Multidrug-resistance P-glycoprotein (MDR1) secretes platelet-activating factor

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The human multidrug-resistance (MDR1) P-glycoprotein (Pgp) is an ATP-binding-cassette transporter (ABCB1) that is ubiquitously expressed. Often its concentration is high in the plasma membrane of cancer cells, where it causes multidrug resistance by pumping lipophilic drugs out of the cell. In addition, MDR1 Pgp can transport analogues of membrane lipids with shortened acyl chains across the plasma membrane. We studied a role for MDR1 Pgp in transport to the cell surface of the signal-transduction molecule platelet-activating factor (PAF). PAF is the natural short-chain phospholipid 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine. [¹⁴C]PAF synthesized intracellularly from exogenous alkylacetyl-glycerol and [¹⁴C]choline became accessible to albumin in the extracellular medium of pig kidney epithelial LLC-PK1 cells in the absence of vesicular transport. Its

translocation across the apical membrane was greatly stimulated by the expression of MDR1 Pgp, and inhibited by the MDR1 inhibitors PSC833 and cyclosporin A. Basolateral translocation was not stimulated by expression of the basolateral drug transporter MRP1 (ABCC1). It was insensitive to the MRP1 inhibitor indomethacin and to depletion of GSH which is required for MRP1 activity. While efficient transport of PAF across the apical plasma membrane may be physiologically relevant in MDR1-expressing epithelia, PAF secretion in multidrug-resistant tumours may stimulate angiogenesis and thereby tumour growth.

Key words: apical membrane, epithelial cells, ether-lipid, inflammatory response, lipid mediator, lipid translocator.

INTRODUCTION

Multidrug resistance (reviewed in [1]) occurs when cancer cells resist chemotherapy by removing a broad range of lipophilic drugs from the cell interior. These cells express high levels of the multidrug-resistance transporters MDR1 P-glycoprotein (Pgp), ABCB1, or MRP1, ABCC1, which pump the drugs from the cytosol across the plasma membrane. Both proteins belong to the ATP-binding-cassette (ABC) transporters [2], but display only 14% amino acid identity [3]. The human MDR3 Pgp, ABCB4, shows 75% identity with MDR1 Pgp [3]. It displays some drug-pumping activity [4], but a role in multidrug resistance has not been documented. Whereas MDR1 Pgp is expressed by most cell types, including fibroblasts, expression of MDR3 Pgp is essentially limited to the bile canalicular membrane of hepatocytes [5,6].

The major physiological function of MDR3 Pgp is the delivery of the phospholipid phosphatidylcholine (PC) into bile [6]. The notion that MDR3 Pgp is an actual PC translocator is supported by an observed increase in PC transport to the surface of MDR3-transfected cells [5], and by the observation that it can translocate a PC analogue with a short fluorescent acyl chain *N*-6[7-nitro-2,1,3-benzoxadiazol-4-yl]amino-hexanoyl (C₆-NBD)-PC, but not the corresponding phosphatidylethanolamine, across the membrane [7,8]. Unexpectedly [7], the multidrug transporters MDR1 Pgp and MRP1 also transport short-chain analogues of membrane lipids across the plasma membrane (reviewed in [9]). MDR1 Pgp mediated the transbilayer movement of C₆-NBD-, C₆- and C₈-short-chain analogues of the diacylglycerol lipids PC

and phosphatidylethanolamine and of the sphingolipids glucosylceramide (GlcCer) and sphingomyelin (SM) [8,10]. MRP1 translocated C₆-NBD-GlcCer and -SM, and this activity, like its drug transport function, depended on cytosolic GSH [11]. While no transport of C₆-NBD-PC or of C₈-NBD-PC was observed in one study [11], MRP1 has been reported to transport C₆-NBD-PC and -phosphatidylserine in another [12], and it has been suggested to contribute to the transbilayer distribution of natural lipids in the erythrocyte membrane [13].

Platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a naturally occurring short-chain PC. Because MDR1 Pgp recognizes short-chain analogues of PC and is expressed in the plasma membrane of virtually every cell, we have suggested that MDR1 Pgp could be responsible for translocating PAF across the plasma membrane [8]. First, experimental support for this notion has been obtained in a special cell system, mesangial cells activated by a Ca²⁺ ionophore ([14]; see the Discussion). PAF is a potent bioactive lipid that is synthesized by a broad range of cells, including circulating inflammatory cells, endothelial cells and epithelial cells (e.g. [15]). It is found in a variety of tissues [16,17], in plasma [18], but also in other extracellular fluids, such as saliva [19] and urine [20]. PAF has a diverse array of biological effects, ranging from activation of inflammatory cells to vascular and other physiological effects. In addition, PAF is involved in various pathological conditions, such as angiogenesis in breast cancers [21], metastasis, shock, sepsis and multiple organ failure (reviewed in [22]).

PAF is synthesized by two intracellular pathways (reviewed in [23]). The ‘*de novo*’ pathway, presumably in the cytosolic leaflet

Abbreviations used: AAG, 1-*O*-alkyl-2-acetyl-*sn*-glycerol; BFA, Brefeldin A; BSO, D,L-butathione-(*S,R*)-sulphoximine; C₆-NBD, *N*-6[7-nitro-2,1,3-benzoxadiazol-4-yl]amino-hexanoyl; GlcCer, glucosylceramide; MDR, multidrug resistance; PAF, platelet-activating factor; PC, phosphatidylcholine; Pgp, P-glycoprotein; SM, sphingomyelin; HBSS, Hanks balanced salt solution.

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of the ER [24], is analogous to the conventional PC biosynthesis and makes 1-*O*-alkyl-2-acetyl-*sn*-glycerol (AAG) as a precursor. This is converted into PAF by a unique CDP-choline:AAG-cholinephosphotransferase [25]. This pathway has been held responsible for constitutive PAF synthesis. In contrast, the 'remodelling' pathway accounts for stimulated PAF synthesis. It involves a phospholipase A₂-catalysed release of arachidonate from the *sn*-2 position of an alkyl-PC, yielding the PAF-precursor lyso-PAF and the eicosanoid precursor arachidonic acid. Lyso-PAF is converted into PAF by the esterification with acetate, which is catalysed by a specific acetyl-CoA:lyso-PAF acetyltransferase [26].

A key role in PAF action is the binding to a unique G-protein-coupled seven transmembrane receptor in the extracellular leaflet of the plasma membrane (reviewed in [23]). In order to bind to the PAF receptor in a target cell, PAF must move from its site of synthesis in the cytosol to the exoplasmic leaflet of the plasma membrane. Once present on the surface, it has been suggested to interact with the PAF receptor on a neighbouring cell via a high-affinity-binding site on the surface of the signalling cell [23]. However, to reach the actual binding site in the receptor, PAF must diffuse as a monomer across the intercellular space. Either way, PAF must be translocated from the cytosolic to the non-cytosolic leaflet of a cellular membrane. This might occur in the ER membrane, after which PAF is transported to the exoplasmic leaflet of the plasma membrane on the luminal side of transport vesicles. However, using a direct assay to measure transport of radiolabelled PAF into the medium, we provide here evidence for an alternative mechanism, where PAF can be translocated directly across the plasma membrane by MDR1 Pgp.

MATERIALS AND METHODS

Materials

Brefeldin A (BFA), D,L-buthionine-(S,R)-sulphoximine (BSO), BSA fraction V, cyclosporin A, GSH-ethyl ester (reduced), and phospholipase C, type IX from *Bacillus cereus*, were from Sigma. [Methyl-¹⁴C]choline chloride (2 GBq/mmol) and indomethacin were purchased from ICN, [¹⁴C]serine (5.59 GBq/mmol) was from Amersham International; PSC833 was kindly provided by Novartis (Basel, Switzerland). 6-[*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl]sphingosine (C₆-NBD-ceramide) was from Molecular Probes. 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) was from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Pefabloc, organic solvents and silica TLC plates were from Merck. Cell culture media were from Gibco BRL. Sep-pak C18 reversed-phase columns were purchased from Waters (Milford, MA, U.S.A.).

Synthesis of AAG

PAF (1.25 mg) was incubated in 2.5 ml of 6.25 mM CaCl₂, 100 mM Tris/HCl, pH 6.3, containing 5.5 units of phospholipase C, for 4 h at 37 °C. After the incubation, lipids were extracted and separated by TLC as for PAF (see below). PAF and AAG were visualized on silica TLC-plates by iodine-staining. AAG was scraped and recovered from silica by extraction for 1 h with 10 ml chloroform/methanol (2:1, v/v). Under these conditions, PAF was completely converted into AAG.

