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ACCELERATED COMMUNICATION

Functional Characterization of Coding Polymorphisms in the Human *MDR*1 Gene Using a Vaccinia Virus Expression System

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ABSTRACT

The human *MDR*1-encoded transporter is a 170-kDa plasma membrane glycoprotein [P-glycoprotein (P-gp)] capable of binding and energy-dependent extrusion of structurally diverse organic compounds and drugs. P-gp seems to play a significant role in uptake, distribution, and excretion of many different drugs. To determine whether common polymorphic forms of P-gp are likely to alter function of P-gp, we characterized five known *MDR*1 coding polymorphisms (N21D, F103L, S400N, A893S, and A998T) using a vaccinia virus-based transient expression system. Cell surface expression of wild-type P-gp was time-dependent over a time course of 5.5 to 34.5 h; highest expression was obtained by 22 to 26.5 h after infection/transfection, indicating that a semiquantitative assay for P-gp expression levels was possible. HeLa cells stained with the P-gp specific monoclonal antibodies MRK-16 and Western blots probed with C219 revealed similar cell surface expression for the polymorphisms and for wild-type protein. Time-dependent P-gp pump function maximal at 22 h after infection/transfection was demonstrated for the following *MDR*1 fluorescence substrates: 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoic acid, succinimidyl ester (bodipy-FL)-verapamil, bodipy-FL–vinblastine, calcein-AM, bodipy-FL–prazosin, bisantrene, and bodipy-FL–forskolin, but not for daunorubicin. Transport studies of all tested substrates indicated that the substrate specificity of the pump was not substantially affected by any of the tested polymorphisms. Cell surface expression and function of double mutants including the more common polymorphisms (N21D-S400N, N21D-A893S, and S400N-A893S) showed no differences from wild-type. These results demonstrate that the common *MDR*1 coding polymorphisms result in P-gps with a cell surface distribution and function similar to wild-type P-gp.

The pharmacokinetics of commonly used drugs varies from person to person, reflecting differences in absorption, distribution, metabolism, and excretion. A family of ATP-dependent ABC transporters has been described, several members of which seem to be involved in drug absorption, distribution, and excretion (Gottesman, 2002). One of the best-characterized members of this superfamily is P-glycoprotein (P-gp), the product of the human *MDR*1 gene (Ambudkar et al., 1999). P-gp was first described as an energy-dependent efflux pump for diverse hydrophobic natural product anticancer drugs such as doxorubicin, vinblastine, and paclitaxel (Taxol) but has since been shown to transport dozens of different commonly used drugs including HIV protease inhibitors (Kim et al., 1998; Lee et al., 1998), cholesterol-lowering statins (Bogman et al., 2001), antihistamines (Chiou et al., 2001), and digoxin (Mayer et al., 1996). The localization of P-gp in the mucosa of the small and large intestine, at blood-brain barrier sites, in biliary hepatocytes, and in proximal tubules of the kidney (Thiebaut et al., 1987; Cordon-Cardo et al., 1989; Thiebaut et al., 1989) together with evidence from *mdr* knockout transgenic mice, indicates a significant role for P-gp in drug pharmacokinetics (Schinkel et al., 1997; Borst et al., 1999, 2000).

Several recent reports indicate that polymorphisms are relatively common in the human *MDR*1 gene (Yoshimoto et al., 1988; Mickley et al., 1998; Decleves et al., 2000; Hoffmeyer et al., 2000; Liu and Hu 2000; Ameyaw et al., 2001; Brinkmann et al., 2001; Cascorbi et al., 2001; Hitzl et al.,

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ABBREVIATIONS: ABC, ATP binding cassette; P-gp, P-glycoprotein; MDR, multidrug resistance; FACS, fluorescence-activated cell sorting; IMDM, Iscove's modified Dulbecco's medium; bodipy-FL, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoic acid, succinimidyl ester.

