Enhanced exposure of phosphatidylserine in human gastric carcinoma cells overexpressing the half-size ABC transporter BCRP (ABCG2)

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Members of the ABC (ATP-binding cassette) transporter superfamily are emerging to be involved in lipid transport. In the present study, we studied the organization of phospholipids in the plasma membrane of EPG85-257 human gastric carcinoma cells overexpressing BCRP (breast cancer resistance protein, ABCG2), a half-size transporter belonging to the ABCG subfamily. A significantly increased plasma membrane association of the PS (phosphatidylserine)-binding probe FITC–Annexin V in comparison with control cells was observed. Treatment of BCRP-overexpressing cells with the inhibitor Tryprostatin A decreased FITC–Annexin V binding almost to the control level. This suggests an enhanced exposure of PS in BCRP-overexpressing cells, which is dependent on functional BCRP. A role of BCRP in the transverse distribution of lipids in the plasma membrane

is supported by the increased outward transport of the lipid analogue C6-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-PS in BCRP-overexpressing EPG85-257 cells and MCF-7 human breast cancer cells. As shown for BCRP-overexpressing EPG85-257 cells, enhanced outward redistribution of the lipid analogue is inhibited by Tryprostatin A as well as by reduction of BCRP expression on transfection with an anti-BCRP-ribozyme. We also observed an enhanced outward transport of C6-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phosphatidylcholine in BCRP-overexpressing EPG85-257 cells, suggesting that the influence of BCRP on transverse lipid organization is not highly specific.

Key words: flippase, lipid asymmetry, phosphatidylserine, plasma membrane.

INTRODUCTION

Over the last couple of years, various lipids and lipid analogues were shown to be transported by members of the ABC (ATP-binding cassette) transporter superfamily.

The ABCB family member MDR3 Pgp (multidrug resistance 3 P-glycoprotein; ABCB4) and its mouse homologue mdr2 Pgp specifically expel PC (phosphatidylcholine) from the cell [1–4], whereas MDR1 Pgp (ABCB1), involved in MDR of tumour cells [5], transports various lipid analogues [4,6–8] and probably also the endogenous lipids platelet-activating factor (a short-chain PC), PS (phosphatidylserine) and glucosylceramide [8–10] from the inner to the outer plasma membrane leaflet. MRP1 (multidrug resistance protein 1, ABCC1), belonging to the ABCC family, was reported to mediate outward transport of C6-NBD-PC [where NBD stands for *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)] and C6-NBD-PS in murine erythrocytes [11,12], and NBD-sphingolipids in epithelial cells [13], whereas the ABCA family member ABCA1 was shown to be involved in the transport of PS in various mammalian cells [14,15].

The potential lipid transporters MDR1 Pgp, MDR3 Pgp, MRP1 and ABCA1 each contain two nucleotide-binding domains and two transmembrane domains (three in MRP1). Half-size ABC transporters containing only one nucleotide-binding domain and one transmembrane domain have recently been implicated with the transport of lipids as well. For example, in humans, White (ABCG1), an ABCG subfamily member has been reported to be involved in the regulation of cholesterol and phospholipid transport in macrophages and possibly in other cells, in which

it is ubiquitously expressed [16]. Disruption of the heterodimer formed by the half-size transporters ABCG5 and ABCG8 has been shown to inhibit greatly the secretion of cholesterol into bile in mice [17]. It has been suggested that ABCG5/ABCG8 may act as a cholesterol flippase and/or facilitates the exposure of membrane-bound cholesterol to bile salts [18].

The recently discovered human half-size transporter BCRP (breast cancer resistance protein, MXR, ABCG2), also a member of the ABCG family, is a 72 kDa protein consisting of 655 amino acids (see overview in [19]). It is supposed to homo- or hetero-dimerize to form an active-transport complex. The BCRP gene is highly overexpressed in the plasma membrane of several drug-resistant cell lines, where it has been shown to confer atypical MDR [20–23]. BCRP transports the antitumour drugs mitoxantrone, topotecan, daunorubicin, doxorubicin and bisantrene [20].

