Transport of phosphatidylserine via MDR1 (multidrug resistance 1) P-glycoprotein in a human gastric carcinoma cell line

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The ATP-binding cassette transporter multidrug resistance 1 P-glycoprotein (MDR1 Pgp) has been implicated with the transport of lipids from the inner to the outer leaflet of the plasma membrane. While this has been unambigously shown for the fluorescent lipid analogues [*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl (C_6-NBD)-phosphatidylcholine, -phosphatidylethanolamine, -sphingomyelin and -glucosylceramide, by using a novel approach we have now found significantly increased outward transport also for C_6-NBD-phosphatidylserine (C_6-NBD-PS) in EPG85-257 human gastric carcinoma cells overexpressing *MDR1* (coding for MDR1 Pgp). The increased transport of C_6-NBD-PS is mediated by MDR1 Pgp, shown by transport reduction nearly to the level of controls in the presence of MDR1 Pgp inhibitors [PSC 833, cyclosporin A and dexniguldipine hydrochloride (Dex)]. Addition of MK 571, a specific inhibitor of the MDR protein MRP1, does not decrease transport in either of the two cell lines. The plasma-membrane association of FITC-annexin V, a fluorescent protein conjugate binding PS, is significantly increased in *MDR1*-overexpressing cells as compared with controls, and can be reduced by an MDR1 Pgp inhibitor. This suggests that MDR1 Pgp transports endogenous PS, the lipid exhibiting the most pronounced transverse asymmetry in the plasma membrane.

Key words: ABCB1, annexin, flippase, lipid asymmetry, plasma membrane.

INTRODUCTION

The lipids in the plasma membrane of eukaryotic cells show a clear asymmetric arrangement, with the majority of glycosphingolipids and phosphatidylcholine (PC) typically in the outer, exoplasmic, leaflet and the aminophospholipids, phosphatidylserine (PS) and phosphatidylethanolamine (PE), in the inner, cytoplasmic, leaflet [1].

Maintenance of this non-random lipid distribution is important for diverse cellular functions. Any change in this distribution generally triggers a physiological event. For example, surface exposure of PS at the surface of activated blood or endothelial cells serves to promote blood coagulation and to signal the removal of injured and apoptotic cells by the reticuloendothelial system [2]. Moreover, regulation of phospholipid asymmetry may also be essential for cellular processes such as membrane budding and fusion, which are required for vesicle trafficking [3].

Maintenance and regulation of the asymmetric lipid distribution across the plasma membrane is governed by the concerted action of specific membrane proteins controlling lipid movement across the bilayer, in addition to passively occurring transmembrane movement of lipids. Under normal conditions, inward movement of PC and sphingomyelin (SM) from the exoplasmic to the cytoplasmic leaflet is a slow, non-mediated process in most cells. In contrast, the aminophospholipids PS and PE are rapidly transported from the exoplasmic to the cytoplasmic leaflet by an active ATP-dependent and protein-mediated process maintaining lipid asymmetry [4,5].

In studies originally related to multidrug resistance (MDR) in cancer cells, members of the ATP-binding cassette (ABC) transporter family have been identified as mediators of phospholipid outward transport. These include MDR3 P-glycoprotein (Pgp) (also designated ABCB4) and its mouse homologue mdr2 Pgp, which specifically transport PC [6–9], as well as MDR1 Pgp (also designated ABCB1), giving rise to a frequent form of pleiotropic resistance in tumour cells [10]. MDR1 Pgp expels a variety of short-chain lipid analogues and amphiphilic drugs from the cell [9,11,12], but was found to be unable to restore transport of PC into the bile of *mdr2* knockout mice [6], suggesting that natural long-chain PC is not an MDR1 Pgp substrate. Yet recent studies revealed MDR1 Pgp mediated outward transport of the short-chain PC platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) [13] and, very likely, glucosylceramide [14], suggesting a possible physiological role for this protein in the distribution of several endogenous lipids in the plasma membrane. MDR1 Pgp, thus surmised to possess a wide specificity for lipid substrates, is believed to act as a drug floppase, transporting amphiphilic molecules integrated into the plasma membrane either from the cytoplasmic to the exoplasmic membrane leaflet, or directly into the extracellular medium, lowering the intracellular drug concentration [15].

Among the various phospholipids of the plasma membrane, PS occupies a particular place: under normal conditions, it is

Abbreviations used: ABC, ATP-binding cassette BFA, brefeldin A; C_6-NBD, [*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl; Cer, ceramide; CMFDA, 5-chloromethylfluorescein diacetate (CellTracker Green); CsA, cyclosporin A; Dex, dexniguldipine hydrochloride, B8509-035; DFP, di-isopropyl fluorophosphate; DG, diacylglycerol; ER, endoplasmic reticulum; GS-MF, glutathione-methylfluorescein; mAb, monoclonal antibody; MDR, multidrug resistance; MDR1 Pgp, MDR1 P-glycoprotein; MK 571, a specific inhibitor of MRP1; mPBS, modified Dulbecco's phosphate buffered saline; MRP1, multidrug resistance protein 1; PA, phosphatidic acid; PAF, platelet activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; Rho 123, Rhodamine 123; SM, sphingomyelin.

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confined almost exclusively to the cytoplasmic leaflet [1]. Remarkably, a number of tumour cell lines have been reported to display 3–7-fold elevated amounts of PS on the exoplasmic membrane leaflet compared with non-tumorigenic cells [16]. Elevated cell-surface exposure of PS could result from impaired inward transport and/or increased outward transport. As MDR1 Pgp is highly expressed at the cell surface of many tumour cells, this raises the question as to whether MDR1 Pgp is involved in the exposure of endogenous PS.

In order to explore the influence of MDR1 Pgp on the transverse distribution of membrane lipids, in particular the asymmetrically distributed PS, we investigated the transbilayer movement of various phospholipid analogues in an *MDR1*-overexpressing subline of the human gastric carcinoma cell line EPG85-257. MDR1 Pgp was confirmed to possess a broad specificity for lipids, transporting not only C_6-NBD analogues of the phospholipids PC and PE, but also significant amounts of a C_6-NBD analogue of PS in mammalian cells, which has been shown here for the first time. In flow-cytometric studies designed to determine the amount of endogenous PS exposed on the outer plasma-membrane leaflet, we found increased association of the PS-binding protein FITC-annexin V with *MDR1*-overexpressing cells compared with controls, suggesting an outwardly directed transport of natural PS by MDR1 Pgp. This implies that MDR1 Pgp mediated MDR may be associated with the emergence of a different lipid pattern in the outer leaflet of the plasma membrane, altering its surface properties, which could have prominent physiological consequences for the cell. Such changes in the phenotype of drug-treated cells might be a hallmark of MDR tumour cells, and could serve for improved diagnostics and treatment strategies of clinical drug resistance.

EXPERIMENTAL

Materials

Brefeldin A (BFA), di-isopropyl fluorophosphate (DFP), fatty acid-free BSA and Rhodamine 123 (Rho 123) were purchased from Sigma–Aldrich (Steinheim, Germany), C219 from Alexis (San Diego, CA, U.S.A.) and cellulose nitrate membranes from Schleicher und Schüll (Dassel, Germany). Chambered coverglasses (Nunc) and Dulbecco's phosphate-buffered saline with Ca^{2+} and Mg^{2+} (PBS) were obtained from Biochrom KG (Berlin, Germany). PBS was supplemented with 24 mM glucose and 10 mM Hepes (mPBS). [*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoylceramide (C_6-NBD-Cer), C_6-NBD-phosphatidic acid (C_6-NBD-PA), C_6-NBD-PC and C_6-NBD-PS were purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.), and high-performance TLC plates were from Merck (Darmstadt, Germany). FITC-annexin V, binding buffer and propidium iodide were bought from Nexins Research (Kattendijke, The Netherlands), CellTracker Green (5-chloromethylfluorescein diacetate, CMFDA) and MitoTracker Red CMXRos were from Molecular Probes (Leiden, The Netherlands). MRPm6 [monoclonal antibody (mAb) for MRP1] was from Sanbio (Uden, The Netherlands), peroxidase-conjugated rabbit antimouse IgG (no. 315-035-003) from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.), and Triton X-100 was from Fluka (Buchs, Switzerland). The following inhibitors of ABC transporters were used: cyclosporin A and glyburide (Sigma–Aldrich, Steinheim, Germany), PSC 833 (Novartis, Basel, Switzerland), Dex (B8509-035; Byk Gulden, Konstanz, Germany), and MK 571 (Merck-Frosst, Pointe-Claire-Dorval, Canada). Cyclosporin A, glyburide, and PSC 833 stocks were prepared in ethanol, MK 571 was prepared in double-distilled water, and Dex was prepared in DMSO. For all experiments, the effect of the solvent was determined.