Cell culture

LLC-PK1 cells transfected with human MDR1 cDNA (LLC-PK1/MDR1) were obtained from A. Schinkel [27]. LLC-PK1 pig kidney epithelial cells transfected with human MRP1 cDNA (LLC-PK1/MRP1) were kindly provided by R. Evers [28].

Cells were cultured (mycoplasma-free) in M199 medium supplemented with 10% fetal calf serum, as described in [11]. For experiments, 2 × 10⁶ cells were seeded on 4.7 cm² filters glued to the bottom of plastic rings (Transwell, 0.4 µm pore diameter; Costar, Cambridge, MA, U.S.A.) and were grown as monolayers for 4 days. Inhibitors were added to the medium 20 min prior to the transport incubation from 1000 × stocks in ethanol. They remained present throughout the transport incubation. GSH depletion was performed as before [11]. Apical medium was 1 ml, basolateral medium was 2 ml throughout.

Assay for lipid transport

AAG

Cell monolayers on filters were preincubated in Hanks balanced salt solution (HBSS) without bicarbonate, 10 mM Hepes, pH 7.4, (HBSS') containing 0.1% w/v BSA (HBSS' + 0.1% BSA) for 1 h at 37 °C to reduce intracellular choline pools. AAG (25 µM final concentration) was complexed to BSA by injection of 10 µl of an ethanolic solution of the precursor into 3 ml of HBSS' + 0.1% BSA. [¹⁴C]Choline (15 KBq/ml) was added and cells were incubated in this medium for 1 h at 37 °C. Subsequently, the apical and basal medium were collected and replaced with HBSS' containing 1% BSA (HBSS' + 1% BSA), and incubated for 1 h at 37 °C in order to allow further synthesis and transport, and depletion of newly synthesized PAF from the cell surface. Apical medium and basal medium were collected, the cells were washed in HBSS' + 1% BSA for 30 min on ice, and the corresponding media were pooled. Pefabloc (150 µM) was present in all media in order to inhibit PAF hydrolysis [29]. The lipids were extracted from media plus wash solutions and from filters (cells), and the radiolabelled lipids were quantitatively analysed (see below). Transport of each lipid class to the apical and basolateral cell surface was calculated as the percentage of that lipid being recovered in the apical and basal medium respectively.

C₆-NBD-ceramide

C₆-NBD-ceramide was complexed to BSA by injection of 10 µl of an ethanolic ceramide stock into 3 ml of HBSS' + 1% BSA (final concentration 5 µM). It was added to both sides of epithelial monolayers on filters for 3 h at 15 °C, as described previously [8]. During the incubation, newly synthesized short-chain GlcCer and SM appearing on the cell surface were depleted from the surface into the medium by BSA. After 3 h the apical medium and basal medium were collected and the cells were washed in HBSS' + 1% BSA for 30 min on ice. The lipids were extracted from media plus wash solutions and from filters, and quantitatively analysed (see below). Transport was calculated as above.

Extraction of PAF from cell homogenates by BSA

Confluent cell monolayers in 3 cm dishes were incubated with AAG and [¹⁴C]choline as described for cells on filters. After 1 h the cells were cooled on ice, scraped in HBSS' without BSA, and disrupted by 20 passages through a 23-gauge needle. The homogenate was incubated in HBSS' with various concentrations of BSA, as indicated in Table 1, for 30 min on ice. After 30 min the solution (3 ml) was layered on top of 2 ml of 0.4 M sucrose in HBSS' and spun at 4 °C for 1 h at 175 000 *g* in a SW-51 rotor. Fractions of 3 ml (BSA fraction), 1 ml (interphase) and 1 ml (membrane fraction) were collected starting from the top, and lipids were extracted and analysed as described below.

Table 1 Extraction of PAF by BSA from membranes

Confluent LLC-PK1 cells in dishes were incubated with 25 μ M AAG and 15 kBq [14 C]choline for 1 h at 37 °C. Cells were scraped and a homogenate was prepared as described in the Materials and methods section. The homogenate was incubated with different concentrations of BSA (0–2%, w/v) for 30 min on ice. After the incubation, membranes were separated from the BSA fraction by pelleting through a sucrose cushion. Lipids from each fraction were extracted and quantified. Data are expressed as the percentage of PAF (a) or PC (b) recovered from each fraction. PAF synthesis was typically 15 Bq/dish and PC synthesis was 450 Bq/dish. Data represent the means $\pm$ S.D. of two independent experiments ($n = 3$ –4).

