Phosphatidylcholine and Phosphatidylethanolamine Behave as Substrates of the Human MDR1 P-Glycoprotein†

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Received October 31, 1996; Revised Manuscript Received February 6, 1997⊗

ABSTRACT: The multidrug resistant cell line CEM/VBL300 and the parental CEM T-lymphoblastic cell line from which it was derived were used to study the accumulation of fluorescent phospholipid analogs of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS). The fluorescent analogs NBD-PC, NBD-PE, and NBD-PS and [^{3}H]PC were delivered in liposomes prepared by ethanol injection. Fluorescence microscopy demonstrated decreased accumulation of the NBD-PC analog in the multidrug resistant cell line compared to the parental cell line. Verapamil enhanced NBD-PC accumulation in the resistant cells. Similar results were obtained with insect cells expressing high levels of recombinant human MDR1. Elimination of NBD fluorescence on the outer leaflet of the plasma membrane with dithionite permitted quantification of the internal cellular fluorescence by FACS analysis. The drug resistant CEM/VBL300 cells accumulated approximately 10% the amount of NBD-PE and 20% the amount of NBD-PC compared to CEM drug sensitive cells. No difference in internal accumulation of NBD-PS was found between the drug resistant and drug sensitive cell lines. The internal accumulation of NBD-PE and NBD-PC was enhanced by the MDR reversal agents verapamil, cyclosporin A, and SDZ PSC 833 in the CEM/VBL300 cells but not in the CEM cells. The increased accumulation was dose dependent, and the relative potency of the reversal agents paralleled their ability to circumvent multidrug resistance. In addition, the monoclonal antibody UIC2 directed against the P-glycoprotein produced similar results. The evidence presented here suggests that PC and PE but not PS behave as substrates for human MDR1 P-glycoprotein.

The human MDR1 P-glycoprotein is a 170 000 Da membrane glycoprotein capable of conveying resistance to multiple chemotherapeutic agents. P-glycoprotein is a member of a large superfamily of membrane transport proteins termed ATP binding cassette (ABC)[1] transporters (Higgins, 1992) or traffic ATPases (Ames et al., 1992). ABC transporters are composed of a highly conserved functional unit which includes two ATP binding domains and approximately 12 transmembrane segments. These transporters move a wide range of compounds across a variety of biological membranes (Croop, 1993; Juranka et al., 1992).

The best-characterized function of the P-glycoprotein is the transport of cytotoxic agents out of cells. Although the precise mechanism of transport remains unclear, a large body of evidence suggests that the energy dependent efflux of drugs across a concentration gradient is responsible for P-glycoprotein-mediated multidrug resistance (Horio et al., 1988; Lankelma et al., 1990; Piwnica-Worms et al., 1993; Ruetz & Gros, 1994a; Ruetz et al., 1993; Shapiro & Ling, 1994). This removal of drugs from the cytoplasm and/or membrane compartments (Croop et al., 1988) may be due to physical translocation (Gottesman & Pastan, 1989), electrochemical gradients (Abraham et al., 1993), or flipase activity (Higgins & Gottesman, 1992). Although P-glycoprotein substrates exhibit a range of physicochemical properties, many of them are hydrophobic, have a protonatable amine group, and are positively charged at neutral pH (Ford & Hait, 1990). Substrates appear to interact at common sites on the polypeptide (Bruggemann et al., 1989; Greenberger, 1993; Zhang et al., 1995) and are capable of stimulating P-glycoprotein ATPase activity (Homolya et al., 1993; Sarkadi et al., 1992). A number of MDR reversal compounds have been identified which inhibit P-glycoprotein dependent transport and reverse multidrug resistance. The pharmacological manipulation of P-glycoprotein function has made it an attractive target for the improvement of anticancer therapies.

The P-glycoproteins are members of a multigene family with two homologues in humans and three in mice. P-glycoprotein homologues that transport drugs are designated as either Class I or II (Juranka et al., 1989). The P-

† This work was supported by grants from Sandoz, the American Cancer Society (JFRA 420), the Dana-Farber Cancer Institute, and the National Institutes of Health (NIH-AI24570).

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⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1997.

[1] Abbreviations: ABC, ATP binding cassette; CsA, cyclosporin A; DES, diethylstilbestrol; FCS, fetal calf serum; $DiOC_2$, 3,3′-dimethylodicarbocyanine iodide; EGF, epidermal growth factor; IgG, immunoglobulin G; MDR, multidrug resistance; NBD-PC, 1-palmitoyl-2-12-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine, PS, phosphatidylserine; pfu, plaque-forming unit; RT-PCR, reverse-transcriptase polymerase chain reaction; PCR, polymerase chain reaction; Rho123, rhodamine 123; 0.2× SSC, 30 mM NaCl, 3 mM sodium citrate; SDS, sodium dodecyl sulfate; Sf, *Spodoptera frugiperda*; $TyTPP^+$, 1-pyrenebutyltriphenylphosphonium bromide.

S0006-2960(96)02728-6 CCC: $14.00

glycoprotein homologues that do not transport cytotoxic agents, human MDR3 and murine Mdr2, are designated as Class III P-glycoproteins. The Class III P-glycoproteins have been shown to function as phospholipid translocases (Ruetz & Gros, 1994b; Smith et al., 1994). Mice deficient in Mdr2 display a phenotype that includes an abnormal hepatic architecture with decreased phospholipid secretion into the bile (Smit et al., 1993). Direct observations of murine Mdr2 expressed in yeast vesicles and human MDR3 expressed in murine fibroblasts indicate that phosphatidylcholine transport across the plasma membrane is mediated by Class III P-glycoprotein homologues (Ruetz & Gros, 1994b; Smith et al., 1994). On the basis of overlapping physicochemical characteristics between hydrophobic cationic drugs which are transported by Class I P-glycoproteins and certain zwitterionic phospholipids, such as PC, which are transported by Class III P-glycoproteins, we decided to study the accumulation of phospholipids in cells expressing human MDR1. The accumulation of PC and PE, zwitterionic phospholipids which include protonable amine groups, was contrasted with PS, an anionic phospholipid containing a negatively charged serine, in cells expressing high levels of human MDR1 P-glycoprotein. We demonstrate that traditionally generated multidrug resistant cell lines, as well as insect cells expressing human MDR1, accumulate less total and internal PC and PE than the parental cell lines. There was no difference in PS accumulation between the cells that differentially express high levels of MDR1. PC and PE accumulation was augmented by the P-glycoprotein reversal agents verapamil, cyclosporin A, and SDZ PSC 833 only in cells expressing human MDR1. We evaluated the efflux of NBD-PC in multidrug resistant cells loaded with fluorescent probe and demonstrate that inhibition of P-glycoprotein results in a significantly higher level of NBD-PC retention. Consistent with these findings, human MDR1 has recently been shown to facilitate translocation of PE and PC generated from precursor C6-NBD-diacylglycerol (van Helvoort et al., 1996). These observations suggest that MDR1 P-glycoprotein mediates one mechanism by which PC and PE are translocated to the outer leaflet of the membrane.

MATERIALS AND METHODS

Cell Lines. The human CEM T-cell leukemia cell line and its multidrug resistant derivative CEM/VBL300 (Arceci et al., 1993, Beck et al., 1979) were maintained at 37 °C in RPMI supplemented with 10% FCS, penicillin, and streptomycin. CEM/VBL300 cells were maintained in 300 ng/mL of vinblastine. Sf9 and Sf21 insect cells were maintained in Grace's media supplemented with 10% fetal calf serum (FCS) and 150 μg/mL of gentamycin (Gibco-BRL, Gaithersburg, MD) at 28 °C.

Expression of P-Glycoproteins in Insect Cells. Baculoviral expression constructs containing the full-length human MDR1 and MDR3 cDNAs (the generous gift of Piet Borst) and mouse Mdr2 (generous gift of Philippe Gros) were engineered in pVL1392 (Invitrogen, San Diego, CA). Restriction enzyme mapping and 5′ end sequencing were used to identify the correct orientation and confirm the start sites for baculovirus expression. High titer viral stocks of recombinant baculovirus carrying the cDNAs were generated using Sf9 cells (ATCC #CRL 1711). Initially Sf9 cells were co-transfected with 0.5 μg of Baculogold DNA (Pharmingen, San Diego, CA) in the presence of Insectin liposomes (Invitrogen) and 3 μg of expression plasmids. Routinely, the viral supernatants were used to infect Sf9 or Sf21 cells with 10 pfu per cell for 48−72 h.