Cells

Culturing of the parental drug-sensitive human gastric-carcinoma cell line (EPG85-257P) and its daunorubicin-selected MDR subline (EPG85-257RDB) was described previously [17,18]. Cells were grown in the absence of cytostatic drugs for 96 h on 35-mm-diameter culture dishes for transport assays and on two-chamber coverglasses for microscopy. Viability was determined at the end of the experiments by Trypan Blue (final concn. 0.5 %) exclusion. Non-viable cells did not exceed 3 %, even when cells were pretreated with inhibitors.

Detection of ABC transporter proteins was done by Western blotting using mouse mAb C219 (for MDR1 Pgp) or MRPm6 (for MRP1) as described previously [19]. Protein–antibody complexes were visualized by enhanced chemoluminescence (ECL® system; Amersham) according to the manufacturer's protocol.

Transport assays

Measurement of Rho 123 and glutathione-methylfluorescein (GS-MF) outward transport

Cells preincubated with or without MDR inhibitors for 10 min on ice were loaded for 15 min at 37 °C with the fluorescence probes 10 μM Rho 123 or 3.2 μM CMFDA, washed with cold mPBS, and incubated for 10 min on ice in mPBS with or without MDR1 Pgp inhibitors. Assays were started by transferring the cell dishes to 37 °C. At indicated time points, media were collected, and cells scraped into mPBS. Fluorescence of media and scraped cells was measured in the presence of 0.5 % Triton X-100 at excitation/emission wavelengths of 470 nm/540 nm and 4 nm/8 nm slit width for Rho 123 and GS-MF, using an Aminco–Bowman (Urbana, IL, U.S.A.) Series 2 fluorescence spectrometer. Intracellular fluorescence after Rho 123 loading was 2 arbitrary units for EPG85-257RDB and 5 arbitrary units for EPG85-257P cells. Intracellular fluorescence after CMFDA loading was 6 arbitrary units for EPG85-257RDB and 8 arbitrary units for EPG85-257P cells.

Measurement of C_6-NBD-PC, -PE and -SM outward transport

Transport of newly synthesized C_6-NBD-PC, -PE and -SM to the exoplasmic leaflet of the plasma membrane was assessed at 15 °C as described by van Helvoort et al. [9]. Cells preincubated in the presence or absence of MDR inhibitors were incubated at 15 °C with 25 μM C_6-NBD-PA (for -PC and -PE synthesis) or 5 μM C_6-NBD-Cer (for -SM synthesis) in 1 % (w/v) BSA in mPBS with or without inhibitors. After 180 min, BSA-containing media were collected and cells subjected to a 30 min back-exchange incubation on ice with 1 % BSA in mPBS, followed by lipid analysis of cells and media. Synthesis of C_6-NBD-PC was about 25 and 40 pmol/dish (1.5×10^6 cells) in EPG85-257RDB and EPG85-257P cells respectively. In both sublines, C_6-NBD-PE synthesis was about 25 pmol, while C_6-NBD-SM synthesis was about 3 pmol in EPG85-257RDB and 15 pmol in EPG85-257P.

Measurement of C_6-NBD-PS and -PC inward transport

After preincubation with or without MDR inhibitors, cells were labelled with 10 μM C_6-NBD-PS or 14 μM C_6-NBD-PC in cold mPBS [20]. After a 15 min incubation on ice, non-inserted analogues were removed by washing with cold mPBS. About

230 pmol of NBD-lipid analogue was inserted per dish (1.5×10^6 cells). The reaction was started by addition of mPBS prewarmed to 20 °C (with or without MDR inhibitors), containing 5 mM DFP (as in all following incubations) to prevent hydrolysis of labelled phospholipid [21]. At indicated time points, culture dishes were transferred on ice, and BSA (final concn. 2 %, w/v) was added to extract NBD-lipid from the cell surface. After 10 min, BSA-containing media were collected and replaced with cold mPBS plus 2 % BSA for 10 min, followed by lipid analysis of cells and media.

Measurement of C_6-NBD-PS outward transport

Cells were preincubated with or without MDR inhibitors, labelled with 5–10 μM C_6-NBD-PS on ice, and incubated at 20 °C for 30 min to allow inward movement of the NBD analogue. C_6-NBD-PS remaining on the cell surface was extracted twice by incubation with 2 % (w/v) BSA in mPBS for 10 min on ice. Before starting the outward transport assay, the medium was removed, and cells were washed with cold mPBS. For $t = 0$ min (i.e. immediately), cold medium containing 2 % (w/v) BSA and 5 mM DFP in mPBS with or without inhibitor was immediately added to the cell dish and incubated for 10 min. To measure C_6-NBD-PS outward transport, prewarmed (15 or 37 °C) mPBS with 2 % (w/v) BSA and 5 mM DFP was added to the dish, and cells were incubated at 15 °C or 37 °C for indicated lengths of time. Removal of the BSA-containing media and a second extraction with 2 % BSA in mPBS for 10 min on ice terminated incubations. Lipid analysis followed.

Lipid analysis

After the incubations, the second BSA back-exchange medium was pooled with the first and cells were scraped into mPBS. For C_6-NBD-PS analysis, lipids from both scraped cells and media were extracted with propan-2-ol/chloroform (5.5:1, v/v) to prevent substantial loss into the aqueous phase. Samples were centrifuged at 780 ***g*** for 5 min and the supernatants transferred into new glass tubes and dried. For C_6-NBD-PC and -PE analysis, lipids from both cells and incubation media were extracted by the method of Bligh and Dyer [22] using 20 mM acetic acid in the aqueous phase. After two-dimensional separation [first direction, chloroform/methanol/25 % ammonium hydroxide (65:25:4, by vol.); second direction, chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, by vol.)] on TLC plates, fluorescent lipid spots were visualized under UV light, scraped off and quantified as described previously [20].

Fluorescence microscopy and photography

For intracellular localization of C_6-NBD-PS, cells grown on chambered coverglasses were preincubated with or without inhibitors, labelled with lipid analogue and back-exchanged with BSA as described above. To obtain mitochondrial labelling [23], cells were incubated with 30 nM MitoTracker Red CMXRos at 37 °C for 30 min. After washing, red CMXRos fluorescence was examined by microscopy. For Golgi labelling, cells were incubated for 10 min on ice with 5 μM C_6-NBD-Cer in mPBS containing 0.1 % (w/v) BSA [24], washed and incubated for 60 min at 37 °C. Thereafter, cells were subjected twice to a 10 min back-exchange with 2 % (w/v) BSA in mPBS on ice, then washed with mPBS. Microscopy was performed with an inverse standard microscope equipped with a Plan-Neofluar 100 × (1.3 numerical aperture) objective. The barrier filter sets (Carl Zeiss, Oberkochen, Germany) used were: green NBD fluorescence: BP 450 to 490 excitation filter, FT 510 dichroic mirror and LP 515 emission filter; red fluorescence: BP 546 excitation filter, FT 580 dichroic mirror and LP 590 emission filter. Photographs were taken using Kodak EPH P 1600 X films push-processed to 3200 ASA. As fluorescence photobleached quickly, different cells were photographed after each treatment.

Annexin assay

To measure exposure of endogenous PS on the cell surface, cells were incubated on ice for 10 min in the dark with 9 nM FITC-annexin V in binding buffer. At 2 min prior to the end of the incubation, propidium iodide (final 1.9 μM) was added. Cells were then washed three times, detached from the dishes by pipetting, and suspended in binding buffer. Measurement was performed with a FACSCalibur flow cytometer (Becton Dickinson, St. Louis, MO, U.S.A.) 5 min after the end of incubation. Forward scatter was set at a linear scale with the voltage set E00 (standard voltage setting of the instrument for forward scatter) and gain 1.39, side scatter with 386 V and gain 1. FITC fluorescence channel was at a logarithmic scale with 646 V, gain 1, and 0.9 % compensation of the propidium iodide signal. The propidium iodide fluorescence channel was at a logarithmic scale with 621 V, gain 1, and 24.9 % compensation of the FITC signal. Data were analysed using Becton–Dickinson CellQuest software.

Statistical analysis

Results are presented as means ± S.E.M. and were analysed statistically using a two-way analysis of variance Tukey test (Jandel SigmaStat 2.0) considering treatment and day of experiment. Differences were considered significant for $P < 0.05$.