(a)			
BSA (%)	[14 C]PAF (%)		
	BSA fraction	Interphase	Membrane fraction
0	4 $\pm$ 1	9 $\pm$ 2	87 $\pm$ 2
0.1	66 $\pm$ 3	21 $\pm$ 4	13 $\pm$ 2
1	87 $\pm$ 5	4 $\pm$ 1	9 $\pm$ 1
2	87 $\pm$ 3	5 $\pm$ 3	8 $\pm$ 1
(b)			
BSA (%)	[14 C]PC (%)		
	BSA fraction	Interphase	Membrane fraction
2	1 $\pm$ 1	0	99 $\pm$ 1

Sphingomyelinase assay

Delivery of SM to the cell surface was assayed by a sphingomyelinase assay [30]. Cellular SM was labelled at 37 °C for 3 h with 1 μ Ci [14 C]serine/ml of HBSS', after which the cells were incubated for 1 h at 15 °C with or without 1 unit of exogenous sphingomyelinase/ml HBSS'. The percentage SM that was hydrolysed is used as a measure of SM transport to the cell surface.

Lipid analysis

AAG

Lipids were extracted from cells and media by a two-phase Bligh and Dyer extraction as described in [31]. The organic phase was dried under N_2 . Lipids were dissolved in 20 μ l of chloroform/methanol (1:2, v/v) and applied to a 2 ml Sep-pak C18 reversed-phase column, which was then air-dried for 5 min. The water phase was also applied to the Sep-pak column in order to recover PAF present in the water phase. Subsequently, lipids were eluted with 8.5 ml of water/methanol (1:7, v/v). This allows for the separation of long-chain lipids, which are retained on the column and short-chain lipids, which are eluted. The collected eluate was dried under N_2 , and the lipids were separated by one-dimensional TLC using chloroform/methanol/water (65:35:5, by vol.). Radiolabelled spots were detected by exposure to phosphor-storage screens and read out on a Storm phosphorimager (Amersham). Spots were identified by comparison with standards and quantified using the Image Quant Software Package (Molecular Dynamics).

C₆-NBD-ceramide

Lipids were extracted from cells and media by a two-phase extraction and lipid products were separated in two dimensions by TLC as described previously [31]. Fluorescent spots were quantified using a Storm Fluorimager (blue fluorescence) and Image Quant.

RESULTS

Synthesis of PAF from exogenous AAG in LLC-PK1 cells

To generate intracellular PAF in LLC-PK1 cells, we adapted an approach to induce synthesis of short-chain PC [32] and incubated cells with the PAF-precursor AAG and [14 C]choline. Because of its short acyl chain AAG should diffuse across aqueous phases, and because its headgroup is not very polar it should rapidly diffuse over membranes. Thus AAG should reach the cytosolic leaflet of the ER membrane, where it should receive [14 C]phosphocholine from CDP-[14 C]choline by means of the cholinephosphotransferase in the PC synthetic pathway yielding radiolabelled PAF. When after 1 h at 37 °C the lipids were extracted from cells and media, fractionated on a Sep-pak C18 column and separated by TLC, PAF synthesis was indeed observed in LLC-PK1 cells, but only when AAG was present in the medium (Figure 1). Increasing the BSA concentration from 0.1% to 1% completely abrogated PAF synthesis (results not shown), probably by reducing the efficiency of AAG uptake. BSA (1%) did not inhibit the uptake of C₆-NBD-phosphatidic acid used as a precursor in C₆-NBD-PC transport experiments [8]. The data show that exogenous AAG triggered PAF synthesis via the *de novo* pathway. PAF synthesis in cells overexpressing MDR1 or MRP1 was similar in control cells. It seems unlikely that AAG is a substrate for MDR1 or MRP1, since, in that case, one might have expected reduced uptake into the cell and a reduced incorporation into PAF. This was not observed (Figure 2).