Western Blot Analysis. Enriched membrane preparations from tissue culture cells were obtained after the cells were rinsed in phosphate-buffered saline and allowed to swell in a solution of 0.25 M sucrose, 10 mM Hepes, pH 7.3, on ice for 10 min. The cells were sonicated on ice, and nuclei and unbroken cells were spun down at 600*g* for 9 min at 4 °C. The supernatant was transferred to a new tube and spun at 100000*g* for 15 min at 4 °C in an RC M120 ultracentrifuge (Sorvall). The pellet was resuspended in 200 μL of 0.25 M sucrose, 10 mM Hepes, pH 7.3, and frozen at −70 °C for further use. Membrane preparations were added to sample buffer with β-mercaptoethanol without heating, fractionated on 10% sodium dodecyl sulfate polyacrylamide gels, and transferred to nitrocellulose in a Hoeffer transfer aparatus using a solution composed of 200 mM glycine, 25 mM Trizma base, and 20% methanol. The nitrocellulose was rinsed in 10 mM Trizma base, pH 8.0, 150 mM sodium chloride, 0.05% Tween 20 (TBST), and the proteins were then blocked in TBST with 5% Carnation instant milk at room temperature for 30 min and then rinsed again in TBST. Monoclonal antibody C219 (Kartner et al., 1985) was diluted 1:200 in TBST and incubated with the nitrocellulose filter at room temperature for 1 h, and excess antibody was washed away in TBST. A 1:7500 dilution of anti-mouse immunoglobulin conjugated to alkaline phosphatase (Promega) was used to detect the primary antibody with NBT/BCIP substrate, as recommended by the manufacturer (Promega).

RNA Analysis. Cellular RNA was isolated from cell lines after NP40 lysis, removal of nuclei and sequential phenol and chloroform extractions (Chang et al., 1984). RNA was fractionated on a 1% formaldehyde gel, electrically transferred to Hybond N (Amersham, Arlington Heights, IL), and cross-linked to the membrane with ultraviolet light (Stratalinker, Stratagene, La Jolla, CA). Human MDR gene specific probes were generated by isolating a 1.0 kb *Bam*HI/*Xba*I fragment from the MDR1 cDNA and a 1.4 kb *Hin*dIII/*Xba*I fragment from the MDR3 cDNA (Van der Bliek et al., 1988). Membranes with transferred RNA were hybridized in 50% formamide, 0.5 M NaCl, 1% SDS, 5% dextran sulfate at 42 °C, washed to 0.2× SSC/0.1% SDS at 55 °C, and exposed to Reflection X-ray film with an intensifying screen (DuPont New England Nuclear, Boston, MA) for 4−48 hours. PCR reactions were performed using cDNA prepared from cellular RNA of CEM and CEM/VBL300 cells. RNA (1 μg) was used to prepare cDNA as template for PCR reactions with specific oligos for the amplification of human MDR3 (Chin et al., 1993). The integrity of the cDNA was confirmed using MDR1 specific primers (Futscher et al., 1993). The PCR was performed for 35 cycles using a denaturing step at 94 °C for 1 min, annealing temperature at 62 °C for 1 min, and an extension temperature of 72 °C for 1 min. Products were analyzed on agarose gels.

Preparation of Liposomes. Donor liposomes containing 40% w/w of NBD-PC were prepared by the addition of 2 mg of 1-palmitoyl-2-[12-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC) (2 mL of 1 mg/mL in chloroform) to 3 mg of egg phosphatidylcholine (Avanti Polar Lipids, Birmingham, AL) (150 μL of 20 mg/mL stock in chloroform) and were dried under nitrogen. The PC mixture was resuspended in 500

μL of ethanol, of which 35 μL was added to 10 mL of media without FCS while stirring. Cells [$(0.5-4) \times 10^6$)] were incubated for 1 h at 37 °C with dilutions of the liposomal suspension ranging from 0.3 to 300 μM. Optimal visualization under the fluorescence microscope, biochemical quantification, and FACS analysis were achieved using the 3 μM concentration. Radiolabeled liposomes composed of PC with either tritiated choline (head-label) or palmitoyl moieties (chain-label) were similarly prepared by the ethanol injection method described above (Furlong et al., 1992). Briefly, 150 μCi of [*choline*-methyl-^{3}H]-L-α-dipalmitoyl phosphatidylcholine (50 Ci/mmol) (DuPont, NEN) was added to 300 μg of egg PC, dried, resuspended in 50 μL of ethanol, and placed in 1 mL of media without FCS with constant stirring. An equal volume of this mixture was incubated with $(0.5-4) \times 10^6$ cells in a final volume of 0.5 mL. 60 μCi of [2-*palmitoyl*-9,10-^{3}H(N)]-L-α-dipalmitoyl phosphatidylcholine (42 Ci/mmol) (New England Nuclear, Boston, MA) was similarly used to prepare radiolabeled liposomes. All lipids were stored in a screw-cap glass tube after exposure to N_2 gas, in chloroform or ethanol solutions at −20 °C.

Extraction and Quantification of Labeled Lipids. Fluorescent or radiolabeled cells were centrifuged, washed two times with phosphate-buffered saline (PBS), and resuspended in 2 mL of PBS. Lipids were extracted with chloroform/methanol as previously described (Folch et al., 1957). The chloroform phase was removed, evaporated under nitrogen, resuspended in 400 μL of fresh chloroform, and quantitated in a Kratos 950 fluorescence detector with a 389 nm excitation filter and a 500 nm emission filter in the linear range of the assay. An aliquot of the methanol/water phase was quantitated directly when needed. Radiolabeled samples were counted in a scintillation counter (Beckman). Four independent experiments were done for fluorometric analysis, and two independent experiments were done for radioactive analysis. All samples were done in duplicate.

Fluorescence Microscopy. Analysis of cell fluorescence was done with a Zeiss AXIOSKOP microscope using epifluorescence. The microscope was equipped with 20× and 40× dry objectives and an MC100 camera system. The exposure times for photographic purposes (5−20 s) were the same for control and experimental cells.

Flow Cytometry. One to three million CEM or CEM/VBL300 cells were pelleted and washed in PBS and resuspended in 1 mL of RPMI without FCS. The cells were incubated at 37 °C for 1 h or as otherwise indicated in the presence of liposomes containing NBD-PC, NBD-PE, or NBD-PS at a concentration of 3 μM. Insect cells were incubated at 26 °C for 1 h in liposomes prepared in Graces' media. The cells were centrifuged, resuspended in 1 mL of PBS at 4 °C, and immediately analyzed. Cells were gated on the basis of size to eliminate dead cells from the analysis. Quantification of fluorescence was done on a Becton-Dickinson FACSCANII using LYSYS or CellQuest software applications. Internal fluorescence was determined by reading the cell fluorescence after a 30 s treatment of 20 mM dithionite (Sigma) at 4 °C (McIntyre & Sleight, 1991). Due to differences in the liposomal preparations, comparison of absolute values of fluorescence between individual experiments was not possible. Relative ratios of fluorescence between control cells and cells expressing P-glycoprotein were generated.

The surface-expression of MDR1 P-glycoprotein was determined by flow cytometry using monoclonal antibody 4E3 at 2 μg/mL and detected with a fluorescein isothiocyanate-conjugated goat anti-mouse as previously described (Arceci et al., 1993).

Antibody Effect on NBD-PC and NBD-PE Accumulation. The monoclonal antibody UIC2, an IgG2a sub-class, has been previously shown to reverse the MDR phenotype and increase the intracellular accumulation of cytotoxic compounds and increase cytotoxicity in cultured cells (Mechetner & Roninson, 1992). One to three million CEM and CEM/VBL300 cells were pre-incubated for 30 min at room temperature in the presence of monoclonal UIC2 (Immunotech, Westbrook, ME) or with the isotype control IgG2a at 10 μg/mL in a volume of 200 μL of media without FCS. Addition of 800 μL of NBD-PC or NBD-PE to a final concentration of 3 μM and incubation at 37 °C for 1 h followed. The cells were washed in PBS at 4 °C and finally resuspended in 1 mL of PBS. FACS analysis was used to quantify total and internal fluorescence.