RESULTS

Elevated synthesis of MDR1 Pgp in the MDR subline EPG85-257RDB

Synthesis of the ABC transporters MDR1 Pgp (ABCB1) and MRP1 (ABCC1) was determined in the parental drug-sensitive human gastric-carcinoma cell line (EPG85-257P) and its MDR subline (EPG85-257RDB) by Western blot. High synthesis of MDR1 Pgp was observed in the EPG85-257RDB subline (Figure 1) (designated *MDR1*-overexpressing line). In the EPG85-257P subline, MDR1 Pgp was below detection level (designated control line). MRP1 synthesis proved to be comparable in both sublines.

To assess activity of MDR1 Pgp and MRP1, cells were loaded with Rho 123 or the GS-MF precursor CMFDA. Upon removal of extracellular substrates, outward transport from the cells into the medium was studied in the presence or absence of various inhibitors. With about 90 %, outward transport of Rho 123 was significantly higher in *MDR1*-overexpressing cells than in control cells, where only about half of the accumulated Rho 123 was expelled into the medium within 30 min at 37 °C (Table 1). MDR1 Pgp inhibitors PSC 833, cyclosporin A and Dex significantly reduced transport of Rho 123 into the medium of *MDR1*-overexpressing cells. PSC 833 decreased transport in *MDR1*-overexpressing cells to the level found in control cells, but had little effect on control cells, while the MRP1 inhibitor MK 571 [25] did not affect Rho 123 outward transport in the *MDR1*-overexpressing subline. Thus the plasma membrane of *MDR1*-overexpressing cells, but not of control cells, exhibits functional (e.g. Rho 123-transporting) MDR1 Pgp.

Loading of cells with CMFDA, which is converted intracellularly into the fluorescent glutathione conjugate GS-MF,

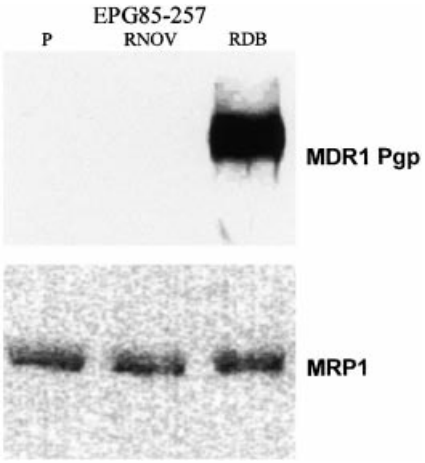

Figure 1 Elevated synthesis of MDR1 Pgp in the MDR subline EPG85-257RDB

Western blot of EPG85-257 cells was performed using C219 mouse mAb directed against human MDR1 Pgp and MRPm6 mouse mAb directed against MRP1. The secondary antibody was peroxidase-conjugated rabbit anti-mouse IgG, visualized by chemoluminescence. The EPG85-257 sublines tested were EPG85-257P (control), EPG85-257 RNOV (not used in the present study), and EPG85-257RDB (*MDR1*-overexpressing).

Table 1 Outward transport of Rho 123 and GS-MF in *MDR1*-overexpressing (MDR1$^+$) and control cells

Cells were preincubated for 10 min on ice in the presence or absence of MDR inhibitors PSC 833 (PSC, 10 μM), cyclosporin A (CsA, 25 μM), Dex (20 μM), and MK 571 (25 μM). After loading of cells with 10 μM Rho 123 or 3.2 μM CMFDA, outward transport was measured as described in the Experimental section. Results are expressed as the percentage of total fluorescence present in the medium after an incubation for 30 min at 37 °C and represent means ± S.E.M.; the number of samples is given in parentheses.

Cells	MDR inhibitor	Proportion of total fluorescence (%) Rho 123	GS-MF
MDR1$^+$	–	91 ± 1 (6)	89 ± 0.4 (6)
	MK	84 ± 4 (4)	47 ± 1 (4)
	PSC	50 ± 2 (6)	56 ± 4 (6)
	CsA	58 (2)	
	Dex	61 (2)	
Control	–	49 ± 4 (6)	86 ± 1 (6)
	MK	37 ± 1 (4)	57 ± 2 (4)
	PSC	38 ± 3 (6)	76 ± 3 (6)

allows detection of MRP1 activity [26]. Both *MDR1*-overexpressing and control cells showed comparable outward transport of GS-MF into the medium, over 85 % of the accumulated GS-MF being expelled into the medium within 30 min at 37 °C. In the presence of the MRP1 inhibitor MK 571, GS-MF transport was pronouncedly reduced and comparable in both sublines, only about half of the GS-MF still reaching the medium. The MDR1 Pgp inhibitor PSC 833 decreased GS-MF outward transport in the *MDR1*-overexpressing subline, while only slightly decreasing transport in control cells, which suggests low transport affinity of MDR1 Pgp for GS-MF. Both cell lines possess similar MRP1 activity, which is specifically inhibited by MK 571, while PSC 833, a potent inhibitor of MDR1 Pgp, also

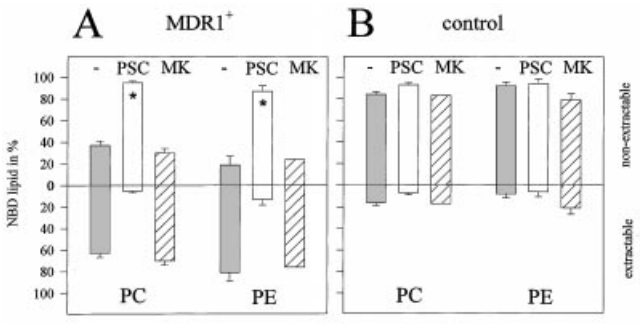

Figure 2 C_6-NBD-PC and -PE outward transport is enhanced in *MDR1*-overexpressing cells

MDR1-overexpressing (MDR1$^+$, **A**) and control (**B**) cells were preincubated for 10 min on ice without inhibitors or with 10 μM PSC 833 or 25 μM MK 571, followed by an incubation with 25 μM C_6-NBD-PA for 180 min at 15 °C in the presence or absence of the respective inhibitor. To trap the fluorescent lipid products (C_6-NBD-PC and C_6-NBD-PE) appearing on the cell surface in the medium, incubation was performed in the presence of 1 % (w/v) BSA. Lipids were quantified as described in the Experimental section. Results are means ± S.E.M. for at least $n = 3$ independent experiments in duplicate; for MK 571 mean ± range of $n = 1$ experiment in duplicate. The asterisks denote a significant difference compared with the respective cells (control or *MDR1*-overexpressing cells) without inhibitor pretreatment ($P < 0.05$).

reveals some efficiency to inhibit MRP1, confirming previous findings [27].

Outward transport of C_6-NBD-PC and -PE is enhanced by MDR1 Pgp

To examine outward transport of C_6-NBD-PC and -PE by MDR1 Pgp in *MDR1*-overexpressing cells and controls, cells were incubated with C_6-NBD-PA. This lipid analogue is partially converted into C_6-NBD-diacylglycerol (C_6-NBD-DG), which rapidly crosses the plasma membrane and becomes available for intracellular synthesis of C_6-NBD-PC and -PE [28]. Transport of the newly synthesized analogues to the cell surface was measured at 15 °C, where vesicular traffic is blocked [29]. After 180 min at 15 °C, about 63 % of C_6-NBD-PC and 81 % of C_6-NBD-PE were extracted in the BSA medium of the *MDR1*-overexpressing subline, while the BSA medium of control cells contained only a small fraction of each lipid (16 % of C_6-NBD-PC and 8 % of C_6-NBD-PE) (Figure 2). Addition of 10 μM PSC 833 caused a significant decrease in the amount of C_6-NBD-PC and -PE extracted from *MDR1*-overexpressing cells, while the amount of the two lipids extracted from control cells was not significantly altered. In both cell lines, 25 μM MK 571 did not influence transport of C_6-NBD-PC and -PE to the cell surface.

