protein or the indirect consequence of hu MDR 1 regulation of ion transport mediated by some other ion transporter(s). This cautious view was motivated by three factors: (1) previous extensive controversy regarding Cl^- channel activity versus Cl^- channel regulator activity of hu MDR 1 (e.g., refs *19* vs *20*], (2) the unusual predicted twofold electrogenic character of apparent Cl^-/H^+ exchange, and (3) our earlier observations of altered ion-exchanger activity and expression in some MDR cell lines (e.g., ref *4*) which made us more suspicious about a variety of possible indirect effects. Thus, part of our motivation for expressing hu MDR 1 protein to high levels in various yeast strains was to begin to distinguish between direct and indirect effects on ion transport. We reason that since the ion-transporter composition and overall bioenergetics of yeast and higher eukaryotic plasma membrane are different, it would be unlikely for an ion-transport regulator (e.g., kinase, phosphatase, etc.) to work similarly in both situations. The fact that apparent ATP- and Na^+-dependent Cl^-/H^+ antiport has been putatively found upon overexpression of hu MDR 1 protein in both membrane systems (LR73 CHO fibroblasts and these yeast) argues that these processes are likely (at least in part) directly mediated by hu MDR 1.

A number of other proposals for direct ion transport by hu MDR 1 by a number of laboratories have generated much controversy over the past several years. In particular, strong differences of opinion and conflicting data have been generated regarding earlier proposals that hu MDR 1 protein might function as a Cl^- channel (*19*, *20*) or as an ATP channel (*21*, *22*). Strong Cl^- gradient effects on H^+ transport by hu MDR 1 presented in this paper suggest movement of Cl^- by hu MDR 1 may indeed occur (however, not necessarily with channel-like kinetics) but also argue that observation of the transport could be complicated by movement of, or gradients in, additional ions (particularly H^+ and Na^+). Considering various possibilities inherent in ion cotransport mechanisms might help to resolve some of the apparent controversies present in the literature. For example, on the basis of the data in this paper, we would argue that pH and Na^+ composition of the electrode and bath solutions used in electrophysiologic measurements should greatly influence the ability to measure Cl^- transport via hu MDR 1 via electrophysiologic methodologies. Interestingly, one study that strongly concluded Cl^- transport was not associated with hu MDR 1 expression (*20*) used Tris-Cl and Cs-Cl as the chloride-containing salts in the electrophysiology bathing and pipet solutions and replaced Tris-Cl with Tris-aspartate when Cl^--free solutions were desired. In contrast, Valverde and colleagues (*19*) used CsCl in the pipet solution, but isotonic NaCl in the bath, and replaced NaCl with Na gluconate when Cl^--free solutions were desired. Not only were experiments by Valverde et al. performed in the presence of Na^+, many were apparently performed in the presence of a Na^+ *gradient*, which, on the basis of our data,

should strongly enhance the ability to measure Cl^- transport mediated by hu MDR 1. Regardless of the precise mechanism, it is important to note that in a general way *all* of these proposals (from a number of laboratories at this point) are consistent with the altered partitioning model for hu MDR 1-mediated multidrug resistance (*4*, *6*, *7*). Although additional work is required to fully define the precise ion-transport mechanism, all of the general ion-transport schemes that have been proposed are capable of instigating pharmacologically relevant perturbations in $\Delta\Psi$ and/or pH_i that would certainly affect accumulation, retention, and subcellular partitioning of chemotherapeutic drugs and other hydrophobic compounds.

We have followed a straightforward interpretation of these acridine data that does not violate the law of enzyme specificity as does the opposing "drug pump" model for hu MDR 1 protein. Although an ion-transport function for hu MDR 1 is more attractive to us, it may not be the preferred interpretation of other investigators. Regardless of the individual interpretation, data such as these will assist further analysis of ion-dependent hu MDR 1 protein function.

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