P-Glycoprotein Reversal Agents. The effect of P-glycoprotein reversal agents and transport substrates on phospholipid accumulation was characterized over a range of concentrations. The contents of two tubes, one with 500 μL of the reversal agent at twice the final concentration and a second tube with three to six million CEM or CEM/VBL300 cells in 500 μL at 6 μM NBD-PC, NBD-PE, or NBD-PS were mixed on ice and then incubated at 37 °C for 1 h. FACS analysis was used to quantify total and internal fluorescence.

Fluorescent P-Glycoprotein Substrates. Accumulation of other fluorescent P-glycoprotein substrates was characterized to compare to the accumulation of NBD-phospholipids. Substrates included BODIPY 564/570-paclitaxel and $PyTPP^+$ (Molecular Probes, Eugene, OR) and Rho123 and DiOc2 (Sigma). Accumulation was measured by incubation of the agents with CEM and CEM/VBL300 cells at concentrations previously described (Altenberg et al., 1994; Chaudhary & Roninson, 1991) or suggested by the manufacturer that ranged from 10 nM to 10 μM. The incubations were for 1 h at 37 °C and total cellular fluorescence was determined by FACS analysis.

Statistical Analysis. Student *t*-test comparisons were utilized for statistical analysis of the samples. The calculation of probability values was determined using StatView SE + Graphic software programs.

RESULTS

Characterization of Cell Lines. Expression of P-glycoprotein on the surface of cells was characterized by FACS analysis using the monoclonal antibody 4E3 directed to an external epitope of human MDR1 (Figure 1A). High levels of human MDR1 were present in the CEM/VBL300 cells. MDR3 expression could not be detected in either CEM or CEM/VBL300 cell lines by Northern blot analysis or RT-PCR with human MDR3 specific oligos (data not shown). RT-PCR using MDR1 specific oligos detected an MDR1 specific product in both cell lines, consistent with the very low levels of MDR1 expression previously shown to exist in the CEM cell line (Arceci et al., 1993).

Human MDR1, MDR3, and mouse Mdr2 cDNAs were expressed in insect cells. High levels of P-glycoprotein were

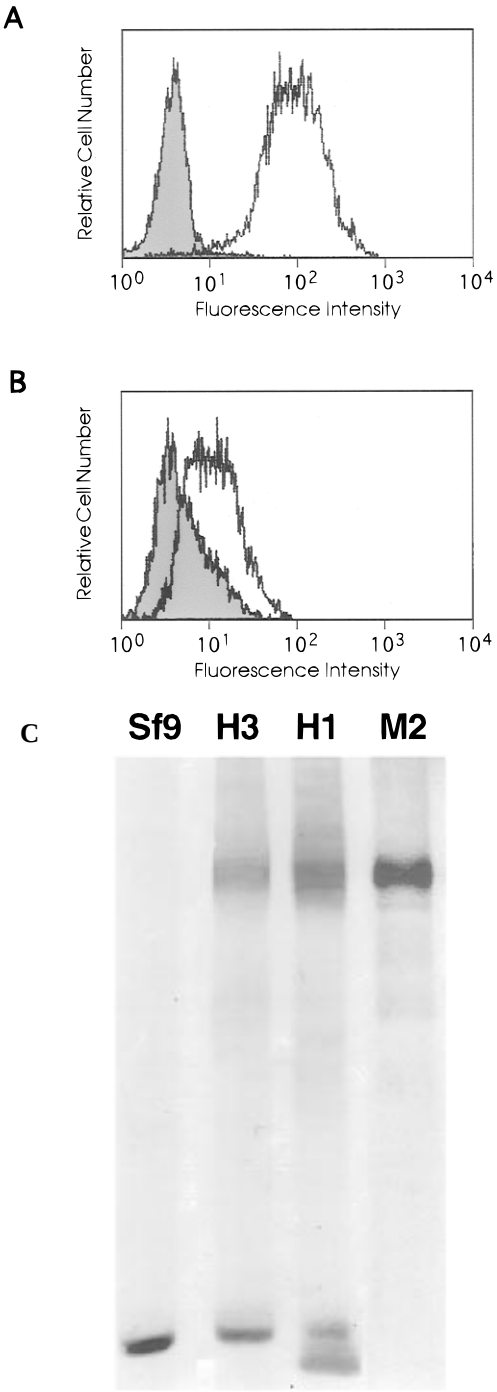

FIGURE 1: P-glycoprotein expression. (A) FACS analysis of live cells to assess surface expression of P-glycoprotein using monoclonal antibody 4E3 in parental CEM cells (gray) and multidrug resistant CEM/VBL300 cell line (white). (B) FACS analysis of uninfected (gray) and human MDR1 infected Sf21 cells (white) using 4E3. Note the high levels of surface expressed P-glycoprotein in CEM/VBL300 cells and insect cells expressing the recombinant baculovirus human MDR1. (C) Western blot analysis. Membrane fractions (20 μg) of uninfected Sf9 cells and Sf9 cells infected with human MDR3 (H3), human MDR1 (H1), and mouse Mdr2 (M2). Monoclonal antibody C219 was used to assess P-glycoprotein expression levels. The major band (150 kDa) corresponds to P-glycoprotein.

observed with all three recombinant viruses using the C219 monoclonal antibody on Western blots (Figure 1C). Sf9 cells infected with recombinant human MDR1 expressed high levels of surface MDR1 P-glycoprotein, although lower than in the CEM/VBL300 cells (Figure 1B). The cells expressing mouse Mdr2 consistently expressed the highest levels of P-glycoprotein followed by human MDR1 and MDR3 by western blot analysis (Figure 1C). Protein expression was found to peak at 48 h post-infection, after which the level of expression remained at high levels (data not shown).

Phospholipid Accumulation Monitored by Fluorescence Microscopy. Accumulation of NBD-PC in the CEM and CEM/VBL300 cell lines was determined by incubation with donor liposomes. When viewed by fluorescence microscopy, the multidrug resistant CEM/VBL300 cell line displayed substantially less fluorescence than the parental CEM cell line (Figure 2). However, in the presence of verapamil (40 μM) the fluorescence levels increased to those observed with CEM cells (Figure 2). Verapamil did not affect NBD-PC accumulation in the parental CEM cells. Similarly, Sf9 or Sf21 cells expressing human MDR1 P-glycoprotein accumulated less NBD-PC compared to uninfected insect cells when analyzed by fluorescence microscopy. Verapamil at 40 μM increased the levels of fluorescence in the insect cells expressing recombinant MDR1 but had no effect on the uninfected cells or cells infected with wild type virus (data not shown).

Quantification of NBD-PC Accumulation. Fluorometric quantitation of NBD-PC in lipids extracted from CEM/VBL300 and CEM cells indicated that the drug sensitive cells accumulated 2.01 $\pm$ 0.001 ($n = 4$) times as much NBD-PC (Figure 3A). FACS analysis indicated that the sensitive cells accumulated 1.88 $\pm$ 0.04 ($n = 3$) times more NBD-PC than the drug resistant cells (Figure 3A). Accumulation of ^{3}H-labeled acyl-chain PC was carried out under similar conditions to confirm that accumulation of NBD-PC was a property of PC and not NBD. The parental sensitive cell line accumulated 2 $\pm$ 0.22 ($n = 2$) times as much of the tritiated probe as the drug resistant cell line (Figure 3A). Similar results were obtained when choline head-labeled PC was used (data not shown). These results demonstrate that the amount of fluorescent and tritiated PC probes in the extracted lipid were consistently higher in the sensitive cells than in the drug resistant cells by a factor of 2 and that the difference was statistically significant for each of the methodologies used ($p < 0.001$).