Extraction of PAF from cellular membranes by BSA

Subsequently, we defined conditions where BSA can be used to monitor the presence of PAF on the epithelial cell surface by quantitatively extracting PAF into the medium [8,33]. LLC-PK1 cells were labelled for 1 h at 37 °C with AAG and [14 C]choline, homogenized, and incubated with BSA (0–2%) for 30 min on ice. Subsequently, membranes were separated from the BSA-containing medium (and cytosol) by pelleting through sucrose. The BSA-, interphase-, and membrane-fractions were collected and analysed for lipid content. Without BSA, approx. 90% of PAF was in the membrane fraction (Table 1). However, 1% BSA in the incubation medium released close to 90% of PAF.

Figure 1 Synthesis of PAF in LLC-PK1 cells

After choline depletion for 1 h, LLC-PK1 cell monolayers were incubated with or without AAG and [14 C]choline for 1 h at 37 °C. After separation of single-chain and short-chain phospholipids from phospholipids containing two long chains on a reversed-phase column, the former lipids were analysed on a TLC plate by comparison with choline-lipid standards ($R_{f,PAF} = 0.24$). PAF synthesis from 15 kBq [14 C]choline was 13 ± 2 Bq ($n = 16$). PC synthesis was 400 ± 40 Bq (means $\pm$ S.D.; $n = 6$).

Figure 2 Transport of newly synthesized PAF to the apical and basolateral surface of LLC-PK1, LLC-PK1/MDR1 and LLC-PK1/MRP1 cells

Cell monolayers on filters were incubated with AAG and [14 C]choline at 37 °C. After 1 h media were collected and replaced for an additional hour at 37 °C by media containing 1% BSA, which was followed by a 30 min wash with 1% BSA on ice. Apical and basolateral media and cells were collected and their lipids were analysed as described in the Materials and methods section. Synthesis of PAF was 13 Bq for the LLC-PK1, 14 Bq for LLC-PK1/MDR1 and 15 Bq for LLC-PK1/MRP1 cells. Data represent the means of 11 independent experiments (with S.D. = 1; n = 10–22). Assuming a similar specific activity for the choline in the two cell types, the pools of PAF were of comparable size.

The fraction of PAF remaining with the membranes (4%) may seem unexpectedly low, since part of PAF might have been protected on the luminal side of closed vesicles. However, it unequivocally shows that PAF is efficiently extracted from membranes by BSA, even at 0 °C, unlike other lipids such as natural long-chain PC that remained fully membrane-associated. The specific extractability of PAF from membranes provides the possibility to study PAF transport to the cell surface by BSA extraction into the medium. Increasing the BSA concentration to 2% did not further increase the extraction efficiency, suggesting that cell surface PAF is extracted quantitatively with 1% BSA, a concentration that was used throughout this study.

Increased transport of PAF to the surface of LLC-PK1/MDR1 cells

In order to study possible roles for MDR1 Pgp and MRP1 in the transport of PAF to the cell surface, we made use of transfected LLC-PK1 cells expressing high levels of MDR1 or MRP1. Confluent monolayers of LLC-PK1, LLC-PK1/MDR1 and LLC-PK1/MRP1 cells were incubated with AAG and [14 C]choline for 1 h at 37 °C. To extract PAF from the outer leaflet of the plasma membrane, the incubation was continued for 1 h in 1% BSA, after which the cells were washed with BSA for 0.5 h on ice. Lipids were extracted from the pooled apical media, the basolateral media and the cells, separated on a C18 column and by TLC, and PAF in each fraction was quantified. In the non-transfected LLC-PK1 cells 15% of total newly synthesized PAF was recovered from the apical medium, and 21% from the basolateral medium (Figure 2). Thus whereas most (64%) of the newly synthesized PAF was present in the cells, a significant fraction had reached the outside medium. The same situation was observed for the LLC-PK1/MRP1 cells (Figure 2). However, apical transport of PAF increased from 15% in LLC-PK1 cells to 63% in the LLC-PK1/MDR1 cells. Basolateral transport was somewhat reduced, showing that the

Figure 3 Effect of MDR1 and MRP1 inhibitors on PAF transport in LLC-PK1 and LLC-PK1/MDR1 cells

Monolayers of LLC-PK1 and LLC-PK1/MDR1 cells were pre-incubated for 10 min at 37 °C without inhibitor, or with 5 μ M PSC833, 10 μ M cyclosporin A or 20 μ M indomethacin, followed by a 2 h transport assay in the presence of the inhibitor. Transport of PAF to the apical or basolateral surface of the cells was assessed as described in the Materials and methods section. The inhibitors had no effect on PAF synthesis (n = 2–12).

increase in PAF transport was specific for the apical membrane where MDR1 Pgp is expressed [27]. No PAF was found in apical medium without BSA (results not shown). These data suggest that MDR1 Pgp can transport PAF across the apical cell membrane.