We have previously demonstrated that MDR1 Pgp mediated outward transport of the PS analogue C6-NBD-PS and of endogenous PS [8] in MDR1-overexpressing EPG85-257 human gastric carcinoma cells. To explore an involvement of BCRP in lipid transport, we have now investigated the transbilayer movement of short-chain fluorescent C6-NBD phospholipid analogues, and exposure of endogenous PS in BCRP-overexpressing EPG85-257 cells in the presence or absence of an anti-BCRP-ribozyme [24], and in BCRP-overexpressing MCF-7 human breast cancer cells. Tryprostatin A was used to inhibit BCRP [25]. Our results suggest an involvement of BCRP in the transport of endogenous phospholipids, potentially causing changes in lipid transbilayer distribution. In the plasma membrane, modified lipid asymmetry

Abbreviations used: ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; MDR, multidrug resistance; MDR1 Pgp, MDR1 P-glycoprotein; mPBB, modified PBS; MRP1, multidrug resistance protein 1; NBD, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl); PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidyl-ethanolamine; PS, phosphatidylserine.

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could influence surface properties and cell-cell interactions, which might allow development of custom-made diagnostics and therapy in *BCRP*-overexpressing MDR tumours in the future.

EXPERIMENTAL

Materials

Di-isopropyl fluorophosphate and fatty acid-free BSA were purchased from Sigma-Aldrich (Steinheim, Germany). Dulbecco's phosphate-buffered saline with Ca^{2+} , Mg^{2+} (PBS) obtained from Biochrom KG (Berlin, Germany) was supplemented with 24 mM glucose and 10 mM Hepes [modified PBS (mPBS)]. C6-NBD-PA (where PA stands for phosphatidic acid) and C6-NBD-PS were purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.), high-performance TLC plates from Merck (Darmstadt, Germany). FITC-Annexin V, binding buffer and propidium iodide were purchased from VPS Diagnostics (Hoeven, The Netherlands). Tryprostatin A (gift from H. Osada, Riken-Institute, Japan) and GF120918 (gift from Glaxo Wellcome, Uxbridge, U.K.) were prepared from methanol stocks. The effect of the solvent was determined for all experiments.

Cell lines and culture conditions

The human breast cancer cell line MCF-7, its atypical multidrug-resistant derivative MCF-7/AdrVp (*BCRP*-overexpressing selection variant MCF-7/AdrVp1000 [26]) and the *BCRP* cDNA-transfected subline MCF-7/*BCRP* clone 8 were kindly provided by Douglass D. Ross (Greenebaum Cancer Center of the University of Maryland, Baltimore, MD, U.S.A.).

The drug-sensitive human gastric carcinoma cell line EPG85-257P (designated EPG85-control) and its multidrug-resistant sublines have been established previously [27–29]. EPG85-257RDB cells (designated EPG85-*MDR1* Pgp), showing a classical *MDR1* Pgp-dependent MDR phenotype, were established *in vitro* by exposure to daunorubicin. EPG85-257RNOV cells (designated EPG85-*BCRP*), exhibiting an atypical MDR phenotype accompanied by enhanced expression of *BCRP* [20], were established *in vitro* by treatment with mitoxantrone.

BCRP expression in EPG85-257RNOV, MCF-7/AdrVp and MCF-7/*BCRP* clone 8 has been previously confirmed using anti-*BCRP* mAb BXP-21 [25]. The *BCRP*-specific signal was highest in EPG85-*BCRP* cells, and lowest in MCF-7/*BCRP* clone 8. In the control lines EPG85-257P and MCF-7, *BCRP* was below the detection level. *MDR1* Pgp and MRP1 syntheses were proved to be comparable in all sublines except for *MDR1* Pgp in the *MDR1*-overexpressing cell line EPG85-257RDB [28].

Expression of *BCRP* in *anti-BCRP*-ribozyme-transfectant EPG85-257RNOV-RzB1 cells (designated EPG85-*anti-BCRP*-ribozyme-transfected) was inhibited by 50 % in comparison with *BCRP*-overexpressing EPG85-257RNOV cells as deduced from the mRNA level [30].