Accumulation of fluorescent NBD-PC was similarly quantitated in insect cells expressing recombinant human MDR1. Uninfected cells accumulated 2.04 $\pm$ 0.18 ($n = 4$) ($p < 0.005$) times as much fluorescence as cells expressing MDR1 as determined by fluorometric quantification of NBD-PC in extracted lipids (Figure 3B). FACS analysis demonstrated 1.35 $\pm$ 0.05 ($n = 3$) ($p < 0.005$) times as much fluorescence in uninfected cells compared to cells expressing MDR1 (Figure 3B).

FACS analysis (Figure 3B) also demonstrated that uninfected cells accumulated 1.92 $\pm$ 0.04 ($n = 2$) times as much NBD-PC than Sf9 cells expressing mouse Mdr2 ($p < 0.005$). There was no difference in NBD-PC accumulation between human MDR3 expressing insect cells and uninfected insect cells (data not shown). Insect cells infected with wild type baculovirus and baculovirus constructs containing a glucose transporter or EGF receptor did not differ in NBD-PC accumulation when compared to uninfected Sf9 cells (data not shown).

Characterization of NBD-Phospholipid Accumulation. The NBD zwitterionic phospholipids, PC and PE, and the NBD-anionic PS probes were used to characterize phospho-

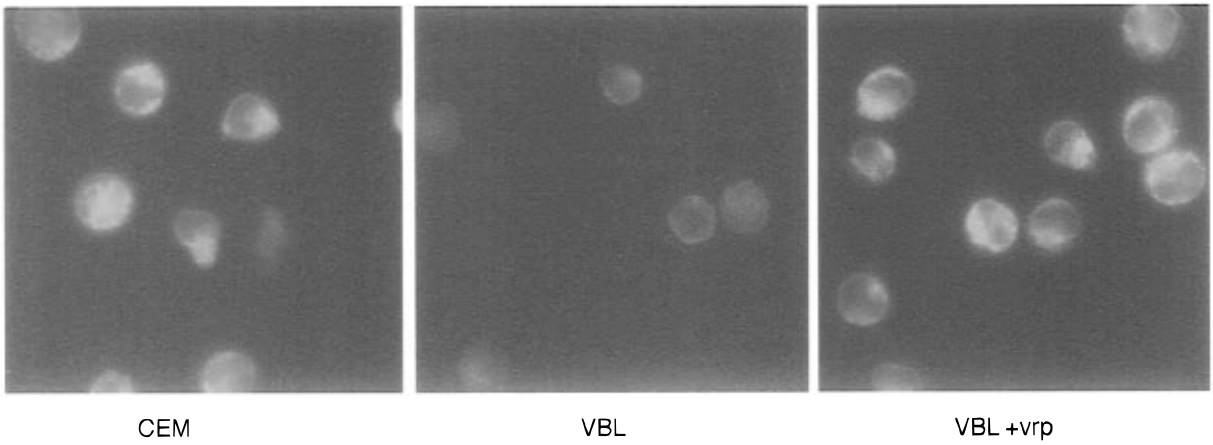

FIGURE 2: Fluorescence microscopy of NBD-PC accumulation. Total fluorescence in CEM cells and drug resistant cell line CEM/VBL300 (VBL) incubated with 3 mM NBD-PC containing liposomes in the absence of verapamil and in the presence of 40 μM verapamil (VBL+vrp). Pictures were taken at the same manual exposure time (15 seconds) using a Zeiss microscope with a 40× objective.

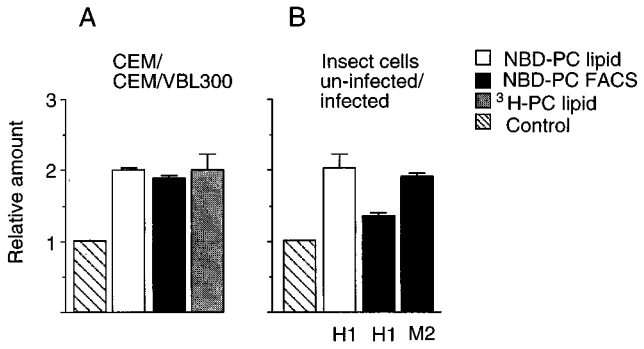

FIGURE 3: Differential accumulation of PC. (A) Ratio of PC probe accumulation in drug sensitive cells CEM to drug resistant cells CEM/VBL300. Fluorescence in the extracted lipid phase of cells incubated with NBD-PC (white); fluorescence detected by FACS analysis of cells incubated with NBD-PC (black); radioactivity in the extracted lipid phase (gray). (B) Ratio of PC probe accumulation in un-infected insect cells to insect cells expressing recombinant human MDR1 (H1) or mouse Mdr2 (M2). Fluorescence in the extracted lipid phase (white) or FACS analysis (black) in cells incubated with NBD-PC. Standard errors are shown for each group. Control (striped) were set at a value of 1.0 for comparison.

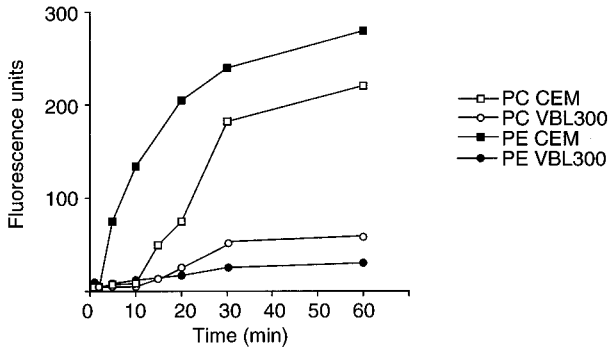

FIGURE 4: Time course of internal phospholipid accumulation. FACS analysis of fluorescence accumulation in CEM (squares) and CEM/VBL300 cells (circles) incubated with liposome containing NBD-PC (white) and NBD-PE (black) after treatment with dithionite. One representative experiment from a total of three is shown.

lipid accumulation in CEM and CEM/VBL300 cells by FACS analysis. Three independent experiments demonstrated 1.88 ± 0.04-fold more fluorescence in CEM cells incubated with NBD-PC and 3.68 ± 0.41-fold more fluorescence when incubated with NBD-PE. The ratio of CEM to CEM/VBL300 fluorescence when incubated with NBD-PS was 1.00 ± 0.07 (data not shown).

Internal NBD fluorescence was characterized in CEM and CEM/VBL300 cells with dithionite treatment followed by FACS analysis. Dithionite chemically reduces NBD, eliminating fluorescence located outside the cell and in the outer leaflet of the plasma membrane (McIntyre & Sleight, 1991). Dithionite treatment revealed a greater differential accumulation of fluorescence between drug sensitive and drug resistant cells than that detected in untreated cells. The time courses of internal fluorescence accumulation of NBD-PC and NBD-PE are shown (Figure 4). The accumulation of fluorescence approached saturation by 60 min. After 1 h, the drug sensitive CEM cells accumulated 9.0 ± 2.1-fold more fluorescence with NBD-PE compared to CEM/VBL300 cells. Similarly, CEM cells accumulated 5.2 ± 0.72-fold more fluorescence with NBD-PC than CEM/VBL300 cells. There was no difference in NBD-PS accumulation between the two cell lines (1.09 ± 0.17, $n = 3$).

Table 1: Differential Accumulation of Fluorescence Probes in CEM and CEM/VBL300 Cells[a]

probe	CEM:CEM/VBL300
NBD-PC	6.55
NBD-PE	12.34
NBD-PS	1.00
$TyTPP^+$	2.74
BODIPY paclitaxel	9.69
Rho123	40.12
$DiOc_2$	129.80

[a] The ratio of CEM:CEM/VBL300 internal fluorescence characterized by FACS analysis is tabulated. The maximal values are listed for the tested concentrations (nM). NBD-PC, NBD-PE, and NBD-PS ($5 \times 10^2-2 \times 10^4$); $TyTPP^+$ ($5 \times 10^2-7 \times 10^3$); BODIPY paclitaxel ($1 \times 10^1-10^4$); Rho123 ($10^1-10^3$); $DiOc_2$ ($4 \times 10^2-4 \times 10^4$).