2001; Ito et al., 2001; Kerb et al., 2001; Kim et al., 2001; Schaeffeler et al., 2001). This finding has stimulated interest in whether common coding polymorphisms affect function of P-gp and/or whether polymorphic variants are linked to altered drug pharmacokinetics. In this study, we examined the five most common P-gp coding polymorphisms previously reported in the literature (N21D, F103L, S400N, A893S, and A998T). We show, using a transient vaccinia expression system to avoid bias resulting from selecting for P-gp expression and optimizing this system to allow for semiquantitative interpretation of results, that none of the common coding polymorphisms alter cell surface localization or transport function of P-gp, as measured using monoclonal antibodies to P-gp and six diverse fluorescent substrates.

Materials and Methods

Cell Line, Cell Culture, and Propagation of the Vaccinia Virus. HeLa cells (cervical epidermoid carcinoma) were maintained as described previously (Ramachandra et al., 1998). Recombinant vaccinia virus encoding bacteriophage T7 RNA polymerase (vTF7-3) was propagated in HeLa cells and purified as described previously (Earl et al., 1991). Titration of the virus was performed on HeLa infected/transfected cells using pTM1-MDR1 vector (Hrycyna et al., 1998).

Vector Construction. The pTM1-MDR1 plasmid (Ramachandra et al., 1998), encoding wild-type P-gp, was used for the vaccinia virus expression system. Five different polymorphisms in the coding region of the human MDR1 cDNA have been described in the literature (references in Table 1). To examine their impact on P-gp cell surface expression and on P-gp function, we generated these changes in the pTM1-MDR1 vector. Using the technique described by Kunkel et al. (1987), five different mutated sites were introduced into the *MDR*1 gene with the following primers: for the N21D (A→G) polymorphism, 5′ TTT TTC ACT TTT ATC GTT CAG TTT AA 3′; for the F103L (C→T) polymorphism, 5′ CAG ATT CAT GAA GAG CCC TGT ATC A 3′; for the S400N (G→T) polymorphism, 5′ TCG AGA TGG GTA ATT GAA GTG AAC AT 3′; for the A893S (G→T) polymorphism, 5′ AGC GAT CTT CCC AGA ACC TTC TAG TT 3′; and for the A998T (G→A) polymorphism, 5′ TAT TTT GGC TTT GGT ATA GTC AGG AGC 3′. Double mutant *MDR*1s were generated using the *Nde*I and *Xho*I restriction enzymes on single mutant templates (N21D-S400N, N21D-A893S, and S400N-A893S). All construct sequences were verified in both directions by automated sequencing with the PRISM Ready Reaction Dye Deoxy Terminator sequencing kit (Applied Biosystems, Foster City, CA).

Infection/Transfection into HeLa Cells and P-gp Expression. Recombinant vaccinia virus encoding bacteriophage T7 RNA polymerase (vTF7-3), which is required for the expression of a gene under the control of a T7 promoter, was obtained from Dr. B. Moss (National Institute of Allergy and Infectious Diseases, Bethesda, MD). Cells were infected/transfected with vTF7-3 and various plasmids as described previously (Hrycyna et al., 1998; Gribar et al., 2000), and incubated for 5.5 to 34.5 h at 32°C, 5% CO_2. Levels of cell surface P-gp were detected by FACS, using monoclonal antibody MRK-16 (Germann et al., 1996). SDS-polyacrylamide gel electrophoresis and immunoblotting using monoclonal antibody C219 to detect P-gp were performed as described previously (Hrycyna et al., 1998).

Drug Accumulation Assays. 5×10^5 cells were harvested after trypsinization by centrifugation and resuspended in 1 ml of Iscove's modified Dulbecco's medium (IMDM), supplemented with 5% fetal bovine serum. The fluorescent substrates bodipy-FL–paclitaxel (0.1 μM), bodipy-FL–verapamil (0.5 μM), daunorubicin (3 μM), bodipy-FL–vinblastine (0.5 μM), calcein-AM (0.5 μM), bodipy-FL–prazosin (0.5 μM), bisantrene (0.5 μM), and bodipy-FL–forskolin (0.5 μM) (Molecular Probes, Eugene, OR) were added to cells in the presence or absence of the P-gp inhibitor cyclosporin A (5 μM; Calbiochem, San Diego, CA), and incubated at 37°C for 40 min. For daunorubicin efflux, an additional incubation with only IMDM or IMDM with cyclosporin A was performed for 40 min at 37°C. The pellet was resuspended in 300 μl of phosphate-buffered saline before FACS analysis (Hrycyna et al., 1998) using CellQuest software (BD Biosciences, San Jose, CA). The difference between the median value of the fluorescence substrate curve and the median value of the fluorescence substrate with an inhibitor was plotted (arbitrary units) versus the harvesting time after infection/transfection.