Rapid inward transport of C_6-NBD-PS, but not of -PC in gastric carcinoma cells

Unlike for C_6-NBD-PC and -PE, the lipid precursor C_6-NBD-PA is not metabolically converted into the respective PS analogue. In order to obtain labelling of the inner plasma-membrane leaflet with C_6-NBD-PS, we tested gastric carcinoma cells for aminophospholipid translocase activity. C_6-NBD lipids were introduced into the exoplasmic leaflet, and probe remaining in the exoplasmic leaflet was removed at various times of incubation using BSA. Indeed, we found rapid C_6-NBD-PS inward transport in both cell lines; only 52 % and 38 % of the analogue still being

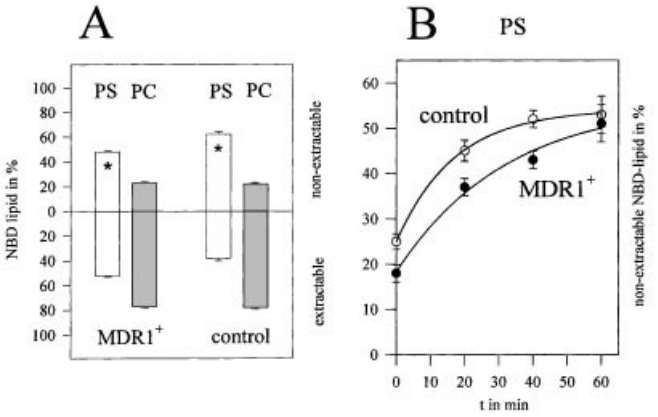

Figure 3 C_6-NBD-PS and -PC inward transport in *MDR1*-overexpressing and control cells

MDR1-overexpressing (MDR1$^+$) and control cells were labelled with 10 μM C_6-NBD-PS or 14 μM C_6-NBD-PC, washed on ice, and BSA back-exchange was performed either immediately ($t = 0$ min) or following incubation at 20 °C for indicated lengths of time. (**A**) Inward transport of C_6-NBD-PS and -PC after 30 min incubation. Lipids were quantified as described in the Experimental section. For C_6-NBD-PS, results are means ± S.E.M. for $n = 3$ independent experiments performed in duplicate. For C_6-NBD-PC, results shown are means ± S.E.M. for $n = 2$ independent experiments performed in duplicate. The asterisks denote a significant difference compared with the respective cells (control or *MDR1*-overexpressing cells) at $t = 0$ min ($P < 0.05$). (**B**) Time course of C_6-NBD-PS inward transport. Lipids were extracted as described in the Experimental section, dried, dissolved in chloroform/methanol (1:1, v/v) and quantified spectroscopically. Results are means ± S.E.M. for at least $n = 2$ independent experiments performed in duplicate (for $t = 60$ min, mean ± range for $n = 1$ experiment performed in duplicate), and were fitted to a monoexponential function. Results shown in (**B**) are independent of those shown in (**A**).

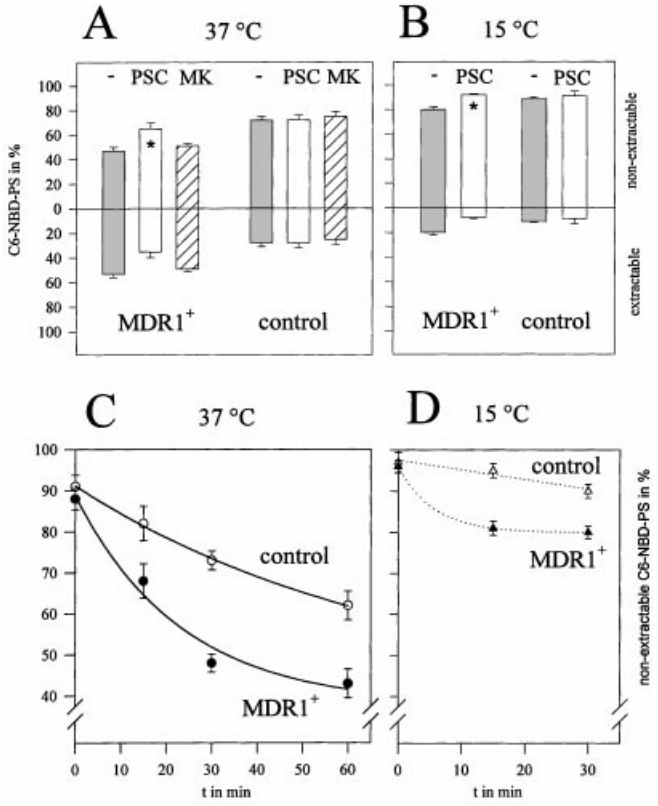

Figure 4 C_6-NBD-PS outward transport is enhanced in *MDR1*-overexpressing cells

MDR1-overexpressing (MDR1$^+$) and control cells were preincubated for 10 min on ice without inhibitors or with 10 μM PSC 833 or 25 μM MK 571, labelled with 5–10 μM C_6-NBD-PS and incubated at 20 °C for 30 min to allow intracellular accumulation of the NBD analogue. C_6-NBD-PS remaining on the cell surface was then extracted twice by incubation with 2% (w/v) BSA in mPBS for 10 min on ice. Then cells were incubated at 37 °C (**A**, **C**) or 15 °C (**B**, **D**) for indicated lengths of time (**C**, **D**) or for 30 min (**A**, **B**) in the presence of 2% (w/v) BSA to extract fluorescent lipids (C_6-NBD-PS and metabolites) appearing on the cell surface into the medium. Lipids were analysed as described in the Experimental section. In (**A**), results represent means ± S.E.M. for at least $n = 3$ independent experiments in duplicate (for MK 571, mean ± S.E.M. for $n = 2$ experiments performed in duplicate. In (**B**), results shown are mean ± S.E.M. for at least $n = 2$ independent experiments in duplicate, for PSC 833 mean ± range of $n = 1$ experiment in duplicate. The asterisks denote a significant difference compared with the respective cells (control or *MDR1*-overexpressing cells) without inhibitor pretreatment ($P < 0.05$). In (**C**) and (**D**), results are shown as means ± S.E.M. for at least $n = 2$ independent experiments performed in duplicate and fitted to a monoexponential function in (**C**).

accessible to BSA extraction after 30 min at 20 °C in *MDR1*-overexpressing cells and control cells respectively (Figure 3A; kinetics shown in Figure 3B). PSC 833 at 10 μM did not affect C_6-NBD-PS inward transport in either subline (results not shown). In contrast with the aminophospholipid analogue C_6-NBD-PS, the bulk (77–78%) of C_6-NBD-PC was accessible to BSA in both cell lines, even after 30 min at 20 °C (versus 87 and 80% immediately after labelling in *MDR1*-overexpressing cells and control cells respectively). Transport was unaffected by PSC 833 (results not shown).

Microscopy confirmed these results (results not shown): upon incubation with C_6-NBD-PS on ice, plasma membranes of *MDR1*-overexpressing cells and controls became highly fluorescent. At 20 °C, C_6-NBD-PS rapidly appeared in the intracellular lumen. After a 30 min incubation and BSA back-exchange, fluorescence was observed in granular fluorescent structures dispersed in the cytosol of both sublines (results not shown), identified as mitochondria using MitoTracker Red CMX Ros.

Control cells additionally accumulated fluorescence in a globular region near the nucleus, while this was rarely observed in *MDR1*-overexpressing cells. Labelling with C_6-NBD-Cer visualized the Golgi [24] as a scattered region in proximity to the nucleus of *MDR1*-overexpressing cells and as a distinct globular region adjacent to the nucleus in controls, suggesting that the globular structure labelled by C_6-NBD-PS in controls represents the Golgi. C_6-NBD-PC, in contrast with C_6-NBD-PS, remained confined to the plasma membrane of both control and *MDR1*-overexpressing cells after incubation for 30 min at room temperature. After back-exchange to BSA, only marginal cytoplasmic labelling was observed.

In conclusion, C_6-NBD-PS, but not -PC, allows substantial intracellular labelling of EPG85-257 cells after a 30 min incubation at 20 °C following incorporation on ice.

Outward transport of C_6-NBD-PS is enhanced by MDR1 Pgp

Subsequent to intracellular labelling of cells with C_6-NBD-PS, its transport back to the exoplasmic leaflet was determined. C_6-NBD-PS and its metabolites appearing on the cell surface were trapped by BSA present in the incubation medium. After a 30 min incubation at 37 °C, lipid analogues were extracted from cells and medium and analysed by two-dimensional TLC. In *MDR1*-overexpressing cells, 53% of C_6-NBD-PS was found in the medium, significantly more than in control cells (28%) (Figure 4A). Even after 60 min, differences remained significant between *MDR1*-overexpressing and control cells (kinetics shown in Figure 4C).

Table 2 Outward transport of C6-NBD-PS at 37 °C in *MDR1*-overexpressing (MDR1$^+$) and control cells with and without inhibitors

Measurement of C6-NBD-PS outward transport within a 30 min incubation at 37 °C was performed in the presence or absence of the MRP1 inhibitor MK 571, the ABCA1 inhibitor glyburide (Gb), or the MDR1 Pgp inhibitors PSC 833 (PSC), cyclosporin A (CsA) and Dex. Lipids were extracted as described in the Experimental section, dried, dissolved in chloroform/methanol (1:1, v/v) and quantified spectroscopically. Results represent means ± S.E.M. (for Gb the mean ± range is given) for independent experiments performed in duplicate; the number of independent experiments is given in parentheses.