The differential accumulation of a variety of other P-glycoprotein transport subtrates was characterized in the CEM and CEM/VBL300 cells for a direct comparison with phospholipid accumulation. Table 1 lists the maximal relative increase of accumulation in CEM cells compared to CEM/VBL300 cells determined over a range of concentrations. The maximal differential fluorescence for NBD-PC was 6.5 and for NBD-PE was 12.3. Maximal differential accumulation for $TyTPP^+$ and BODIPY-paclitaxel were 2.7 and 9.6, respectively, and for Rho123 and $DiOc_2$, 40.1 and 129.8, respectively. The differential accumulation of PC and PE was similar to known P-glycoprotein transport substrates.

Inhibition of Phospholipid Accumulation. Compounds which inhibit P-glycoprotein function or that are transported

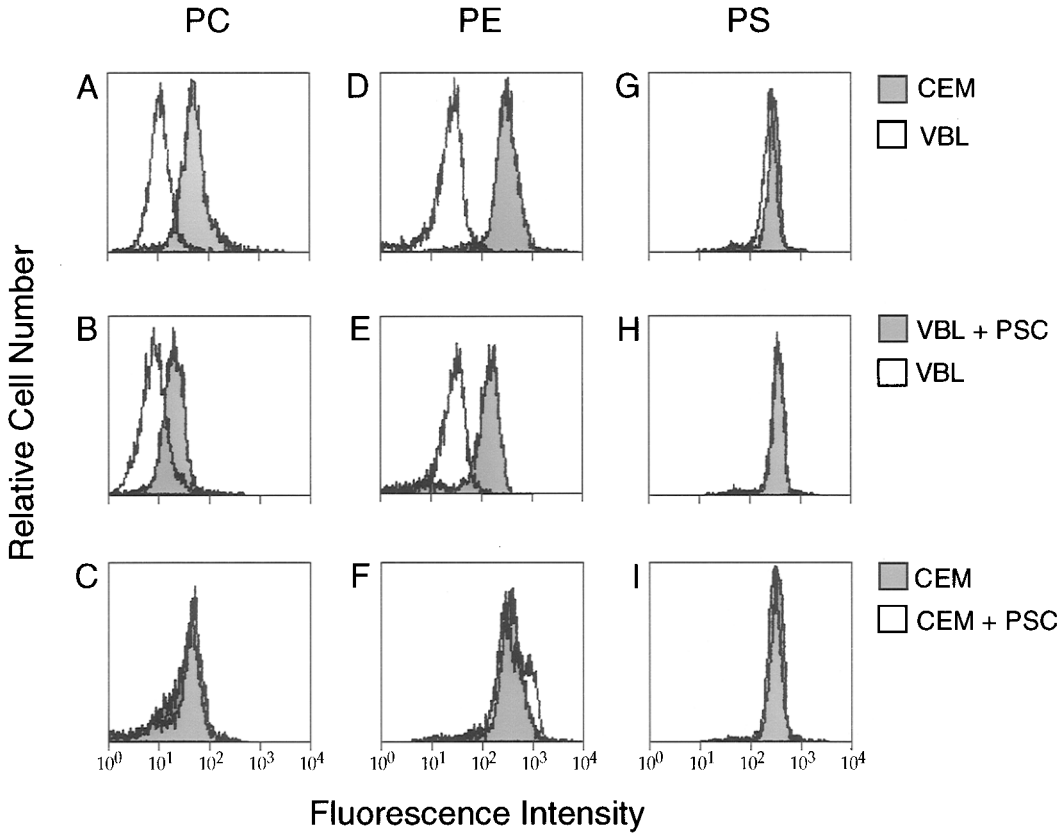

FIGURE 5: FACS analysis of internal NBD-phospholipid accumulation. Effect of 2 μM SDZ PSC 833 on phospholipid accumulation from liposomes at 60 min incubation followed by treatment with dithionite. Fluorescence of CEM (gray) and CEM/VBL300 (white) cell lines after incubation with NBD-PC (A), NBD-PE (D), and NBD-PS (G). Fluorescence of CEM/VBL300 cells in the absence (white) or in the presence of SDZ PSC 833 (gray) incubated with NBD-PC (B), NBD-PE (E), and NBD-PS (H). CEM cells in the absence (gray) or in the presence of SDZ PSC 833 (white) incubated with NBD-PC (C), NBD-PE (F), and NBD-PS (I).

by P-glycoprotein were characterized for their ability to modulate accumulation of the fluorescent phospholipid probes NBD-PC, NBD-PE, and NBD-PS. Figure 5 shows the FACS analysis of internal fluorescence in the presence and absence of 2 μM SDZ PSC 833. The accumulation of both PC and PE in the CEM/VBL300 cells approaches the value of CEM when incubated in the presence of SDZ PSC 833 (Figure 5B and E). The reversal agent does not affect the accumulation of phospholipids in the CEM cells (Figure 5C, F, and I) nor the accumulation of NBD-PS in the resistant cell line (Figure 5H).

Fluorescence quantification using FACS analysis as depicted in Figure 5 was used to generate dose−response curves for P-glycoprotein reversal agents. Each of the reversal agents tested, verapamil, cyclosporin A, and SDZ PSC 833, increased the accumulation of NBD-PE and NBD-PC in a dose dependent fashion (Figure 6A−C). Increasing concentrations of the reversal agents resulted in higher levels of accumulation of the fluorescent phospholipid probe in the multidrug resistant cell line but not in the parental cell line. The concentrations of reversal agents required to increase accumulation of the phospholipid probes (0.5−10 μM) were comparable to levels which increase accumulation of traditional P-glycoprotein transport substrates (Hait et al., 1989; Piwnica-Worms et al., 1995). Amiloride, an inhibitor of Na^+/H^+ antiporter, did not affect the accumulation of the phospholipid probes in the resistant or sensitive cells (Figure 6D).

Table 2 lists the concentration of each compound required to achieve 50% of the maximal increase of the fluorescent

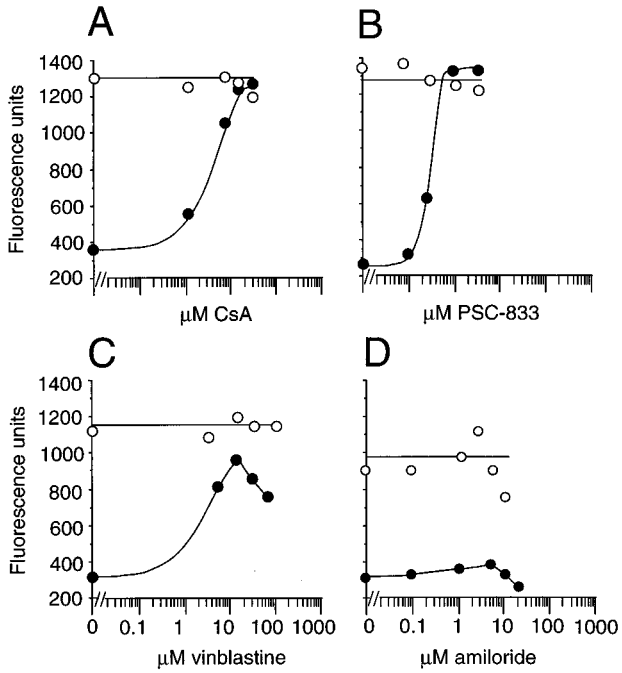

FIGURE 6: Effect of reversal agents on total NBD-PC and NBD-PE accumulation. Dose−response curves were generated from FACS analysis. Effects of (A) cyclosporin A and (B) SDZ PSC 833 on NBD-PE accumulation. Effects of (C) verapamil and (D) amiloride on NBD-PC accumulation. CEM (open circles), CEM/VBL300 (solid circles).

phospholipid probe in the CEM/VBL300 cell line (EC_{50}). The rank order found for SDZ PSC 833, cyclosporin A, and verapamil for increasing phospholipid accumulation is the

Table 2: Effect of P-Glycoprotein Reversal Agents and Other Drugs on Phospholipid Accumulation in CEM/VBL300 Cells[a]

drug	C16-C12-NBD-phospholipid	$EC_{50} \pm SE$ (μM)
SDZ PSC 833	NBD-PC	0.63 ± 0.04
	NBD-PE	0.72 ± 0.12
cyclosporin A	NBD-PC	2.75 ± 0.25
	NBD-PE	2.57 ± 0.38
verapamil	NBD-PC	20
	NBD-PE	10.25 ± 1.42
vinblastine	NBD-PC	2
amiloride	NBD-PC	NS

[a] FACS analysis of phospholipid accumulation in the absence and presence of reversal agent at concentrations from 10^{-8} to 10^{-3} M in CEM/VBL300 cells. The half-maximal effective concentration (EC_{50}) is the concentration required to increase accumulation to half of the maximal fluorescence. NS, no significant effect. Means of the values of two independent experiments ± standard errors are shown.