Results

Time Course of Expression of Wild-Type P-Glycoprotein Using a Vaccinia Virus Expression System. Wild-type MDR1 cDNA is under the control of a T7 promoter and downstream from an internal ribosome entry site in the pTM1 vector. As described previously, high levels of expression of wild-type MDR1 protein can be obtained in mammalian cells 24 h after infection with the vTF7-3 recombinant vaccinia virus encoding bacteriophage T7 RNA polymerase followed by transfection with the pTM1-MDR1 vector (Ramachandra et al., 1998; Hrycyna et al., 1998). We have chosen HeLa cells for these studies because of their low level of endogenous P-gp expression, their ability to express high

TABLE 1
Common *MDR*1 polymorphisms that change amino acids

Location		Polymorphic Variant	Heterozygous Frequency	Reference
Exon	Nucleotide			
			%	
2	61	N21D	17.6	Hoffmeyer et al. (2000)
			11.2	Cascorbi et al. (2001)
			5.7	Decleves et al. (2000)
5	307	F103L	1.2	Hoffmeyer et al. (2000)
10	1107	G369P	0.2	Cascorbi et al. (2001)
11	1199	S400N	12.9	Hoffmeyer et al. (2000)
			5.5	Cascorbi et al. (2001)
		A893S	43.0	Mickley et al. (1998)
		A893T	41.6	Cascorbi et al. (2001)
21	2677	A893S	62.0[a], 13.0[b]	Kim et al. (2001)
		A893G	56.4	Cascorbi et al. (2001)
24	2995	A998T	11.0	Mickley et al. (1998)

[a] European Americans.
[b] African Americans.

levels of wild-type and mutant P-gp after vaccinia infection/transfection, and their relative ease of transfection (Hrycyna et al., 1998; Ramachandra et al., 1998; Gribar et al., 2000). To develop a more quantitative assay to detect minor changes in function or in cell surface expression, we performed time course studies to find a time after transfection when the amount of P-gp on the cell surface and P-gp function were in a linear range.

An immunoblot analysis with monoclonal antibody C219 shows an increase in expression levels for total P-gp (Fig. 1A) from 5.5 to 32.5 h after infection/transfection. The specific increase in P-gp cell surface expression as measured by FACS with MRK-16 staining was also determined (Fig. 1B). Cells infected with the control plasmid show very low levels of endogenous P-gp expression. As shown in Fig. 1B, P-gp is detectable 5.5 h after infection/transfection but approximately 4-fold less than at 26.5 h after infection/transfection, when it is expressed at highest levels. In contrast to the Western blots that show continuing total cell accumulation of P-gp, P-gp cell surface expression decreases after 26.5 h. Any time between 5.5 and 26.5 h after infection, during which cell surface expression of P-gp increases linearly, was deemed optimal for assays of cell surface P-gp.

Functional Assays for P-gp with Substrate. Fig. 2 summarizes the results obtained from functional assays for P-gp with the substrates bodipy-FL–paclitaxel, bodipy-FL–verapamil, and bodipy-FL–prazosin. Consistent with the expression results, we find increases between 5.5 and 26.5 h and P-gp function decreases in this system ~26 h post infection/transfection for most substrates. However, with daunorubicin, we could demonstrate little or no change in P-gp function during the time course, despite changes in cell surface P-gp. Therefore, for daunorubicin, the functional assay is not a quantitative measure of the amount of P-gp on the cell surface.

We conclude from these results that to detect minor differences between different *MDR*1 polymorphic forms, cell surface expression and functional assays should be done between 5.5 and 26.5 h after infection/transfection. We chose 13.5 h after transfection/infection to optimize the sensitivity of the assay.