Cells	Inhibitor	NBD-lipid (% extractable)
MDR1$^+$	–	50 ± 1 (16)
	MK	48 ± 2 (4)
	PSC	28 ± 2 (7)
	CsA	37 ± 4 (2)
	Dex	39 ± 4 (2)
	Gb	49 ± 5 (1)
Control	–	29 ± 1 (15)
	MK	22 ± 2 (5)
	PSC	25 ± 2 (6)
	CsA	24 ± 4 (2)
	Dex	30 ± 4 (2)
	Gb	23 ± 5 (1)

Pretreatment with PSC 833 caused a sharp and highly significant decrease in the amount of C_6-NBD-PS in the BSA medium of *MDR1*-overexpressing cells (35 %), while not affecting control cells. Other MDR1 Pgp inhibitors (25 μM cyclosporin A, 20 μM Dex) also blocked outward movement of C_6-NBD-PS, while 25 μM MRP1 inhibitor MK 571, as well as 200 μM glyburide, shown to inhibit ABCA1 [30], had no effect (Table 2). Expression of ABCA1 is low in both sublines, modulated only very slightly in MDR1-overexpressing cells, as indicated by RT-PCR analysis (D. Kerbiriou-Nabias and I. Laude, personal communication).

Increase of vesicular transport has been shown to be one mechanism by which some cells increase their drug tolerance [17]. To investigate whether C_6-NBD-PS transport observed at 37 °C could be due to increased membrane traffic in the *MDR1*-overexpressing subline, experiments were performed at 15 °C. Although outward transport of the PS analogue is reduced at this temperature, a clear difference in transport between control and *MDR1*-overexpressing cells was observed: 20 and 11 % of C_6-NBD-PS were found in the medium of the *MDR1*-overexpressing subline and the control cell line respectively (Figure 4B, kinetics shown in Figure 4D). PSC 833 reduced the fraction of C_6-NBD-PS in the medium of *MDR1*-overexpressing cells to the level of controls, while no change was observed when control cells were treated with PSC 833.

To exclude the possibility that C_6-NBD-PS was not accessible to outward redistribution due to confinement to the Golgi compartment, in particular in control cells, the antibiotic BFA was employed. BFA is known to induce fusion of the Golgi with the endoplasmic reticulum (ER), thereby allowing redistribution of NBD-lipids across the ER–Golgi membrane [30–32]. Thus BFA treatment permits NBD-lipids from the ER–Golgi compartment to reach the inner leaflet of the plasma membrane. In an experiment performed to evaluate the effect of BFA on the gastric carcinoma cell line used in the present study, synthesis of C_6-NBD-SM from the precursor C_6-NBD-Cer was shown to be

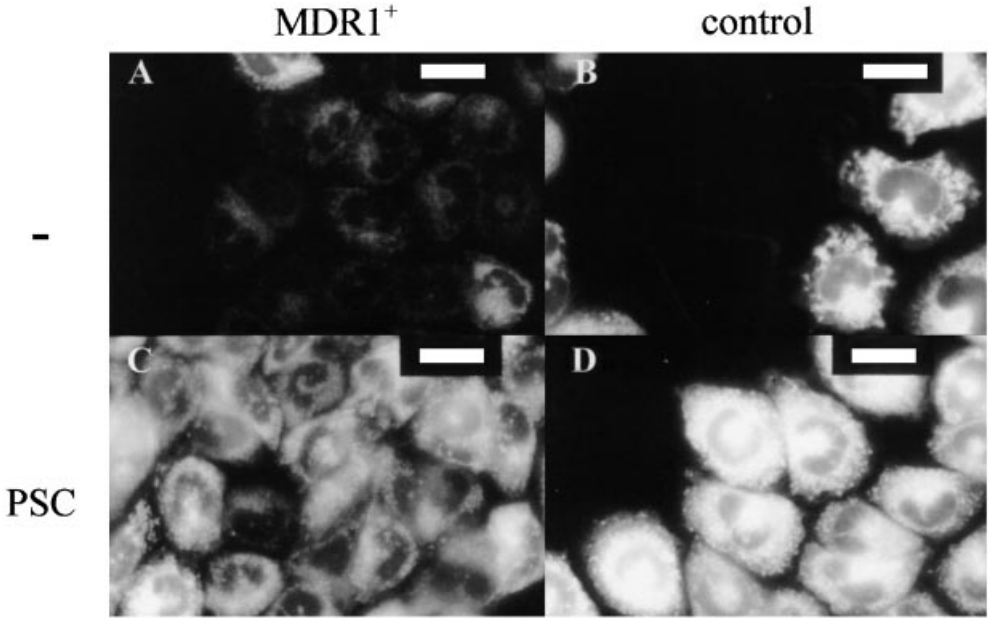

Figure 5 Comparison of C_6-NBD-PS outward transport in *MDR1*-overexpressing (MDR1$^+$) and control cells

After labelling of the plasma membrane with C_6-NBD-PS, the analogue was allowed to accumulate intracellularly. Subsequently, incubation for outward transport was performed without inhibitors (**A** and **B**) or in the presence of 10 μM PSC 833 (**C** and **D**) at 37 °C as described in the Experimental section. For microscopic examination of *MDR1*-overexpressing (**A**, **C**) and control (**B**, **D**) cells, BSA was removed and cells were washed twice. The major metabolic products of C_6-NBD-PS were C_6-NBD-PE (24% in *MDR1*-overexpressing cells and 35% in controls) and C_6-NBD-PA (4% in *MDR1*-overexpressing cells and 3% in controls). Labelling concentrations and exposure times are identical for all images shown. The bars represent 20 μm.

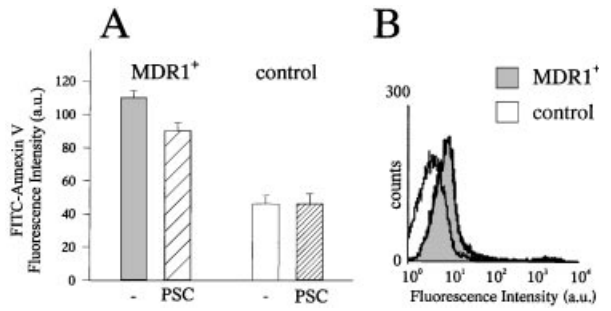

Figure 6 *MDR1*-overexpressing (MDR1$^+$) cells expose elevated amounts of endogenous PS

Flow-cytometric analysis of FITC-annexin V binding to the cell surface of *MDR1*-overexpressing and control human gastric carcinoma EPG85-257 cells. Following a 30 min incubation with or without 10 μM PSC 833 at 37 °C, cells were co-labelled with FITC-annexin V and the membrane-impermeable nucleic acid stain propidium iodide as described in the Experimental section. Cells showing elevated propidium iodide staining were excluded. A total of 10000 cells were counted per sample. In the histogram (**A**), the means $\pm$ S.E.M. for $n = 7$ independent experiments are shown; in (**B**), FITC-annexin V binding to *MDR1*-overexpressing cells (grey) and control cells (white) is shown for one typical experiment.

increased in both sublines upon addition of 5 μM BFA (control, 4.3-fold increase; *MDR1*-overexpressing cells, 4-fold increase), a criterion for BFA-induced morphological changes [33]. Moreover, under these conditions, C_6-NBD-SM synthesized in the lumen of the Golgi appeared in the BSA medium of *MDR1*-overexpressing cells (results not shown), having become accessible to MDR1 Pgp in the plasma membrane after BFA treatment [9,32]. C_6-NBD-PS outward transport, however, was not affected significantly in either subline upon preincubation with 5 μM BFA at 37 °C for 30 min and BFA present in the outward transport assay (results not shown).

Fluorescence microscopy confirmed efficient outward transport of C_6-NBD-PS in *MDR1*-overexpressing cells in comparison with control cells. After labelling of the exoplasmic leaflet and subsequent inward transport of the analogue at 20 °C, *MDR1*-overexpressing and control cells showed bright intracellular fluorescence. Subsequently, cells were incubated for 1 h at 37 °C in the presence of BSA and washed prior to examination by fluorescence microscopy. *MDR1*-overexpressing cells lost most of the analogue into the medium (Figure 5A), while intracellular fluorescence decreased only slightly in control cells (Figure 5B). In the presence of PSC 833, strong intracellular fluorescence was maintained in both *MDR1*-overexpressing cells (Figure 5C) and controls (Figure 5D). Meanwhile, the intracellular distribution of C_6-NBD-PS to cell organelles did not change in either of the two sublines in response to PSC 833. Lipid metabolism was not responsible for the observed differences between control and *MDR1*-overexpressing cells (see the legend to Figure 5).