Cell lines were grown in a humidified atmosphere of 5 % CO_2 at 37 °C in Leibovitz L15 medium (BioWhittaker, Grand Island, NY, U.S.A.), containing 1 mM L-glutamine, 6.25 mg/ml fetuin, 80 IE/l insulin, 2.5 mg/ml transferrin, 1 g/l glucose, 1.1 g/l NaHCO_3 , 1 % minimal essential vitamins and 20 000 kIE/l trasylol, supplemented with 2.5 $\mu\text{g}/\text{ml}$ daunorubicin for EPG85-257 and with 0.2 $\mu\text{g}/\text{ml}$ mitoxantrone for EPG85-257RNOV cells. The drug-resistant MCF-7/AdrVp cell line was continuously maintained in the presence of 1 $\mu\text{g}/\text{ml}$ doxorubicin and 2.5 $\mu\text{g}/\text{ml}$ verapamil. In addition, the medium used for the cultivation of transfected MCF-7 sublines contained 400 $\mu\text{g}/\text{ml}$ G418. Before

transport assays, cells were grown in the absence of cytostatic drugs for 96 h on 12-well plates.

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 before the end of the incubation, propidium iodide (final concentration 1.9 μM) was added. Cells were then washed three times, detached from the dishes through 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. Regions were set to exclude subcellular particles, and only single cells were counted (10 000 cells/sample). Another gate excluded damaged cells with increased propidium iodide staining. Data were analysed by Becton Dickinson CellQuest software.

Measurement of C6-NBD-PS outward transport

To measure the transport of C6-NBD-PS from the cytoplasmic to the exoplasmic leaflet, labelling of the cytoplasmic leaflet is a prerequisite. As described previously [8], cells were labelled with 5 μM (EPG85-257 cell variants) or 10 μM (MCF-7 cell variants) C6-NBD-PS, and incubated at 20 °C for 30 min to allow inward movement of the NBD analogue. C6-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 ice-cold mPBS. At $t = 0$ min, ice-cold medium containing 2 % BSA and 5 mM di-isopropyl fluorophosphate in mPBS with or without inhibitor was immediately added to the cell dish for 10 min. To measure the C6-NBD-PS outward transport, the medium prewarmed to 37 °C was added to the dish, and incubated at 37 °C for 30 min (EPG85-257 cell variants) or 10 min (MCF-7 cell variants) respectively. Removal of the BSA-containing media and a second extraction with 2 % BSA in mPBS for 10 min terminated the incubations. Subsequently, lipid analysis was performed.

Measurement of C6-NBD-PC outward transport

Transport of newly synthesized C6-NBD-PC to the exoplasmic leaflet of the plasma membrane was assessed at 15 °C as described by van Helvoort et al. [4]. To this end, cells were incubated at 15 °C with 25 μM of the precursor analogue C6-NBD-PA in 1 % BSA in mPBS. 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.

Lipid analysis

After the incubations, the second BSA back-exchange medium was pooled with the first, and cells scraped into mPBS. For analysis of outward transport and of metabolic conversion of C6-NBD-PS, 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 10 min, the supernatant transferred to new glass tubes and dried, dissolved in chloroform/methanol (1:1) and quantified by TLC and fluorescence spectroscopy. For C6-NBD-PC

analysis, lipids from both cells and incubation media were extracted by the method of Bligh and Dyer [31], using 20 mM acetic acid in the aqueous phase. After two-dimensional separation [I: chloroform/methanol/25 % ammonium hydroxide (65:25:4, by vol.); II: 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 and quantified as described before [32].

Miscellaneous

Results are presented as means $\pm$ S.E.M.