Effect of Five Common *MDR*1 Polymorphisms on P-Gp Cell Surface Expression and Function. HeLa cells were infected/transfected with the pTM1-MDR1 vector harboring the *MDR*1 polymorphisms N21D, F103L, S400N, A893S, and A998T (described in Table 1). MRK-16 staining was performed on all infected/transfected cells at 9, 13.5, and 26 h after infection/transfection. Figure 3 shows that the amount and efficiency of P-gp cell surface expression for all five polymorphic variants is superimposable on the data for wild-type P-gp. Western blot analysis of P-gp expression for all polymorphisms gave similar results (data not shown).

We measured P-gp function using the fluorescent substrates bodipy-FL–verapamil, daunorubicin, bodipy-FL–vinblastine, calcein-AM, bodipy-FL–prazosin, bisantrene, bodipy-FL–paclitaxel, and bodipy-FL–forskolin, all with or without the P-gp inhibitor cyclosporin A. Figure 4, A to C, shows very similar P-gp cell surface expression for all mutants as well as for wild-type *MDR*1 with the fluorescent compounds calcein AM, bodipy-FL–forskolin, bodipy-FL–verapamil, and bodipy-FL–paclitaxel. Results for the other fluorescent substrates (bodipy-FL–vinblastine, bodipy-FL–prazosin, bisantrene) are not shown but were similar. For bodipy-FL–paclitaxel, the efflux by wild-type P-gp was slightly more then the efflux seen with the plasmids carrying each of the polymorphic variants (Fig. 4D). All cells infected/transfected with double mutants (N21D-S400N, N21D-A893S, and S400N-A893S) revealed results similar to those of the single mutants (data not shown).

Discussion

In this study a transient vaccinia expression system was used to determine the effect of five known coding human *MDR*1 polymorphisms on P-gp function: N21D, F103L, S400N, A893S, and A998T. Using this system, we were able