Table 3: Effect of UIC2 on Phospholipid Accumulation[a]

probe	CEM:CEM/VBL300	*p* value
NBD-PC IgG2a	2.28 ± 0.04	$p < 0.05$
NBD-PC UIC2	1.53 ± 0.03	
NBD-PE IgG2a	5.9 ± 1.4	$p < 0.0005$
NBD-PE UIC2	2.8 ± 0.15	

[a] Ratios of internal fluorescence in CEM:CEM/VBL300 determined by FACS analysis. The values are the average ± standard errors, and the number of experiments is indicated.

same order of potency found for increasing cytotoxicity of traditional P-glycoprotein substrates in multidrug resistant cell lines (Hait et al., 1989; Piwnica-Worms et al., 1995). Different maximal levels of accumulation were achieved with the reversal agents. SDZ PSC833 or CsA resulted in CEM/VBL300 cells reaching 100% of the level of fluorescence of CEM cells. Verapamil inhibition resulted in only a 50−75% increase in fluorescence relative to the level found in CEM cells (Figure 6C). Increasing concentrations of the P-glycoprotein transport substrate vinblastine similarly enhanced accumulation of the phospholipid probes in the resistant cell line to the same levels found in CEM cells (Table 2).

The monoclonal antibody UIC2 was utilized as a second means to inhibit P-glycoprotein transport activity. UIC2, an IgG2a antibody subclass, has been previously shown to reverse the MDR phenotype and increase the intracellular accumulation of cytotoxic compounds (Mechetner & Roninson, 1992). The relative accumulation of both NBD-PC and NBD-PE was increased approximately 2-fold in the CEM/VBL300 cell line in the presence of UIC2 (Table 3). The control IgG2a antibody did not significantly affect phospholipid accumulation.

Characterization of Phospholipid Retention. The efflux of NBD-PC was characterized by FACS analysis in CEM and CEM/VBL300 cells which had been loaded with the fluorescent phospholipid probe by incubation in the presence of SDZ PSC 833. As expected the CEM/VBL300 cells incubated in SDZ PSC 833 accumulated more NBD-PC fluorescence than those incubated without the reversal agent (Figure 7A). The cells treated with SDZ PSC833 were then treated with dithionite, washed, and incubated in the presence or absence of 10 μM SDZ PSC 833 (Figure 7B). After 1 h, CEM/VBL300 cells retained 62.9% more fluorescence in the presence of the reversal agent ($p < 0.0005$, $n = 3$). This suggested that by interfering with P-glycoprotein function, SDZ PSC 833 was inhibiting the efflux of the fluorescent phospholipid probe. The decrease in fluorescence was the same in CEM cells in the absence or in the presence of SDZ PSC 833 (Figure 7B). Similar results were obtained with the same experimental design using verapamil (40 μM) as the reversal agent to inhibit P-glycoprotein efflux. CEM/VBL300 cells treated with verapamil ultimately retained $18.47 \pm 3.3\%$ ($n = 2$) more fluorescence. No difference was observed in the retention of fluorescence for CEM cells in the presence or absence of verapamil (data not shown). These data suggest that efflux of PC is inhibitable by MDR reversal agents and that MDR1-mediated translocation is one of the mechanisms by which PC moves to the outer leaflet of the plasma membrane.

DISCUSSION

The observations presented here suggest that PC and PE are transport substrates for the human MDR1 P-glycoprotein. These phospholipids, in contrast to the anionic PS, behave like traditional P-glycoprotein transport substrates in cells expressing high levels of human MDR1 when delivered via donor liposomes. There is less accumulation of the zwitterionic phospholipids PC and PE in cells expressing human MDR1. Traditional P-glycoprotein reversal agents both increase accumulation and inhibit efflux of these phospholipids. Furthermore, the concentrations of the reversal agents required to enhance phospholipid accumulation are comparable to the level required to enhance cytotoxic agent accumulation. The potency of the reversal agents for increasing phospholipid accumulation follows the same rank order found for cytotoxic compounds (Hait et al., 1989; Piwnica-Worms et al., 1995). This pharmacologic specificity further supports a role for P-glycoprotein in phospholipid transport.

Both traditionally generated multidrug resistant cells and insect cells overexpressing recombinant human MDR1 P-glycoprotein accumulated less PC than control cells after incubation with donor liposomes. This was demonstrated using both fluorescent analogs and radiolabeled derivatives. Quantitative results using either type of PC lipid probe were remarkably consistent. Incubation of the drug resistant cell line CEM/VBL300 in the presence of the fluorescent analog NBD-PC resulted in less accumulation than the parental CEM drug sensitive cells. Quantitation of internal NBD-PC and NBD-PE accumulation using dithionite indicated that the CEM/VBL300 cell line accumulated only 20% and 10% of the levels detected in the parental CEM cells, respectively. By contrast, there was no difference in accumulation of NBD-PS between the resistant and the sensitive cell lines.

Insect cells expressing recombinant P-glycoproteins were utilized to confirm that human MDR1 was responsible for the difference in PC accumulation. Control insect cells accumulated 2-fold more fluorescence than insect cells expressing human MDR1, indicating that human MDR1 alone can be responsible for decreased accumulation of PC. Insect cells expressing recombinant protein are undergoing a lytic viral infection which has the potential to affect membrane permeability. However, we have previously demonstrated that this system is suitable for evaluation of P-glycoprotein mediated transport activity. Patch-clamp analysis of insect cells expressing recombinant P-glycoprotein identified ATP transport (Bosch et al., 1996) similar to

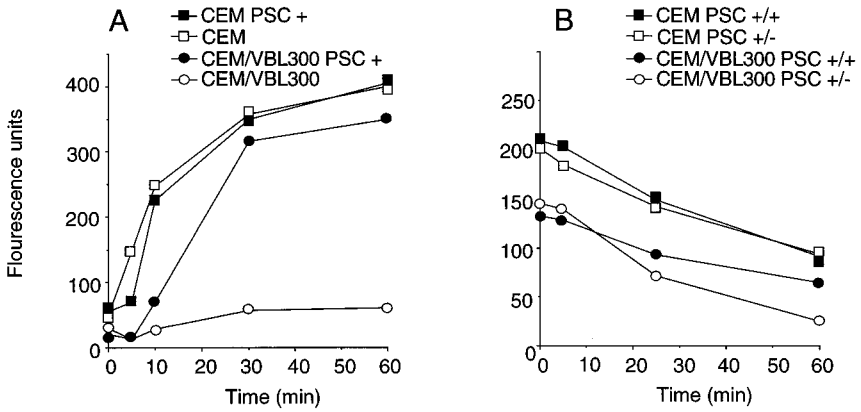

FIGURE 7: Cellular retention of NBD-PC in the presence of SDZ PSC 833. (A) Total fluorescence of NBD-PC accumulation in the presence (solid symbols) or absence of 10 μM SDZ PSC 833 (open symbols) for CEM/VBL300 (circles) and CEM cells (squares). (B) Retention of internal fluorescence in the presence or absence of SDZ PSC 833. All cells were loaded in the presence of SDZ PSC 833 (A), treated with dithionite, washed and further incubated in the presence or absence of SDZ PSC 833 (PSC +/+ or PSC +/−). Phospholipid-loaded CEM (squares) and CEM/VBL300 cells (circles) incubated for 60 min in the presence (solid) or in the absence (open) of SDZ PSC 833. Higher levels of NBD-PC were retained with SDZ PSC 833 in CEM/VBL300 cells ($p < 0.0005$, $n = 3$) but not in CEM cells.

observations in mammalian cells (Abraham et al., 1993). In addition, insect cells expressing recombinant human MDR1 accumulated less of the P-glycoprotein substrate [^{99m}Tc]-Sestamibi than control Sf9 cells, confirming that it is a functional transporter [Rao et al. (1994) and data not shown]. Insect cells infected with wild type virus as well as baculovirus constructs encoding plama membrane proteins such as a glucose transporter and the EGF receptor did not display differences in NBD-PC accumulation from uninfected cells (data not shown).