Reduction of PAF transport by MDR1 inhibitors

As an independent test for an involvement of MDR1 Pgp, the PAF-transport experiments were repeated in the presence of the MDR1 inhibitors PSC833 and cyclosporin A [8,34,35], or of indomethacin, an inhibitor of MRPs but not of MDR1 Pgp ([11]; Figure 3). The MDR1 Pgp inhibitors reduced transport of PAF into the apical medium of LLC-PK1/MDR1 cells by more than 80%, whereas PAF transport to the basolateral medium was unaffected. Interestingly, also in the control cells, transport of PAF to the apical medium was inhibited by the MDR1 drugs (Figure 3), suggesting that translocation of PAF in these cells occurred by an endogenous transporter with similar properties to MDR1 Pgp, probably the pig *mdr1* Pgp. Although a small increase in transport of PAF to the basolateral surface of LLC-PK1/MRP1 cells was observed (Figure 2), indomethacin did not inhibit transport of PAF to either apical or basolateral membrane. MRP1 was fully active, as shown in a control experiment using C_6 -NBD-ceramide carried out as described in the Materials and methods section. Indomethacin inhibited transport of the MRP1-substrate C_6 -NBD-GlcCer to the basolateral surface from $34 \pm 5\%$ to $11 \pm 3\%$ (means $\pm$ S.D., n = 4) without inhibition of apical transport and without effect on the transport of C_6 -NBD-SM. In addition, depletion of GSH, which is required for C_6 -NBD-lipid transport by MRP1 in LLC-PK1/MRP1 cells [11], for 24 h by 25 μ M BSO inhibited basolateral transport of C_6 -NBD-GlcCer by more than 60%, from $34 \pm 5\%$ in control cells to $14 \pm 2\%$ (n = 4). However, transport of PAF to either apical or basolateral membrane was not affected. Together, these data show that PAF transport to the cell surface correlated with the activity of MDR1 Pgp, but not with the activity of MRP1.

Figure 4 PAF transport in the absence of vesicular traffic

Monolayers of LLC-PK1/MDR1 cells were pre-incubated for 20 min at 37 °C with or without 1 µg/ml BFA. Transport of PAF (open bars) to the apical and basolateral cell surface in the presence or absence of BFA was measured as in Figure 2. Transport of SM (hatched bars) to the surface was determined after 2 h at 37 °C by measuring its accessibility to sphingomyelinase at 15 °C (see the Materials and methods section). PAF synthesis in the absence of BFA was typically 14 ± 2 Bq, whereas synthesis in the presence of BFA was somewhat lower (11 ± 1 Bq; $n = 8$).

MDR1 translocates PAF at the plasma membrane

The appearance of PAF on the surface of LLC-PK1/MDR1 cells may reflect the direct translocation of PAF across the plasma membrane bilayer by MDR1 Pgp. Alternatively, PAF might have been translocated by MDR1 Pgp across the membrane of an intracellular organelle, followed by transport to the cell surface in the luminal leaflet of transport vesicles and delivery to the outer leaflet of the plasma membrane by fusion. In order to discriminate between these possibilities, PAF transport was determined in the absence of vesicular traffic. For this, LLC-PK1/MDR1 cells were pretreated with 1 µg/ml BFA. BFA induces retrograde transport of Golgi enzymes to the ER and inhibits vesicular transport from this mixed compartment [36]. For comparison, a sphingomyelinase assay was used to determine the transport of SM to the plasma membrane. SM is synthesized in the luminal leaflet of the Golgi and is transported by vesicles [37]. SM transport to both the apical (25–30 %) and the basolateral plasma membrane (40–45 %) was almost completely inhibited by BFA, whereas BFA did not alter the efficiency of transport of newly synthesized PAF to the cell surface (Figure 4). Both in the presence and in the absence of BFA, PSC833 inhibited PAF transport by more than 80 %. Thus in the absence of vesicular transport PAF reached the outer leaflet of the apical plasma membrane by a mechanism sensitive to an MDR1 Pgp inhibitor. This shows that PAF was translocated across the plasma membrane. In addition, transport of PAF from the cytosolic leaflet of the ER membrane to the inner leaflet of the plasma membrane did not depend on vesicular transport.