MDR1-overexpressing cells expose more endogenous PS on the cell surface than controls

Exposure of endogenous PS in *MDR1*-overexpressing cells and controls was tested by flow cytometry. PS present on the outer plasma-membrane leaflet of cells with intact cell membranes was detected by labelling with both FITC-annexin V, a high-affinity PS-binding protein [34], and the membrane-impermeable nucleic acid stain propidium iodide. Regions were set to exclude subcellular particles, and only single cells were counted (10000 cells/sample). A further gate excluded damaged cells with elevated propidium iodide staining. Few ungated cells with propidium iodide-permeable membranes were detected, slightly more in *MDR1*-overexpressing cells (5 %) than in controls (1 %). Cells from the *MDR1*-overexpressing subline bound over 2-fold more FITC-annexin V than control cells. The mean FITC-annexin V fluorescence intensity was 110 ± 4 arbitrary units for *MDR1*-overexpressing cells, and 46 ± 5 arbitrary units for control cells ($n = 10000$) (Figure 6), the differences being highly significant. The FITC-annexin V histogram shape was similar for both cell lines, showing that the amount of PS present on the outer plasma-membrane leaflet in *MDR1*-overexpressing cells was generally increased for all cells in this population.

To assess whether FITC-annexin V binding was reduced upon inhibition of MDR1 Pgp, cells were preincubated with 10 μM PSC 833 for 30 min at 37 °C. The mean FITC-annexin V fluorescence intensity was significantly decreased in *MDR1*-overexpressing cells (90 ± 5 arbitrary units) and remained unchanged in controls (46 ± 6 arbitrary units). However, FITC-annexin V binding to PSC 833 treated *MDR1*-overexpressing cells remained significantly higher in comparison with controls.

DISCUSSION

MDR1 Pgp has been implicated with the transport of lipids from the inner to the outer leaflet of the plasma membrane. In the present study we have investigated the transport of lipids to the cell surface in *MDR1*-overexpressing EPG85-257 human gastric carcinoma cells compared with control cells.

Incubation of both sublines with C_6-NBD-PA at 15 °C permitted us to investigate the transport of the newly synthesized C_6-NBD-PC and -PE from the cytoplasmic to the exoplasmic plasma membrane leaflet. Compared with controls, very strongly increased outward transport of C_6-NBD-PC and -PE was found in *MDR1*-overexpressing cells, surpassing previous findings in MDR1 transfected pig kidney epithelial cells [9] and supporting a role for MDR1 Pgp in the transport of C_6-NBD-PC and -PE to the cell surface. Consistent with this, outward transport of C_6-NBD-PC and -PE was sensitive to MDR1 Pgp inhibitors, while an inhibitor of MRP1, effectively blocking the outward transport of the MRP1 substrate GS-MF, had no effect on the appearance of either NBD lipid on the cell surface.

Unlike in experiments regarding outward transport of C_6-NBD-PC and -PE (see above and [9]), C_6-NBD-PA is not a suitable precursor to label the cytoplasmic leaflet with C_6-NBD-PS. Therefore we aimed to insert C_6-NBD-PS into the cytoplasmic leaflet of the plasma membrane via the aminophospholipid translocase activity, a ubiquitous fast mechanism transporting PS from the outer to the inner plasma-membrane leaflet. Indeed, inward-movement measurements at 20 °C revealed fast internalization of C_6-NBD-PS, while C_6-NBD-PC remained mostly confined to the exoplasmic leaflet of the plasma membrane. Preferential internalization of the PS analogue by transbilayer movement was confirmed by fluorescence microscopy and suggests the presence of an aminophospholipid translocase activity. This inward-directed transport activity mediated rapid and extensive labelling of the inner plasma-membrane leaflet with C_6-NBD-PS. Together with the BSA back-exchange technique, it offers a new tool to study C_6-NBD-PS outward transport. Continuous incubation of cells in the presence of BSA permits monitoring of C_6-NBD-analogue outward movement without needing to take into account simultaneous inward movement. BSA acts as an extracellular sink for short-chain

lipids by rapidly extracting C_6-NBD-phospholipid analogues [35], excluding analogues from inward transport via the aminophospholipid translocase.

Within 30 min at 37 °C, a significantly higher percentage of C_6-NBD-PS became accessible to BSA in *MDR1*-overexpressing cells compared with controls. This was confirmed by fluorescence microscopy. When vesicular transport was inhibited at 15 °C [29] or by BFA [32], *MDR1*-overexpressing cells still exhibited significantly higher transport of C_6-NBD-PS to the cell surface than did control cells. Fusion of the Golgi with the ER induced by BFA did not influence outward transport of C_6-NBD-PS in either subline. We could therefore exclude that variations in the intracellular localization of C_6-NBD-PS between both sublines gave rise to differences in C_6-NBD-PS outward movement. Outward-directed transport of C_6-NBD-PS was efficiently decreased by MDR1 Pgp inhibitors, marking the involvement of MDR1 Pgp.

Recently, two other ABC transporters, MRP1 (ABCC1) and ABCA1, have emerged as further candidate lipid translocases. In studies with *MRP1* knockout mice, MRP1-mediated transport of C_6-NBD-PC and -PS was reported in erythrocytes, but no changes in the distribution of endogenous PS were detected [36,37]. Raggers et al. [38] observed transport of lipid analogues in *MRP1*-transfected epithelial cells for sphingolipids with an NBD moiety. Using the MRP1 specific inhibitor MK 571, our studies clearly demonstrate that this ABC transporter is not involved in transport of C_6-NBD-PS to the cell surface of *MDR1*-overexpressing EPG85-257 cells. ABCA1-dependent transport of PS has recently been shown for various mammalian cells [39,40]. However, we have no indication for involvement of ABCA1 in the enhanced transport of C_6-NBD-PS in *MDR1*-overexpressing EPG85-257 cells. In both sublines used, ABCA1 mRNA is barely detectable and C_6-NBD-PS transport was not affected by glyburide, a compound reported to inhibit ABCA1-dependent exposure of PS [39].

Taken together, our results show MDR1 Pgp to mediate outwardly directed transport of C_6-NBD-PS. In a previous study, Bosch et al. [11] were not able to detect transport of the long-chain (dodecanoyl) analogue C_{12}-NBD-PS by MDR1 Pgp, assessing the accumulation of C_{12}-NBD-phospholipid analogues in *MDR1*-overexpressing and control cells. Unlike what was found in our study, the aminophospholipid translocase activity could not be eliminated in the experimental set-up used by Bosch et al. [11]. While we cannot exclude the possibility that C_{12}-NBD-PS might not be a substrate for MDR1 Pgp, C_{12}-NBD analogues of PE and PC were shown to be transported by MDR1 Pgp [11]. This suggests that a longer fatty acid chain in the *sn*-2 position does not prevent recognition of the analogues by MDR1 Pgp. Since the affinity of the aminophospholipid translocase is about ten times lower for analogues of PE than for PS [41], it is possible that MDR1 Pgp-mediated transport of C_{12}-NBD-PS, but not C_{12}-NBD-PE, is masked by an aminophospholipid translocase activity. C_{12}-NBD-PS is unfortunately not suitable for our set-up to measure outward movement of PS analogues, as extraction of this analogue by BSA is less efficient than of C_6-NBD-PS [42].