RESULTS

BCRP-overexpressing cells expose more endogenous PS when compared with controls

Exposure of endogenous PS in EPG85-BCRP cells and EPG85-control cells was assessed by flow cytometry. Labelling with both FITC-Annexin V, a high-affinity PS-binding protein [33], and the membrane-impermeant nucleic acid stain propidium iodide, allows detection of PS present on the outer plasma membrane leaflet of cells with intact cell membranes. Figure 1(A) shows the result of a typical experiment, whereas the results of several experiments are summarized in Figure 1(B). Since variation between independent experiments was significant (ANOVA, $P < 0.05$), the FITC-Annexin V signal of the respective cell line in the absence of any inhibitor (see below) was set to 100 %. Binding of Annexin V was clearly enhanced for EPG85-BCRP cells when compared with control cells. As revealed by ANOVA of original data, the differences were highly significant ($P = 0.016$) and were not dependent on the experiment ($P = 0.638$). For EPG85-anti-BCRP-ribozyme-transfected cells, binding of Annexin V was intermediate between values of EPG85-BCRP and control cells (ANOVA, $P = 0.181$). Preincubation of EPG85-BCRP cells with the BCRP-inhibiting substance Tryprostatin A [25] decreased the level of Annexin V binding to that of control cells without inhibitor. Annexin V binding to control cells was not affected by treatment with Tryprostatin A (results not shown).

Outward transport of C6-NBD-PS is enhanced in BCRP-overexpressing cells

To assess whether an enhanced PS exposure could be associated with the modulation of transbilayer dynamics of lipids in BCRP-overexpressing cells, we investigated the outward transport of the lipid analogue C6-NBD-PS. To this end, the inner plasma membrane leaflet of EPG85-257 human gastric carcinoma cells was labelled with C6-NBD-PS, utilizing the presence of an aminophospholipid translocase activity (see [34,35] for reviews) in these cells, which we have described previously [8]. Subsequent to the labelling of the cytoplasmic side, cells were incubated for 30 min at 37 °C in BSA-containing medium. C6-NBD-PS and its metabolites, which appear on the cell surface during the incubation, were extracted by BSA. Thereafter, lipid analogues were extracted from the cells and the medium, and were quantified by fluorescence spectroscopy. Inward transport of the analogue, e.g. via the aminophospholipid translocase, does not interfere with the assay, since BSA rapidly extracts C6-NBD-phospholipid analogues [1,36].

In EPG85-BCRP cells, 46 % of C6-NBD-PS was extracted into the medium, significantly more than in control cells (34 %) (Figure 2A). In EPG85-anti-BCRP-ribozyme-transfected cells, the outward transport of the PS analogue (42 %) was lower in

Figure 1 Flow-cytometric analysis of PS exposure on the outer-membrane leaflet by FITC-Annexin V binding to EPG85-BCRP cells, EPG85-anti-BCRP-ribozyme-transfected cells and control cells

(A) Histograms show fluorescence intensity due to FITC-Annexin binding at the surface of EPG85-BCRP cells in the absence of Tryprostatin A (grey-filled graph) and after preincubation with 5 μ M Tryprostatin A for 30 min at 37 °C (white-filled graph). The histogram of control cells (EPG85-257P) was similar to that of BCRP-overexpressing cells in the presence of Tryprostatin A (not shown). (B) FITC-Annexin V binding to BCRP-overexpressing cells was decreased to the level of control cells after preincubation with 5 μ M Tryprostatin A. Cells were co-labelled with the membrane-impermeant nucleic acid stain propidium iodide. Cells showing increased propidium iodide staining were excluded. A total of 10 000 cells were counted for each sample. Data are the means $\pm$ S.E.M. of $n = 3$ or $n = 2$ (anti-BCRP-ribozyme-transfected cells) independent experiments of duplicate or triplicate measurements. For each experiment, the control was set to 100 % (see the Results section).

comparison with EPG85-BCRP cells, but still higher when compared with control cells.

Pretreatment with 10 μ M of the BCRP inhibiting Tryprostatin A [25] or the MDR-modulator GF120918 (inhibiting MDR1 Pgp and BCRP) [37] caused a significant decrease in the amount of C6-NBD-PS in the BSA-containing medium of EPG85-BCRP cells (Figure 3). Inhibitors did not significantly affect PS transport in control cells.