DISCUSSION

PAF synthesis and transport

The signalling lipid PAF mediates a range of physiological processes. Primarily, it activates target cells by binding to PAF receptors on their surface. Possible intracellular roles for

PAF have not been rigorously characterized [23]. To learn how PAF signalling between cells is regulated, we must understand the processes that determine the concentration of PAF at the surface of the target cell. These include: synthesis in the signalling cell, transport to the outer leaflet of the plasma membrane and diffusion across the aqueous phase to the target cell, and, finally, hydrolysis. PAF is hydrolysed at various locations by two families of PAF acetylhydrolases [38].

A constitutive low level of PAF is synthesized by the *de novo* pathway and is thought to contribute to normal cellular function. The process may be regulated at the level of either lipid synthesis or at the dedicated lysoglycerophosphate acetyltransferase and cholinephosphotransferase [25]. In contrast, the stimulated PAF synthesis via the remodelling pathway [26] is induced by cell-specific stimuli such as the inflammatory mediator thrombin [23]. It can be triggered non-specifically by Ca^{2+} ionophores. After synthesis on the cytosolic side of the ER [24], PAF can, in principle, reach the cell surface by translocation across the ER membrane followed by vesicular traffic. However, the fact that PAF transport is not inhibited by BFA, while SM transport via vesicles is virtually abolished (Figure 4), shows that PAF has free access to the cytosolic surface of the plasma membrane by a vesicle-independent pathway, probably as a monomer. From here, PAF can, in principle, reach the cell surface by translocation across the plasma membrane, and evidence has been provided that this may occur by several mechanisms.

Translocation of PAF across the plasma membrane by MDR1 Pgp

It was recently reported [14] that PAF secretion into the medium of Ca^{2+} ionophore A23187-activated human mesangial cells was inhibited, when (i) the cells were cultured in the presence of various inhibitors of human MDR1 Pgp; and (ii) the expression level of MDR1 Pgp had been reduced by antisense oligonucleotides. Unfortunately, transport was measured as the absolute amount of PAF (ELISA) in the medium without quantitation of PAF synthesis. Reduced synthesis may, therefore, have contributed to the decrease in PAF secretion. In addition, 0.1 % BSA was present in the medium, which is suboptimal for PAF-transport measurements, as it has been reported [33] that at 0.1 % albumin PAF secretion was four times lower than at 1 % or 2 %. Finally, concomitant A23187-induced scrambling of the membrane phospholipids [33] was not studied.

In the present paper, we report that MDR1 Pgp mediates the transbilayer movement of chemically defined radiolabelled PAF in unstimulated kidney epithelial cells. In accordance with its apical localization [27], MDR1 Pgp in transfected cells stimulated PAF transport across the apical plasma membrane domain. PAF transport was independent of vesicular traffic and was inhibited by the MDR1 Pgp inhibitors PSC833 and cyclosporin A. Also, in control LLC-PK1 cells, PAF transport to the apical surface was inhibited by the MDR1 Pgp inhibitors (Figure 3), suggesting that the endogenous MDR1 Pgp is sufficiently active to translocate PAF. If the inhibition of MDR1 by PSC833 was complete, 50 % of the PAF on the apical surface of control cells reached this surface via MDR1 Pgp, whereas the other 50 % used a different pathway, possibly intracellular translocation across the ER membrane followed by vesicular transport. In contrast, MRP1 expressed at the basolateral surface did not translocate the short-chain phospholipid PAF.

PC translocation by ABC transporters

MDR3 Pgp was found to be a PC translocator in the bile canaliculus membrane when mice lacking *Mdr2* (the mouse homologue of human MDR3 Pgp) displayed a deficiency in

transport of PC into the bile. Mouse Mdr1 Pgp (the homologue of human MDR1 Pgp) did not rescue PC transport, suggesting that Mdr1 Pgp cannot mediate the translocation of PCs [6]. Indeed, MDR1 Pgp, in contrast with the PC translocator MDR3 Pgp, seemed unable to translocate short-chain C_6 -NBD-PC [7]. However, later studies showed that human MDR1 and mouse Mdr1a Pgp do mediate the translocation of C_6 -NBD-PC [8], C_{12} -NBD-PC [10] and C_8C_8 -PC, and other classes of short-chain lipids [8]. In the present study we show that, in addition, MDR1 Pgp translocates the natural short-chain PC 1-*O*-alkyl-2-acetyl-PC, PAF. The fact that MDR1 Pgp is able to translocate a variety of short-chain PCs predicts that other short-chain PCs with PAF activity, such as peroxidation products [23], are natural substrates as well.