Insect cells expressing mouse Mdr2 similarly accumulated less NBD-PC than control cells, indicating that the methodology of liposome delivery corroborates the PC translocase activity of Mdr2 (Ruetz & Gros, 1994b). However, we were unable to demonstrate a difference in NBD-PC accumulation in insect cells expressing human MDR3. Whether this is related to the level of protein expression or protein processing is not clear. Recombinant plasma membrane proteins expressed in insect cells may accumulate in intracellular compartments preventing proper functional localization. The human MDR3 translocase activity has been shown previously in two separate experimental models (Smith et al., 1994; Helvoort et al., 1996).

P-glycoprotein reversal agents were utilized to substantiate evidence that PC and PE accumulation were mediated by human MDR1. Verapamil inhibition of NBD-PC accumulation could easily be detected by fluorescence microscopy in both mammalian and insect cells expressing human MDR1. Quantitative FACS analysis of NBD-PC and NBD-PE accumulation in the presence of SDZ PSC 833, CsA and verapamil demonstrated that the drug resistant CEM/VBL300 cells increased accumulation of the fluorescent phospholipid probes. The increase in fluorescence was dose dependent and of the same rank order of potency as observed for inhibition of drug transport with reversal agents. The drug sensitive CEM cells did not show an increase in fluorescence in the presence of reversal agents suggesting that the accumulation of NBD-PC and NBD-PE was modulated by P-glycoprotein. Similar increases in NBD-PC and NBD-PE accumulation were obtained with the P-glycoprotein transport substrate vinblastine. Monoclonal antibody UIC2 which recognizes an external epitope of human MDR1 P-glycoprotein was utilized as another specific method to inhibit P-glycoprotein transport function (Mechetner & Roninson, 1992). UIC2 similarly increased the accumulation of NBD-PC and NBD-PE in CEM/VBL300. Increased accumulation of the phospholipid analogs by UIC2 further suggests a specific role for human MDR1 P-glycoprotein participation in phospholipid transport.

SDZ PSC 833 and verapamil were used to characterize NBD-PC movement out of the cells. CEM and CEM/VBL300 cells were loaded with the fluorescent probe and the amount of fluorescence which was retained after further incubation in the presence or absence of the reversal agents was determined. The results indicate that continued incubation in the reversal agents resulted in higher levels of retention of the fluorescent probe in the multidrug resistant, but not the drug sensitive, cell line. Dithionite treatment provided a mechanism to ensure monitoring of internal fluorescence alone. The higher levels of retained fluorescence in CEM/VBL300 cells in the presence of SDZ PSC 833 suggests that the NBD-PC probe is moving out of the cell (Figure 7B). Since there is no dithionite in the media at this time, the decrease fluorescence must be due to removal of the fluorescent phospholipid analog from the cell. The inhibition of P-glycoprotein function by reversal agents appears to modulate this efflux activity. The initial rate of efflux appears similar for the CEM/VBL300 cells with or without SDZ PSC 833 as well for the parental CEM cell line. This may be in part due to the prolonged effects of SDZ PSC 833 even after removal from the media (Archinal-Mattheis et al., 1995) or a relatively slow removal rate in the presence of a high intracellular content of fluorescent phospholipids which may not be accessible to the P-glycoprotein. It is not until 30 min that there is sufficient differential accumulation of NBD-PC in the treated and untreated CEM/VBL300 cells to indicate that phospholipid efflux is mediated by P-glycoprotein. There is no difference in phospholipid accumulation in the treated and untreated CEM cells. These observations further support the concept that inhibition of P-glycoprotein function interferes with efflux of NBD-PC and NBD-PE.

The internalization of fluorescent phospholipid analogs has been investigated in a variety of cultured cells and has provided insights about their subcellular distribution. Although all three phospholipids are internalized, two distinct

pathways have been proposed. PC and PE enter the cell via endocytosis while PS is internalized by an energy independent translocation. After internalization, PC and PE localize to the plasma membrane with a cell specific asymmetric distribution between the two leaflets, whereas PS localizes in the mitochondrial membrane (Moreau & Cassagne, 1994). It is possible that the inability to demonstrate a differential PS accumulation between the drug sensitive and drug resistant cells may be due to the fact that PS is not accessible for P-glycoprotein export as opposed to physicochemical differences.

Previous studies have suggested that MDR1 P-glycoprotein directly interacts with lipids present in the plasma membrane. Cytotoxic agents encapsulated in liposomes made of phospholipid mixtures have been shown to circumvent P-glycoprotein mediated drug resistance (Oubard et al., 1991; Rahman et al., 1992; Thierry et al., 1989). Although the mechanism is not entirely clear, at least part of the increased cytotoxicity appears to be due to increased intracellular concentrations of the cytotoxic agents suggesting inhibition of P-glycoprotein mediated efflux (Thierry et al., 1989). Liposomes composed of phospholipids inhibited [^{3}H]vinblastine binding to membranes from multidrug resistant cell lines (Thierry et al., 1989) and increased the intracellular concentration of [^{3}H]vinblastine in multidrug resistant cell lines (Warren et al., 1992). Furthermore, [^{3}H]azidopine labeling of P-glycoprotein in membranes from multidrug resistant cell lines can be inhibited by liposomes (Rahman et al., 1992).

Finally, previous data have shown that the lipid composition in drug resistant cells is altered. Metabolic labeling with [^{3}H]choline indicates a lower concentration of radiolabeled PC in the drug resistant cells compared to drug sensitive cells (Ramu et al., 1984). Although differential synthesis of PC could account for this difference, increased efflux of PC from the resistant cells remains a possibility in light of the observations presented here.

Several studies have indicated that membrane lipid composition is important for P-glycoprotein function. The ATPase activity of P-glycoprotein in reconstituted vesicles can be modified by the type of membrane phospholipids. PC was the most effective phospholipid in restoring P-glycoprotein ATPase activity after delipidation (Doige et al., 1993; Saeki et al., 1992). Although it is not clear how P-glycoprotein function is mediated by the surrounding lipids, a direct lipid interaction appears to be necessary for activity. Furthermore, the role of CFTR in membrane recycling (Bradbury et al., 1992) and the identification of P-type membrane ATPases which translocate aminophospholipids PE and PS (Tang et al., 1996) indicate that additional members of this superfamily as well as other membrane ATPases have a range of lipid associated activities.

Recently, human MDR1 P-glycoprotein has been demonstrated to be a phospholipid translocase with broad specificity (van Helvoort et al., 1996). Short chain C6-NBD-diacylglycerol was used as a precursor for the formation of PC and PE. Cell expressing human MDR1 translocated the phospholipids across the membrane, an activity which could be inhibited by verapamil. These observations are in agreement with the conclusions drawn from the data presented in this manuscript. Thus, two different assay systems confirm that human MDR1 can function as a phospholipid translocase. Liposome delivery allows the analysis of defined phospholipids, including long chain fatty acids, the prevalent physiological phospholipids, which appear to be transported like the short chain species.

The data presented here suggests that certain phospholipids are substrates of the human MDR1. The effects of lipids on P-glycoprotein function are not yet well defined. The interaction of human MDR1 with phospholipids has already been shown to have significant implications in circumventing drug resistance in cancer chemotherapy (Mickisch et al., 1992; Rahman et al., 1992). This report suggests that phospholipids may be physiological substrates of the human MDR1 P-glycoprotein and further emphasizes their potential therapeutic use.

ACKNOWLEDGMENT

The authors thank E. S. Dinsmore for help in preparing the figures, Patrick Skelly and Hema Ramachandran for providing control baculovirus stocks, and D. Sylvain and S. Arciniegas for technical help and advice. We thank Dr. B. Hall and Dr. J. Dinsmore for their critical reading of the manuscript.