A significant part of internalized C6-NBD-PS is metabolized in EPG85-257 human gastric carcinoma cells [8], the major metabolic product being C6-NBD-PE (where PE stands for phosphatidylethanolamine). Approximately 45 and 55 % of C6-NBD-PS were converted into C6-NBD-PE in the BCRP-overexpressing and control EPG85-257 cells respectively. However,

Figure 2 Outward transport of C6-NBD-PS in *BCRP*-overexpressing cells

(A) EPG85-control cells (control), EPG85-*BCRP* cells (BCRP), EPG85-*anti-BCRP*-ribozyme-transfected cells (*anti-BCRP*-ribozyme); (B) MCF-7 cells (control) and MCF-7/AdrVp1000 (AdrVp); (C) MCF-7 control cells (control) and MCF-7/BCRP clone 8 cells (clone 8). Cells were labelled with C6-NBD-PS and incubated at 20 °C to allow intracellular accumulation of the NBD analogue. C6-NBD-PS remaining on the cell surface was then extracted twice by BSA. Cells were then incubated at 37 °C for 30 min (A) or 10 min (B, C). Fluorescent lipids (C6-NBD-PS and metabolites) appearing on the cell surface were extracted and quantified as described in the Experimental section. Data represent the means $\pm$ S.E.M. of at least $n = 2$ independent experiments performed in duplicate.

Figure 3 Inhibition of C6-NBD-PS outward transport in EPG85-*BCRP* cells (BCRP), EPG85-*MDR1 Pgp* cells (MDR1 Pgp) and EPG85 control cells (control) at 37 °C by Tryprostatin A and GF120918

Cells were preincubated for 10 min on ice in the absence of BCRP inhibitors (—) and in the presence of 10 μ M Tryprostatin A (T) or GF120918 (G), labelled with 5 μ M C6-NBD-PS and were incubated again at 20 °C for 30 min to allow intracellular accumulation of the NBD analogue. C6-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. Cells were then incubated at 37 °C for 30 min. Fluorescent lipids (C6-NBD-PS and metabolites) appearing on the cell surface were extracted and determined as described in the Experimental section. Data represent the means $\pm$ S.E.M. of at least $n = 2$ independent experiments performed in duplicate. Data were analysed by a two-way ANOVA Tukey test (Jandel SigmaStat 2.0) considering treatment and day of experiment. *Significant difference compared with the respective cells without inhibitor pretreatment ($P < 0.05$).

we did not observe an enhanced outward transport of C6-NBD-PE in EPG85-*BCRP* cells (results not shown).

When C6-NBD-PS experiments were performed at 15 °C, outward transport of the PS analogue was too low to observe clear differences in transport between EPG85-257 control and EPG85-*BCRP* cells (results not shown).

To determine whether Tryprostatin A and GF120918 may also inhibit lipid-transport activity of MDR1 Pgp, we measured the outward transport of C6-NBD-PS in the *MDR1*-overexpressing subline (EPG85-MDR1 Pgp) (Figure 3). Whereas GF120918 caused a significant decrease in the outward transport of the PS analogue, Tryprostatin A did not affect the MDR1 Pgp lipid-

transport activity. This supports our previous finding about the strong inhibition of BCRP by Tryprostatin A [25], whereas GF120918 inhibits ABC transporters less specifically.

A role of BCRP in lipid transport was sustained by the enhanced outward transport of C6-NBD-PS in two different *BCRP*-overexpressing breast cancer cell lines, MCF-7/AdrVp and MCF-7/BCRP clone 8 (Figures 2B and 2C). The amount of extractable analogues was higher by 1.69 ± 0.05 and 1.26 ± 0.04 for MCF-7/AdrVp and MCF-7/BCRP cells respectively in comparison with control cells. In both these cases, incubation with Tryprostatin A abolished the difference between *BCRP*-overexpressing and control MCF-7 cells. The ratios of extracted analogues between *BCRP*-overexpressing cells and control cells in the presence of Tryprostatin A were 0.98 ± 0.05 and 0.99 ± 0.04 for MCF-7/AdrVp and MCF-7/BCRP cells respectively ($n = 2$ independent experiments in duplicate). Owing to high outward transport of the PS analogue in MCF-7 control cells, transport was measured after a 10 min incubation of the cells at 37 °C in the presence of BSA.