Also MRP1 has been reported to translocate short-chain C_6 -NBD-PC [12], and might be involved in regulating the asymmetric distribution of PC and SM in the erythrocyte membrane [13]. However, we observed no translocation of C_6 -NBD-PC nor of C_8C_8 -PC in LLC-PK1/MRP1 cells [11], and MRP1 that was functionally expressed in the basolateral membrane of these cells, as evidenced by transport of C_6 -NBD-GlcCer, did not mediate the transbilayer movement of the short-chain PC PAF. Although the observation that exogenous PAF inhibited drug transport by MDR1 Pgp and not by MRP1 [14] would be in line with this, this finding cannot be directly extrapolated to imply that PAF is an MDR1 substrate because it was not established that PAF inhibited directly.

Alternative mechanisms of PAF translocation

The agents that activate cells and stimulate PAF synthesis via the remodelling pathway also induced rapid transbilayer movement of PAF across the plasma membrane of endothelial cells [39] and neutrophils [33]. These agents, the action of which can be mimicked by the Ca^{2+} ionophore A23187, have been shown to disrupt the asymmetrical arrangement of the plasma membrane lipids exposing the pro-coagulant and pro-apoptotic phosphatidylserine on the cell surface [33], probably via activation of a scramblase. Like phosphatidylserine, PAF may reach the outer leaflet of the plasma membrane via the lipid-scrambling event.

In the present study, a significant amount of PAF reached the basolateral medium of all LLC-PK1 cells (Figures 2–4). This was not due to PAF synthesis on the cell surface [32], since on a short timescale [^{14}C]choline is selectively incorporated intracellularly by the *de novo* pathway. Basolateral transport that continued in the absence of vesicular transport (Figures 2 and 4) must have been mediated by a basolateral PAF translocator. Notably, this experiment does not exclude the possibility that part of the PAF transport in the absence of BFA is mediated by vesicles. Translocation in the presence of BFA was independent of the presence or activity of MDR1 Pgp and MRP1, because it was not inhibited by PSC833, cyclosporin A and indomethacin, nor by GSH depletion. Possibly, the translocation of PAF across the basolateral membrane is related to the MDR-independent outward PC translocation ($t_{1/2} = 20$ min) that was measured in erythrocytes [40]. In contrast, inward translocation of both PAF and PC across the erythrocyte membrane is very slow ($t_{1/2} > 10$ h; [41,42]).

Physiological relevance of PAF translocation by MDR1 Pgp

MDR1 Pgp is expressed in a wide variety of cells, but occurs at high levels in the apical membrane of endothelial and epithelial cells. It is, therefore, quite possible that MDR1 Pgp translocates PAF from the endothelium towards the blood and from the

epithelia into the lumen of the various organs, such as the lung [43], the kidney and the intestine. Interestingly, the absence of Mdr1a in a knock-out mouse has been related to inflammatory bowel disease [44]. In contrast to the scramblase, MDR1 Pgp would be involved in the secretion of constitutively synthesized PAF from unstimulated cells. It is unclear whether MDR1 Pgp activity can be regulated e.g. by post-translational modifications, but phosphorylation/dephosphorylation mechanisms do not seem to play an essential role in regulation of MDR1 Pgp activity and the concomitant MDR phenotype [45,46]. In the case that MDR1 Pgp activity is not regulated, MDR1 Pgp transport of PAF to the cell surface may be constitutive and regulated by the level of synthesis of PAF and/or MDR1 Pgp.

MDR1 Pgp is recognized for its overexpression in various tumours. This overexpression may increase PAF transport to the surface of these cells, and thereby enhance PAF signalling. It was shown that the medium of the breast epithelial tumour cell line MCF7, known to overexpress MDR1 Pgp [47], induced angiogenesis, and that PAF, synthesized by the cells and present in the medium of these cells, was responsible. It is tempting to speculate that the presence of PAF in the medium was due to the high MDR1 Pgp expression in these cells. Inhibitors of MDR1 Pgp may, therefore, not only make the cells more sensitive to chemotherapy but also may interfere with angiogenesis as well.

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