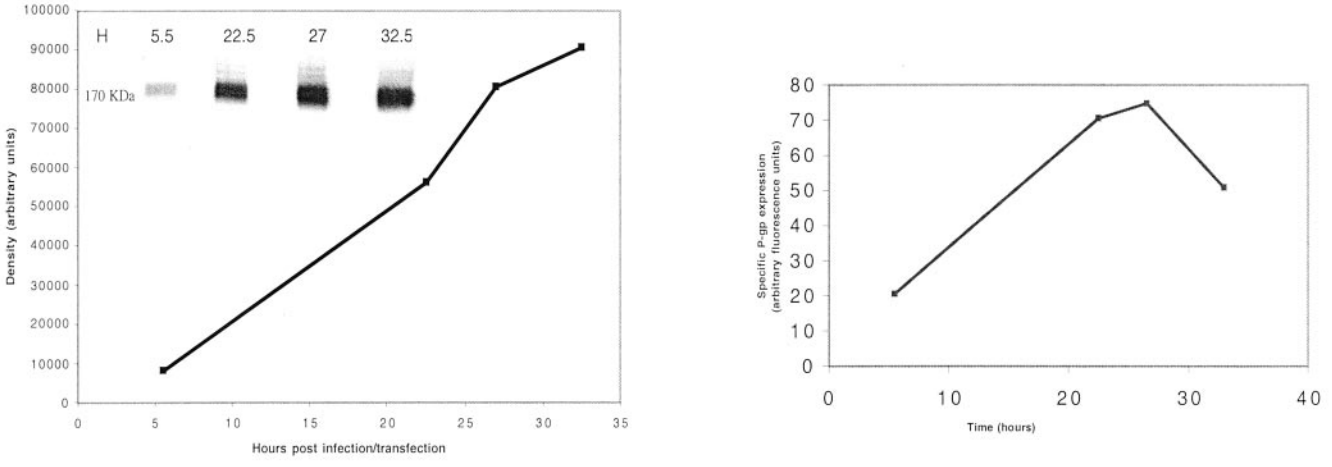

Fig. 1. Time course for cell surface level of wild-type P-gp expressed in vaccinia system. A, an immunoblot analysis 5.5, 22.5, 27, and 32.5 h post infection/transfection (2 μg protein/lane) was done using the monoclonal antibody C219. Peroxidase-conjugated anti-mouse secondary antibody (see *Materials and Methods*) was used to detect antigen-antibody complexes by ECL. The specific density (arbitrary units) of each band was quantified by subtracting the density value of the background from the density value of that band (using Eagle Eye system; Stratagene, La Jolla, CA), and was plotted against the time (in hours) after infection/transfection. B, infected/transfected HeLa cells were incubated and analyzed by FACS as described under *Materials and Methods*, with MRK-16 monoclonal antibody 5.5, 9, 13.5, and 26 h after infection/transfection. The specific change in P-gp cell surface expression (the difference between the median value of the control antibody curve and the median value of the MRK-16 curve (arbitrary units) was plotted against the time (in hours) after infection/transfection.

to demonstrate semiquantitative functional assays for six different fluorescent substrates, as well as for cell surface expression of P-gp using monoclonal antibody MRK-16. Comparison between each of these polymorphisms and wild-type *MDR*1 revealed no modification of cell surface localization and expression and no measurable change in transport function of P-gp by these polymorphisms.

*MDR*1 encodes a 170-kDa transmembrane transporter, P-gp, which confers energy-dependent resistance to a number of naturally occurring, structurally unrelated chemotherapeutic agents (Gottesman et al., 1995). Some recent studies have reported several different polymorphisms in the *MDR*1 coding region; some of these were "silent" and caused no amino acid change but others were found to change an amino acid (Brinkmann et al., 2001). Several of the polymorphisms were found to be relatively common in the population under study. However, different studies revealed variability in the frequencies of these polymorphisms in different populations (Decleves et al., 2000; Hoffmeyer et al., 2000; Cascorbi et al., 2001). These might be caused by a founder effect (e.g., predominant German population versus American population) or by undetermined subpopulations in the U.S.

Another interesting phenomenon in P-gp is a polymorphic site 893 with 3 different amino acid changes. Mickley et al. (1998), Cascorbi et al. (2001), and Kim et al. (2001) found different allelic frequencies in their study populations. This variability might reflect the relative insignificance of this specific amino acid at this location, its selective advantage in some populations, or, given its location in a region of repetitive DNA sequence, a failure in sequencing at this position (2677 bp, amino acid 893). Our data (C. Kimchi-Sarfaty, J. Gribar, M. Edmondsen, J. Kelley, unpublished observations) indicate multiple different substitutions at this site with sequencing data of reasonably high quality.

P-gp drug accumulation assays reflect both the level of gene expression, and the ability of the protein to function. The effect of any changes in the coding sequence may affect overall structure, stability, and subcellular localization of the protein and the affinity and/or efficiency of transport of individual substrates. Currently, only a few articles have re-