A different approach to selectively investigate the activity of MDR1 Pgp, excluding other potential lipid transporters, is to reconstitute MDR1 Pgp in liposomes. In a recent paper, Romsicki and Sharom [43] studied transport of a number of short-chain and long-chain NBD analogues of PC, PE, PS and SM in proteoliposomes reconstituted with MDR1 Pgp. The reconstituted MDR1 Pgp was predominantly oriented inside-out into the liposome membranes. Presumably for steric reasons, the transmembrane distribution of nearly all NBD lipids was shifted to the outer leaflet in the presence of MDR1 Pgp, prohibiting a direct comparison with protein-free liposomes. Nevertheless, when ATP was added, the portion of lipid analogues in the inner leaflet of MDR1 Pgp containing liposomes increased. While this is principally in line with our observation, the increase in the inner leaflet by a few per cent (maximum 5 %) [43] was much less in comparison with the transport activity found for the *MDR1*-expressing cell line. This may raise concerns with respect to the preservation of MDR1 Pgp function in proteoliposomes, as indicated by a recent study of Rothnie and Theron [44]. In that report, the transport of short-chain NBD analogues of PC, PE and Cer was studied in MDR1 Pgp-reconstituted liposomes. A low percentage of the analogues (up to about 6 %) also appeared to reorient across the membrane in the presence of MDR1 Pgp in a process that was, surprisingly, found to be ATP-independent. However, the authors noted that the proteoliposome assay used may be subject to important technical limitations (e.g. inhibition of further lipid transport by increased lateral pressure in the inner membrane leaflet following initial transport). Nevertheless, such studies can give insight into the biophysical restrictions for MDR1 Pgp-mediated transport: as found by Rothnie and Theron, physiological concentrations of cholesterol seem to be required to partially compensate for membrane perturbations by MDR1 Pgp. This interplay of MDR1 Pgp with specific lipid compounds might be a prerequisite for the transport of amphiphilic substrates. Moreover, an effect of lateral pressure in the membrane on MDR1 Pgp-mediated lipid transport could either act on MDR1 Pgp directly (mechanosensitivity) or on the substrate lipids' propensity to redistribute towards the layer exhibiting lower packing.

Having established that MDR1 Pgp can transport C_6-NBD-PS to the outer plasma-membrane leaflet, we studied the exposure of endogenous PS on the cell surface. Consistent with a role of MDR1 Pgp in the transport of endogenous PS, binding of FITC-annexin V to the cell surface of *MDR1*-overexpressing cells was significantly higher than binding to control cells, and inhibition of MDR1 Pgp decreased FITC-annexin V binding in *MDR1*-overexpressing cells, but not at all in controls. MDR1 Pgp-mediated transport of endogenous PS might provide an explanation for increased PS exposure on tumorigenic keratinocytes reported previously [16]. However, our approach does not allow quantification of endogenous PS in the exoplasmic leaflet. The FITC-annexin V binding assay has been reported to be sensitive to as little as 5 mol % PS [45], and the disturbance of PS asymmetry here is most likely only partial. It remains to be shown whether exposure of PS in the exoplasmic leaflet of *MDR1*-overexpressing cells is sufficient to trigger subsequent biological processes, such as recognition by macrophages [39]. Detailed information is now needed on the transbilayer dynamics of endogenous PS in these cells, e.g. on the transport of this lipid by the aminophospholipid translocase.

In 1996, van Helvoort et al. speculated on the ability of MDR1 Pgp to transport endogenous lipids, coming to the conclusion that this is unlikely, as it might lead to a futile ATP-utilizing cycle when the same substrates transported inside by the aminophospholipid translocase are moved in the opposite direction by MDR1 Pgp [9]. However, *MDR1* has only a low expression in most cells [46], where MDR1 Pgp would not be an important antagonist to the aminophospholipid translocase. In several studies, *MDR1* expression was found to become increased in the presence of cytostatic drugs [47]. One might speculate that the regulation of MDR1 Pgp according to the current demand could make PS transport an (energetically) expensive side-effect of MDR1 Pgp's detoxifying action, being tolerable only when limited in time. In some cell types with moderate *MDR1*

expression, MDR1 Pgp-mediated outward transport of endogenous PS could be a desired function. It is conceivable that the aminophospholipid translocase activity could be down-regulated in such cells to avoid futile energy consumption (see [48] for an example of controlled regulation of lipid inward and outward transport in yeast). In fact, without co-ordinated regulation of the two transporters, high expression of *MDR1* could lead to excessive energy consumption by antagonistic transport of the same substrate. An elevated rate of glucose uptake is typically found in many tumour cells [49]. Tumor cells, differently from untransformed cells, might keep up their resistance against cytostatic drugs by accelerated metabolism, this being one of a number of instances where tumours disregard economic restraints.

As analogues of endogenous lipids, short-chain NBD lipids only reach a certain degree of accordance due to the short fatty acid chain and the presence of the bulky reporter group. Since van Helvoort et al. [9] could demonstrate transport of short-chain analogues of PC, PE and glucosylceramide lack the NBD group in their *MDR1*-transfected cell line, transport by MDR1 Pgp does not appear to be due to the modification on the level of one of the fatty acid chains. Recently, Ernest and Bells-Reuss [13] have shown PAF to be an endogenous substrate of MDR1 Pgp in human mesangial cells. In our study, we provide evidence that endogenous PS is possibly also recognized and transported by MDR1 Pgp. Addition of PS and its analogues to the circle of MDR1 Pgp lipid substrates further supports the idea of MDR1 Pgp acting as a floppase with extremely low substrate specificity, which implies first of all the integration of the substrate into the membrane with little regard to the chemistry of the polar head group. Yet, different affinities for various lipid substrates can give rise to a certain degree of specificity for transport. Detailed studies on the affinity of MDR1 Pgp for lipids are necessary to understand modulation of MDR1 Pgp-mediated MDR by the ubiquitous lipids. Very likely these questions can be addressed successfully only in a defined, yet adequately physiological system, e.g. transfected cells with a high level of *MDR1* expression and a natural membrane environment, or MDR1 Pgp reconstituted into giant liposomes with a distinct lipid composition and a marginal degree of membrane curvature similar to that of a cell, avoiding the generation of high lateral pressure that could potentially inhibit lipid transport.