Outward transport of C6-NBD-PC is enhanced in *BCRP*-overexpressing EPG85-257 gastric carcinoma cell variants

To investigate whether transbilayer dynamics of other lipids might also be affected in *BCRP*-overexpressing cells, C6-NBD-PC outward transport was examined in EPG85-*BCRP* and control cells after incubation with C6-NBD-PA. C6-NBD-PA is partially converted into C6-NBD-diacylglycerol [38], which rapidly crosses the plasma membrane and becomes available for intracellular synthesis of C6-NBD-PC and C6-NBD-PE. When transport of the newly synthesized analogues to the cell surface was measured at 15 °C, vesicular traffic could be excluded, as it would be blocked at this temperature [39].

After 180 min at 15 °C, 44% of C6-NBD-PC was extracted in the BSA medium of EPG85-*BCRP* cells, whereas the BSA medium of control cells contained only 24% of C6-NBD-PC (Figure 4). To verify that the enhanced outward transport of C6-NBD-PC was due to overexpression of *BCRP*, we investigated outward transport in EPG85-*anti-BCRP*-ribozyme-transfected cells. Indeed, we found that the outward transport of C6-NBD-PC in *anti-BCRP*-transfected cells (35%) to be significantly decreased in comparison with *BCRP*-overexpressing cells, whereas it remained higher than in controls.

Figure 4 C6-NBD-PC outward transport in EPG85-BCRP gastric carcinoma cells (EPG85-257RNOV), EPG85-anti-BCRP-ribozyme-transfected (EPG85-257RNOV-RzB1) and EPG85 control cells (EPG85-257P)

Cells were incubated with 25 μ M C6-NBD-PA for 180 min at 15 °C. To trap the fluorescent lipid products (C6-NBD-PC) appearing on the cell surface in the extracellular medium, incubation was performed in the presence of 1% BSA. Data represent the means $\pm$ S.E.M. of at least $n = 2$ independent experiments performed in duplicate.

In all three EPG85-257 sublines, synthesis of C6-NBD-PE from the respective PA precursor was too low for the determination of specific outward transport.

DISCUSSION

Previously, it has been shown that some half-size transporters are functionally involved in phospholipid and cholesterol trafficking in macrophages [16,40]. However, although several full-size ABC transporters have been shown to mediate transport of various lipids from the cytoplasmic to the exoplasmic leaflet of the plasma membrane, it is not known whether half-size ABC proteins may have similar lipid-transport activity.

In the present study, we found that the human half-size ABC transporter BCRP promotes the exofacial exposure of lipid analogues and endogenous PS. To study the exposure of endogenous PS at the cell surface, binding of fluorescent Annexin V was assessed in EPG85-257 human gastric carcinoma cells. In EPG85-BCRP cells, binding of this PS probe to the cell surface was significantly higher than for control cells, whereas binding to EPG85-anti-BCRP-ribozyme-transfected cells with a lower expression of BCRP was only slightly enhanced with respect to control cells. In the presence of the inhibitor Tryprostatin A, binding of Annexin V to EPG85-BCRP cells was decreased to the level found for control cells. This suggests that translocation of endogenous PS to the outer plasma membrane leaflet is affected by expression of BCRP.

On labelling of the cytoplasmic leaflet with the fluorescent lipid analogue C6-NBD-PS [8], BCRP-overexpressing EPG85-257 cells showed significantly increased outward transport of this lipid analogue compared with controls. Using precursor labelling, outward transport of C6-NBD-PC was also found to be increased in these cells. Transport of the PC and PS analogues was decreased in EPG85-anti-BCRP-ribozyme-transfected cells with respect to EPG85-BCRP cells, but was still higher than in control cells, supporting an influence of BCRP on the transport of C6-NBD-PC and C6-NBD-PS to the cell surface. Consistent with this, outward transport was sensitive to BCRP inhibitors Tryprostatin A and GF120918 as shown for the PS analogue.