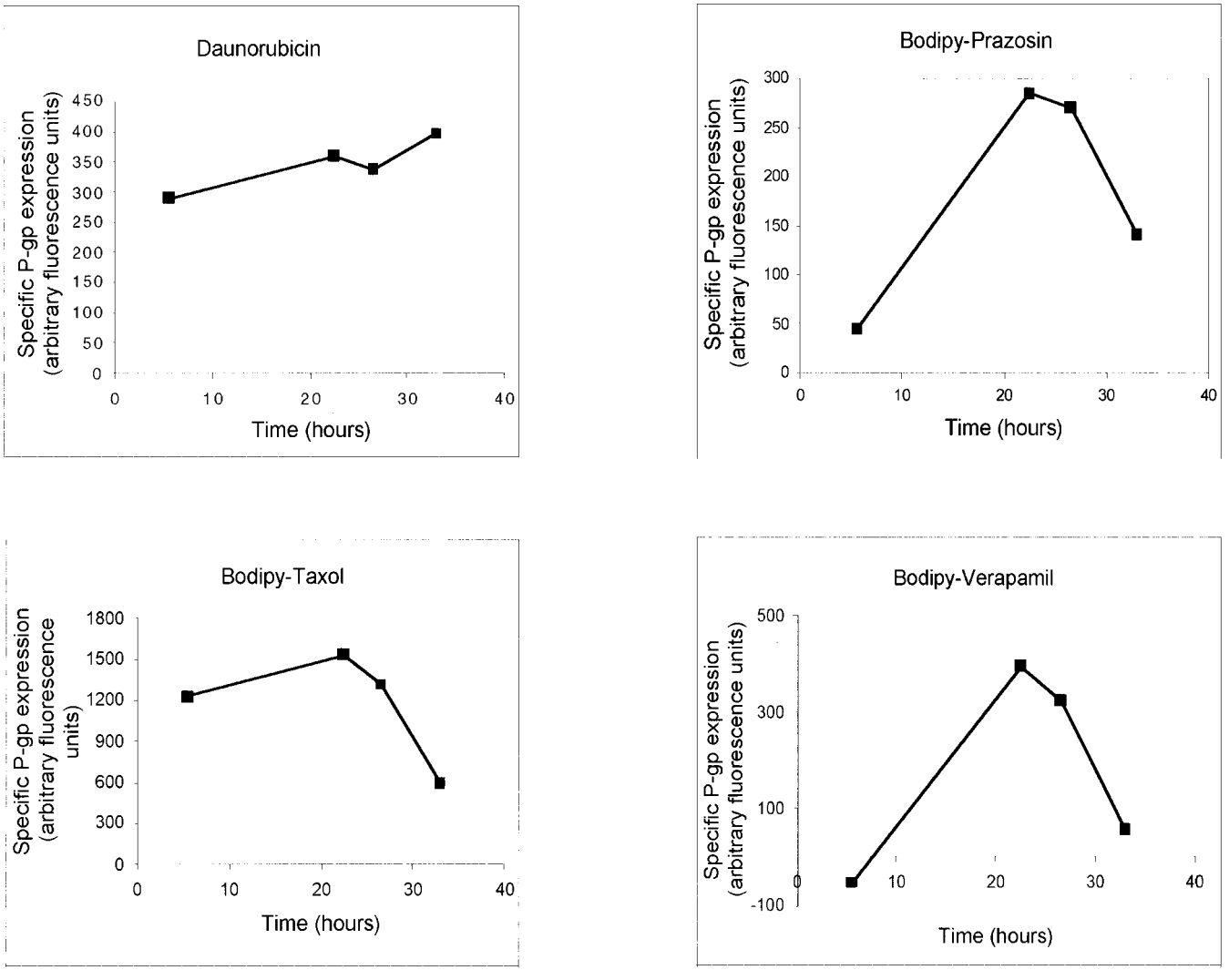

Fig. 2. Time course for efflux of fluorescent substrates by wild-type P-gp. Infected/transfected HeLa cells were incubated and analyzed by FACS (as described under *Materials and Methods*), with daunorubicin, bodipy-FL–prazosin, bodipy-FL–paclitaxel and bodipy-FL–verapamil 5.5, 9, 13.5, and 26 h after infection/transfection. The specific pump function of P-gp (the difference between the median value of the fluorescence substrate curve and the median value of the fluorescence substrate with the inhibitor cyclosporin A) was plotted against time in hours after infection/transfection.

ported any correlation between a polymorphism and a functional alteration in levels of expression of P-gp. Persons who carry the reported wobble polymorphism in exon 26, position 3435 (C3435T), which does not change the coding sequence, had significantly lower duodenal *MDR*1 expression and high digoxin plasma levels, reflecting higher efficiency of absorption and possibly reduced excretion of digoxin (Hoffmeyer et al., 2000; Cascorbi et al., 2001). Leukocytes were isolated from carriers of the above polymorphism and were assayed for rhodamine 123 transport (Hitzl et al., 2001); persons with the homozygous CC genotype revealed higher transport of this substrate. For the C3435T polymorphism, Hoffmeyer et al. (2000), Ameyaw et al. (2001), Cascorbi et al. (2001), and Schaeffeler et al. (2001) found differences in frequencies among different populations in their reports, which might reflect a founder effect and/or an advantage for the heterozygotes.