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REFERENCES

1 Zachowski, A. (1993) Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. Biochem. J. **294**, 1–14
2 Zwaal, R. and Schroit, A. (1997) Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. Blood **89**, 1121–1132
3 Devaux, P. (2000) Is lipid translocation involved during endo- and exocytosis? Biochimie **82**, 497–509
4 Seigneuret, M. and Devaux, P. F. (1984) ATP-dependent asymmetric distribution of spin labelled phospholipids in the erythrocyte membrane: relation to shape changes. Proc. Natl. Acad. Sci. U.S.A. **81**, 3751–3755
5 Daleke, D. L. and Lyles, J. V. (2000) Identification and purification of aminophospholipid flippases. Biochim. Biophys. Acta **1486**, 108–127
6 Smit, J., Schinkel, A., Oude Elferink, R., Groen, A., Wagenaar, E., van Deemter, L., Mol, C., Ottenhoff, R., van der Lugt, N., van Roon, M., van der Valk, M., Offerhaus, G. et al. (1993) Homozygous disruption of the murine mdr2 P-Glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. Cell **75**, 451–462
7 Ruetz, S. and Gros, P. (1994) Phosphatidylcholine translocase: a physiological role for the mdr2 gene. Cell **77**, 1071–1081
8 Smith, A., Timmermans-Hereijgers, J., Roelofsen, B., Wirtz, K., van Blitterswijk, W., Smit, J., Schinkel, A. and Borst, P. (1994) The human MDR3 P-glycoprotein promotes translocation of phosphatidylcholine through the plasma membrane of fibroblasts from transgenic mice. FEBS Lett. **354**, 263–266
9 van Helvoort, A., Smith, A., Sprong, H., Fritzsche, I., Schinkel, A., Borst, P. and van Meer, G. (1996) MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. Cell **87**, 507–517
10 Cardarelli, C., Aksentijevich, I., Pastan, I. and Gottesman, M. (1995) Differential effects of P-glycoprotein inhibitors on NIH3T3 cells transfected with wild-type (G185) or mutant (V185) multidrug transporters. Cancer Res. **55**, 1086–1091
11 Bosch, I., Dunussi-Joannopoulos, K., Wu, R., Furlong, S. and Croop, J. (1997) Phosphatidylcholine and phosphatidylethanolamine behave as substrates of the human MDR1 P-glycoprotein. Biochemistry **36**, 5685–5694
12 Abulrob, A. and Gumbleton, M. (1999) Transport of phosphatidylcholine in MDR3-negative epithelial cell lines via drug-induced MDR1 P-glycoprotein. Biochem. Biophys. Res. Commun. **262**, 121–126
13 Ernest, S. and Bello-Reuss, E. (1999) Secretion of platelet-activating factor is mediated by MDR1 Pglycoprotein in cultured human mesangial cells. J. Am. Soc. Nephrol. **10**, 2306–2313
14 Raggers, R. J., Pomorski, T., Holthuis, J. C. M., Kalin, N. and van Meer, G. (2000) Lipid traffic: the ABC of transbilayer movement. Traffic **1**, 226–234
15 Higgins, C. and Gottesman, M. (1992) Is the multidrug transporter a flippase? Trends Biochem. Sci. **17**, 18–21
16 Utsugi, T., Schroit, A., Connor, J., Bucana, C. and Fidler, I. (1991) Elevated expression of phosphatidylserine in the outer leaflet of human tumor cells and recognition by activated human blood monocytes. Cancer Res. **51**, 3062–3066
17 Dietel, M., Arps, H., Lage, H. and Niendorf, A. (1990) Membrane vesicle formation due to acquired mitoxantrone resistance in human gastric carcinoma cell line EPG85-EP257. Cancer Res. **50**, 6100–6106
18 Lage, H., Jordan, A., Scholz, R. and Dietel, M. (2000) Thermosensitivity of multidrug-resistant gastric and pancreatic carcinoma cells. Int. J. Hyperther. **16**, 291–303
19 Lage, H., Perlitz, C., Abele, R., Tampé, R., Dietel, M., Schadendorf, D. and Sinha, P. (2001) Enhanced expression of human ABC-transporter TAP is associated with cellular resistance to mitoxantrone. FEBS Lett. **503**, 179–184
20 Pomorski, T., Müller, P., Zimmermann, B., Burger, K., Devaux, P. and Herrmann, A. (1996) Transbilayer movement of fluorescent and spin-labeled phospholipids in the plasma membrane of human fibroblasts: a quantitative approach. J. Cell Sci. **109**, 687–698
21 Colleau, M., Hervé, P., Fellmann, P. and Devaux, P. (1991) Transmembrane diffusion of fluorescent phospholipids in human erythrocytes. Chem. Phys. Lipids **57**, 29–37
22 Bligh, E. and Dyer, W. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**, 911–917
23 Poot, M., Zhang, Y., Krämer, J., Wells, K., Jones, L., Hanzel, D., Lugade, A., Singer, V. and Haugland, R. (1996) Analysis of mitochondrial morphology and function with novel fixable fluorescent stains. J. Histochem. Cytochem. **44**, 1363–1372
24 Lipsky, N. and Pagano, R. (1985) A vital stain for the Golgi apparatus. Science **228**, 745–747
25 Jones, T., Zamboni, R., Belley, M., Champion, E., Charette, L., Ford-Hutchinson, A., Frenette, R., Gauthier, J., Leger, S., Masson, P. et al. (1989) Pharmacology of L-660,711 (MK-571): a novel potent and selective leukotriene D4 receptor antagonist. Can. J. Physiol. Pharmacol. **67**, 17–28
26 Roelofsen, H., Vos, T., Schippers, I., Kuipers, F., Koning, H., Moshage, H., Jansen, P. and Mülller, M. (1997) Increased levels of the multidrug resistance protein in lateral membranes of proliferating hepatocytederived cells. Gastroenterology **112**, 511–521
27 Lehne, G., Morkrid, L., den Boer, M. and Rugstad, H. (2000) Diverse effects of P-glycoprotein inhibitory agents on human leukemia cells expressing the multidrug resistance protein (MRP). Int. J. Clin. Pharmacol. Ther. **38**, 187–195
28 Pagano, R., Martin, O., Schroit, A. and Struck, D. (1981) Formation of asymmetric phospholipid membranes via spontaneous transfer of fluorescent lipid analogueues between vesicle populations. Biochemistry **20**, 4920–4927
29 van Genderen, I. and van Meer, G. (1995) Differential targeting of glucosylceramide and galactosylceramide analogueues after synthesis but not during transcytosis in Madin–Darby canine kidney cells. J. Cell Biol. **131**, 645–654

30 Lippincott-Schwartz, J., Donaldson, J., Schweizer, A., Berger, E., Hauri, H., Yuan, L. and Klausner, R. (1990) Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. Cell **60**, 821–836
31 Kok, J., Hoekstra, K., Eskelinen, S. and Hoekstra, D. (1992) Recycling pathways of glucosylceramide in BHK cells: distinct involvement of early and late endosomes. J. Cell Sci. **103**, 1139–1152
32 van Helvoort, A., Giudici, M., Thielemans, M. and van Meer, G. (1997) Transport of sphingomyelin to the cell surface is inhibited by brefeldin A and in mitosis, where C_6-NBD-sphingomyelin is translocated across the plasma membrane by a multidrug transporter activity. J. Cell Sci. **110**, 75–83
33 Brüning, A., Karrenbauer, A., Schnabel, E. and Wieland, F. (1992) Brefeldin A-induced increase of sphingomyelin synthesis. J. Biol. Chem. **267**, 5052–5055
34 Dachary-Prigent, J., Freyssinet, J., Pasquet, J. and Nurden, A. (1993) Annexin V as a probe of aminophospholipid exposure and platelet membrane vesiculation: a flow cytometry study showing a role for free sulfhydryl groups. Blood **81**, 2554–2565
35 Marx, U., Laßmann, G., Holzhütter, H.-G., Wüstner, D., Müller, P., Höhlig, A., Kubelt, J. and Herrmann, A. (2000) Rapid flip-flop of phospholipids in endoplasmic reticulum membranes studied by a stopped-flow approach. Biophys. J. **78**, 2628–2640
36 Dekkers, D., Comfurius, P., Schroit, A., Bevers, E. and Zwaal, R. (1998) Transbilayer movement of NBD-labelled phospholipids in red blood cell membranes: outward-directed transport by the multidrug resistance protein 1 (MRP1). Biochemistry **37**, 14833–14837
37 Kamp, D. and Haest, C. (1998) Evidence for a role of the multidrug resistance protein (MRP) in the outward translocation of NBD-phospholipids in the erythrocyte membrane. Biochim. Biophys. Acta **1372**, 91–101
38 Raggers, R., van Helvoort, A., Evers, R. and van Meer, G. (1999) The human multidrug resistance protein MRP1 translocates sphingolipid analogues across the plasma membrane. J. Cell Sci. **112**, 415–422
39 Marguet, D., Luciani, M. F., Moynault, A., Williamson, P. and Chimini, G. (1999) Engulfment of apoptotic cells involves the redistribution of membrane phosphatidylserine on both phagocyte and prey. Nat. Cell Biol. **1**, 454–456

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40 Hamon, Y., Broccardo, C., Chambenoit, O., Luciani, M.-F., Toti, F., Chaslin, S., Freyssinet, J.-M., Devaux, P. F., Neish, J., Marguet, D. and Chimini, G. (2000) ABC1 promotes engulfment of apoptotic cells and transbilayer redistribution of phosphatidylserine. Nat. Cell Biol. **2**, 399–406
41 Zachowski, A., Favre, E., Cribier, S., Herve, P. and Devaux, P. (1986) Outside–inside translocation of aminophospholipids in the human erythrocyte membrane is mediated by a specific enzyme. Biochemistry **25**, 2585–2590
42 Wüstner, D., Pomorski, T., Herrmann, A. and Müller, P. (1998) Release of phospholipids from erythrocyte membranes by taurocholate is determined by their transbilayer orientation and hydrophobic backbone. Biochemistry **37**, 17093–17103
43 Romsicki, Y. and Sharom, F. (2001) Phospholipid flippase activity of the reconstituted P-glycoprotein multidrug transporter. Biochemistry **40**, 6937–6947
44 Rothnie, A. and Theron, D. (2001) The importance of cholesterol in maintenance of P-glycoprotein activity and its membrane perturbing influence. Eur. Biophys. J. **30**, 430–442
45 Stuart, M. C. A., Bevers, E. M., Comfurius, P., Zwaal, R. F. A., Reutelingsperger, C. P. M. and Frederik, P. M. (1995) Ultrastructural detection of surface exposed phosphatidylserine on activated blood platelets. Thromb. Haemostasis **74**, 1145–1151
46 Cordon-Cardo, C., O'Brien, J. P., Boccia, J., Casals, D., Bertino, J. R. and Melamed, M. R. (1990) Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. J. Histochem. Cytochem. **38**, 1277–1287
47 Hu, X. F., Slater, A., Wall, D. M., Kantharidis, P., Parkin, J. D., Cowman, A. and Zalcberg, J. R. (1995) Rapid upregulation of mdr1 expression by anthracyclines in a classical multidrug-resistant cell line. Br. J. Cancer **71**, 931–936
48 Hanson, P. K. and Nichols, J. W. (2001) Energy-dependent flip of fluorescence-labeled phospholipids is regulated by nutrient starvation and transcription factors, PDR1 and PDR3. J. Biol. Chem. **276**, 9861–9867
49 Kawamura, T., Kusakabe, T., Sugino, T., Watanabe, K., Fukuda, T., Nashimoto, A., Honma, K. and Suzuki, T. (2001) Expression of glucose transporter-1 in human gastric carcinoma: association with tumor aggressiveness, metastasis and patient survival. Cancer **92**, 634–641