Increased outward transport of C6-NBD-PS in BCRP-overexpressing cells could be confirmed in the human breast cancer cell lines MCF-7/AdrVp and MCF-7/BCRP clone 8 using BCRP

inhibitors. Although the EPG85-BCRP cells showed a more pronounced BCRP protein expression level than the cell line MCF-7/AdrVp [25], the outward redistributed fraction of C6-NBD-PS was higher for MCF-7/AdrVp cells than for EPG85-BCRP cells. In comparison with the respective control cells, the amount of extracted analogues was approx. 1.7- and 1.4-fold enhanced for MCF-7/AdrVp and EPG85-BCRP cells respectively. This phenomenon may be explained by the polymorphism at position 482 of the amino acid sequence. BCRP protein synthesized in the cell line MCF-7/AdrVp contains a threonine at this site [41], whereas EPG85-257RNOV cells express a wild-type BCRP with arginine at this position (E. L. Volk and E. Schneider, personal communication).

As deduced from experiments at 15 and 37 °C, the affinity for and/or transport activity of BCRP appears to differ between both analogues depending on the temperature. Whereas both lipid analogues were found to be transported at 37 °C in EPG85-257 cells, BCRP-mediated outward transport was observed for C6-NBD-PC, but not for C6-NBD-PS, when experiments were performed at 15 °C.

Amongst several full-size ABC transporters, MDR1 Pgp, MDR2/3 Pgp, MRP1 and ABCA1 have been found to mediate lipid transport [4,10–12,14,15]. We could rule out that the established lipid-transport activity of MDR1 Pgp is responsible for the outward directed transport of lipid analogues in BCRP-overexpressing cells seen here. Furthermore, using inhibitors for MRP1 and ABCA1, MK 571 and glyburide, we have shown previously that outward transport of lipid analogues observed with the present method cannot be ascribed to the activity of MRP1 or ABCA1 in EPG85-257 control cells as well as in EPG85-257 cells overexpressing ABC transporters as MDR1 Pgp [8].

Thus BCRP is directly or indirectly involved in the outward-directed transport of several fluorescent lipid analogues and of endogenous PS.

In contrast with the bacterial half-size ABC transporter LmrA of *Lactococcus lactis*, which mediates transbilayer movement of C6-NBD-PE and not C6-NBD-PC [42], we did not observe BCRP-dependent outward movement of C6-NBD-PE formed intracellularly as a metabolic product of C6-NBD-PS. This may suggest differences in the substrate specificity among half-size transporters. Nevertheless, the enhanced outward movement of two different phospholipid analogues, C6-NBD-PC and C6-NBD-PS, in BCRP-overexpressing cells indicates that the BCRP-dependent lipid transport might be of relatively broad lipid specificity. The latter is supported by observations of Litman et al. [22] suggesting that a fluorescent analogue of ceramide (BODIPY C5-ceramide) might be a substrate for BCRP, based on the intracellular distribution of the analogue in a BCRP-expressing breast-cancer cell line. Moreover, very recently, transport of the sterol oestradiol by BCRP expressed in *L. lactis* has been reported [43]. Broad specificity towards lipid transport substrates may not be surprising, and has been documented for the full-size ABC transporter MDR1 Pgp.

In mammalian cells, PS is restricted to the inner leaflet of the plasma membrane [44]. This asymmetric lipid arrangement is maintained by the activity of an ATP-dependent aminophospholipid translocase, rapidly pumping aminophospholipids as PS from the exoplasmic to the cytoplasmic leaflet of the plasma membrane [35,36,45]. Alterations of this non-random lipid distribution may have pivotal physiological consequences, such as removal of apoptotic cells by macrophages upon surface exposure of PS [46]. Increased exposure of endogenous PS has been reported previously for tumour cells versus non-tumourigenic cells [47] and for MDR1-overexpressing EPG85-257RDB cells versus controls [8].

Further studies on lipid transport by BCRP are warranted. In particular, it has to be elucidated whether BCRP forms homodimers [48,49], or rather heterodimers with other ABCG family members, to mediate lipid transport. Furthermore, additional information is needed to determine whether the lipid-transport activity of BCRP is comparable with that of MDR1 Pgp, and the role it might play in BCRP-mediated MDR.

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