Kim et al. (2001) reported enhanced efflux of digoxin by cells expressing the coding polymorphism A893S. In this study, however, cells were selected for expression of P-gp after transduction by a retroviral vector until 100% of the cells expressed the gene. Other changes in these cells after this selection, including possible selection of cells expressing other ABC transporters, were not monitored. As noted above, position 893 has four different variants (Table 1), which could be interpreted to suggest a nonessential role for that position. Moreover, using the same variant, our results demonstrate that function and specificity is unaltered for six other drugs, none of which are from the same family of drugs as digoxin. This discrepancy could reflect a difference in A893S P-gp pump function for digoxin or a difference in the selected cells as noted above.

The drugs we used for the analysis (bodipy-FL–paclitaxel, bodipy-FL–verapamil, bodipy-FL–vinblastine, bodipy-FL–prazosin, bisantrene, bodipy-FL–forskolin, and calcein-AM dye) were chosen because these drugs are analogs for a large spectrum of known *MDR*1 substrates and modulators (Gottesman et al., 1995). However, four of them include a fluorescent bodipy group to make them fluorescent. Such a bulky modification may affect substrate specificity and reduce the apparent heterogeneity of the drugs we tested. We believe that this is not the case because previous studies from this lab have indicated that mutant forms of P-gp have differential altered activity toward these bodipy-substrates (Hafkemeyer et al., 1998). Another potential limitation of our study is that we have not directly determined other biochemical parameters for P-gp, including ATP binding and substrate binding. However, these are unlikely to be significantly altered if overall pump function is unchanged.

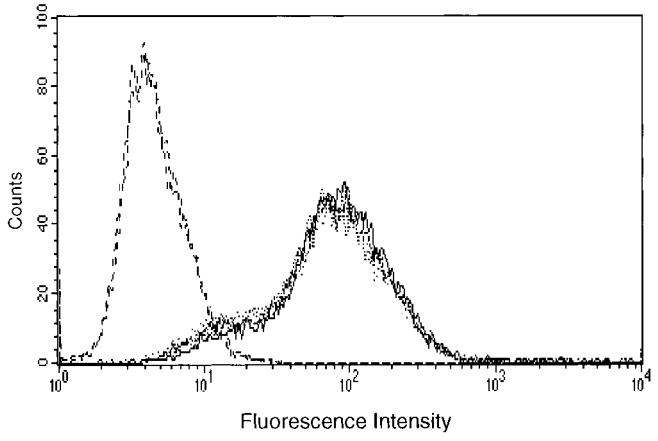

Fig. 3. Assessment of cell surface expression of wild-type and five *MDR*1 polymorphisms. Infected/transfected HeLa cells with wild-type pTM1-MDR1 (——), pTM1-MDR1-N21D (- -·- -·), pTM1-MDR1-F103L (····), pTM1-MDR1-S400N (-·-·-), pTM1-MDR1-A998T (- - - -), and pTM1-MDR1-A893S (···-···) were incubated and analyzed by FACS as described under *Materials and Methods*, with MRK-16 or control IGg2aκ monoclonal antibodies (-·-·-) 13.5 h after infection/transfection.