REFERENCES

Abraham, E. H., Prat, A. G., Gerweck, L., Seneveratne, T., Arceci, R., Kramer, R., Guidotti, G., & Cantiello, H. F. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 312−316.
Altenberg, G. A., Vanoye, C. G., Han, E. S., Deitmer, J. W., & Reuss, L. (1994) *J. Biol. Chem. 269*, 7145−7149.
Ames, G. F., Mimura, C. S., Holbrook, S. R., & Shyamala, V. (1992) *Adv. Enzymol. Rel. Areas Mol. Biol. 65*, 1−47.
Arceci, R., Stieglitz, K., Bras, J., Schinkel, A., Baas, F., & Croop, J. (1993) *Cancer Res. 53*, 310−317.
Archinal-Mattheis, A., Rzepka, R. W., Watanabe, T., Kokubu, N., Itoh, Y., Combates, N. J., Bair, K. W., & Cohen, D. (1995) *Oncol. Res. 7*, 603−610.
Beck, W. T., Mueller, T. J., & Tanzer, L. R. (1979) *Cancer Res. 39*, 2070−2076.
Bosch, I., Jackson, G. R., Croop, J. M., & Cantiello, H. F. (1996) *Am. J. Physiol. 40*, C1527−C1538.
Bradbury, N., Jilling, T., Berta, G., Sorscher, E., Bridges, R., & Kirk, K. (1992) *Science 256*, 530−532.
Bruggemann, E. P., Germann, U. A., Gottesman, M. M., & Pastan, I. (1989) *J. Biol. Chem. 264*, 15483−15488.
Chang, K., Warren, Z., Bergsma, D., Dodgson, J., & Schwartz, R. (1984) *Mol. Cell. Biol. 4*, 2498−2508.
Chaudhary, P. M., & Roninson, I. B. (1991) *Cell 66*, 85−94.
Chin, K. V., Pastan, I., & Gottesman, M. M. (1993) *Cancer Res. 60*, 157−180.
Croop, J. (1993) *Cytotechnology 12*, 1−32.
Croop, J. M., Gros, P., & Housman, D. E. (1988) *J. Clin. Invest. 81*, 1303−1309.
Doige, C. A., Yu, X., & Sharom, F. J. (1993) *Biochim. Biophys. Acta 1146*, 65−72.
Folch, J., Lees, M., & Sloane, S. (1957) *J. Biol. Chem. 226*, 497−509.
Ford, J. M., & Hait, W. N. (1990) *Pharmacol. Rev. 42*, 155−199.
Furlong, S. T., Thibault, K. S., & Rogers, R. A. (1992) *J. Cell Sci. 103*, 823−830.
Futscher, B. W., Blake, L. L., Gerlach, J. H., Grogan, T. M., & Dalton, W. S. (1993) *Anal. Biochem. 213*, 414−421.
Gottesman, M., & Pastan, I. (1989) *J. Biol. Chem. 263*, 12163−12166.
Greenberger, L. M. (1993) *J. Biol. Chem. 268*, 11417−11425.
Hait, W. N., Stein, J. M., Koletsky, A. J., Harding, M. W., & Handschumacher, R. E. (1989) *Cancer Commun. 1*, 35−43.
Higgins, C. F. (1992) *Annu. Rev. Cell Biol. 8*, 67−113.
Higgins, C. F., & Gottesman, M. M. (1992) *Trends Biochem. Sci. 17*, 18−21.
Homolya, L., Hollo, Z., Germann, U. A., Pastan, I., Gottesman, M. M., & Sarkadi, B. (1993) *J. Biol. Chem. 268*, 21493−21496.

Horio, M., Gottesman, M. M., & Pastan, I. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 3580−3584.
Juranka, P., Zhang, F., Kulpa, J., Endicott, J., Blight, M., Holland, I. B., & Ling, V. (1992) *J. Biol. Chem. 267*, 3764−3770.
Juranka, P. F., Zastawny, R. L., & Ling, V. (1989) *FASEB J. 3*, 2583−2592.
Kartner, N., Evernden-Porelle, D., Bradley, G., & Ling, V. (1985) *Nature 316*, 820−823.
Lankelma, J., Spoelstra, E. C., Dekker, H., & Broxterman, H. J. (1990) *Biochim. Biophys. Acta 1055*, 217−222.
McIntyre, J. C., & Sleight, R. G. (1991) *Biochemistry 30*, 11819−11827.
Mechetner, E. B., & Roninson, I. B. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 5824−5828.
Mickisch, G. H., Rahman, A., Pastan, I., & Gottesman, M. M. (1992) *J. Natl. Cancer Inst. 84*, 804−805.
Moreau, P., & Cassagne, C. (1994) *Biochem. Biophys. Acta 1197*, 257−290.
Oubard, S., Thierry, A., Jorgensen, T., & Rhaman, A. (1991) *Cancer Chem. Pharmacol. 28*, 259−265.
Piwnica-Worms, D., Chiu, M. L., Budding, M., Kronauge, J. F., Kramer, R. A., & Croop, J. M. (1993) *Cancer Res. 53*, 977−984.
Piwnica-Worms, D., Rao, V. V., Kronauge, J. F., & Croop, J. M. (1995) *Biochemistry 34*, 12210−12220.
Rahman, A., Husain, S. R., Siddiqui, J., Verma, M., Agresti, M., Center, M., Safa, A. R., & Glazer, R. I. (1992) *J. Natl. Cancer Inst. 84*, 1909−15.
Ramu, A., Glaubiger, D., & Weintraub, H. (1984) *Cancer Treat. Rep. 68*, 637−641.
Rao, V., Chiu, M., Kronauge, J., & Piwnica-Worms, D. (1994) *J. Nuclear Med. 35*, 510−515.
Ruetz, S., & Gros, P. (1994a) *J. Biol. Chem. 269*, 12277−12284.
Ruetz, S., & Gros, P. (1994b) *Cell 77*, 1071−1081.
Ruetz, S., Raymond, M., & Gros, P. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 11588−11592.
Saeki, T., Shimabuku, A. M., Ueda, K., & Komano, T. (1992) *Biochim. Biophys. Acta 1107*, 105−110.
Sarkadi, B., Price, E. M., Boucher, R. C., Germann, U. A., & Scarborough, G. A. (1992) *J. Biol. Chem. 267*, 4854−4858.
Shapiro, A. B., & Ling, V. (1994) *J. Biol. Chem. 269*, 3745−3754.
Smit, J. J., Schinkel, A. H., Oude Elferink, R. P., Groen, A. K., Waganaaf, E., van Deemter, L., Mol, C. A., Ottenhofer, R., van der Lugt, N. M., van Room, M. A., van der Valk, M. A., Offerhous, G. J., Berns, A. J., & Borst, P. (1993) *Cell 75*, 451−462.
Smith, A. J., Timmermans-Hereijger, J., Roelofsen, B., Wirtz, K., van Blitterswijk, W. J., Smit, J. J. M., Schinkel, A. H., & Borst, P. (1994) *FEBS Lett. 354*, 263−266.
Tang, X., Halleck, M., Schlegel, R., & Williamson, P. (1996) *Science 272*, 1495−1497.
Thierry, A. R., Jorgensen, T. J., Forst, D., Belli, J. A., Dritschilo, A., & Rahman, A. (1989) *Cancer Commun. 1*, 311−316.
Van der Bliek, A. M., Baas, F., Van der Velde Koerts, T., Biedler, J. L., Meyers, M. B., Ozols, R. F., Hamilton, T. C., Joenje, H., & Borst, P. (1988) *Cancer Res. 48*, 5927−5932.
van Helvoort, A., Smith, A., Sprong, H., Fritzsche, I., Schinkel, A., Borst, P., & van Meer, G. (1996) *Cell 87*, 507−517.
Warren, L., Jardillier, J. C., Malarska, A., & Akeli, M. G. (1992) *Cancer Res. 52*, 3241−3245.
Zhang, X., Collins, K. I., & Greenberger, L. M. (1995) *J. Biol. Chem. 270*, 5441−5448.

BI962728R