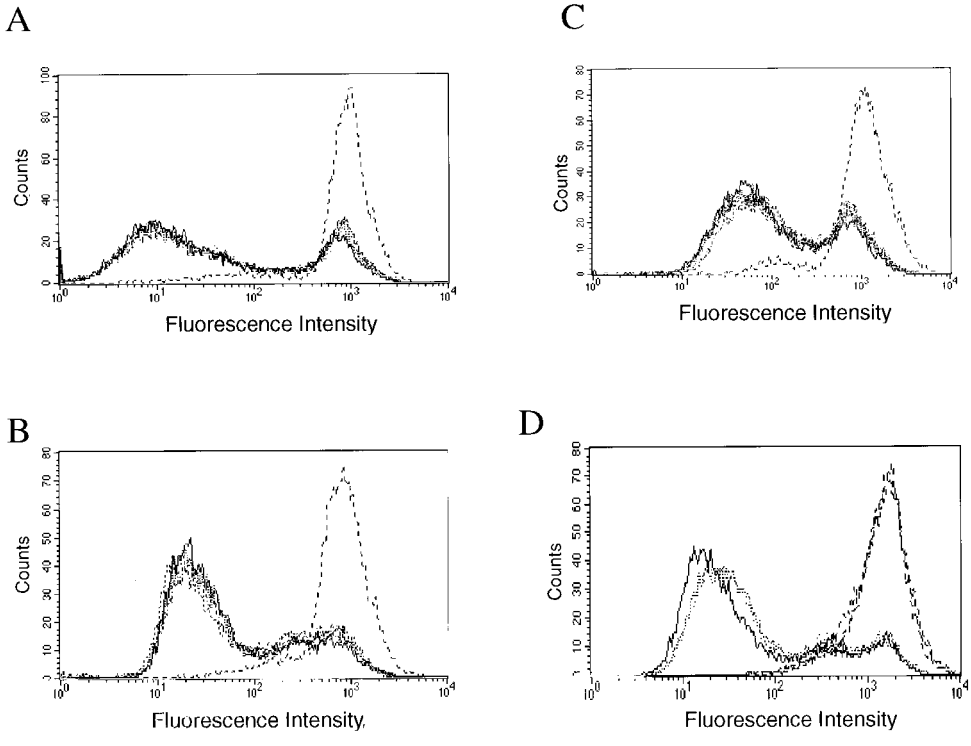

Fig. 4. Drug transport function of wild-type and five *MDR*1 polymorphisms. The drug accumulation of vaccinia virus infected-transfected HeLa cells was determined by FACS analysis. Cells were transfected with pTM1 (control), pTM1-MDR1, (wild-type P-gp), pTM1-MDR1-N21D, pTM1-MDR1-F103L, pTM1-MDR1-S400N, pTM1-MDR1-A893S, and pTM1-MDR1-A998T. At 13.5 h after infection, cells were harvested, washed and loaded for 20 min. with A, 0.5 μM Calcein-AM: wild-type (——), N21D (- -·- -·), and S400N (-·-·-), in the presence of an inhibitor, 5 μM cyclosporin A (- - -); B, 0.5 μM bodipy-FL–forskolin: wild-type (——), N21D (- -·- -·), F103L (····), and S400N (-·-·-), in the presence of an inhibitor, 5 μM cyclosporin A (- - -); C, 0.5 μM bodipy-FL–verapamil: wild-type (——), N21D (- -·- -·), F103L (····), and S400N (-·-·-), in the presence of an inhibitor, 5 μM cyclosporin A (- - -); D, 0.1 μM bodipy-FL–paclitaxel: wild-type (——), A893S (- -·- -·), in the presence of an inhibitor, 5 μM cyclosporin A for the wild-type (- -), and for A893S (- - -).

The five polymorphisms we studied are located in exons 2, 5, 11, 21, and 24 of the *MDR*1 gene. Based on the predicted secondary structure of P-gp, these exons correspond to extracellular and intracellular as well as transmembrane regions, none of which has previously been shown to affect substrate specificity or protein stability. The finding that cell surface localization and expression of these polymorphisms was not altered even in the double polymorphisms is not, therefore, surprising. We have no explanation for the slight decrease observed in their ability to efflux bodipy-FL–paclitaxel. It might be that the paclitaxel transport assay is more sensitive to these structural alterations. Paclitaxel is a preferred substrate for P-gp, and perhaps wild-type P-gp has a minor advantage compared with all of the tested polymorphic forms.

It remains to be determined whether these polymorphisms, or perhaps polymorphisms not yet tested, will be altered in their ability to transport one or more of the many specific P-gp substrates. However, these studies indicate that common polymorphisms that affect P-gp do not have global effects on its pump function. Taken together with the recent work of Hoffmeyer et al. (2000) and Kerb et al. (2001), polymorphisms that are linked to alterations in P-gp expression are more likely to be clinically significant than those studied here, which change coding sequence.

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