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The human MDR3 P-glycoprotein promotes translocation of phosphatidylcholine through the plasma membrane of fibroblasts from transgenic mice

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Abstract The mouse mdr2 P-glycoprotein (P-gp) and its human MDR3 homologue are present in high concentrations in the canalicular membrane of hepatocytes. Mice lacking this protein are unable to secrete phosphatidylcholine (PC) into bile, suggesting that this P-gp is a PC translocator. We have tested this in fibroblasts from transgenic mice expressing the *MDR3* gene under a vimentin promoter. Transgenic and control fibroblasts were incubated with [^{14}C]choline to label PC. When the labeled cells were incubated with a PC transfer protein and acceptor liposomes, transfer of radioactive PC was enhanced in transgenic cells relative to the wild type controls. We conclude that the MDR3 P-glycoprotein is able to promote the transfer of PC from the inner to the outer leaflet of the plasma membrane, supporting the idea that this protein functions as a PC flippase.

Key words: MDR3; P-glycoprotein; Phosphatidylcholine; Flippase

1. Introduction

Upon selection with a single cytotoxic drug mammalian cells can become resistant to a variety of structurally and functionally unrelated drugs, a phenomenon called Multidrug Resistance (MDR). The best known form of MDR is caused by (over)expression of P-glycoproteins (P-gps), 140–170 kDa transmembrane proteins that actively extrude drugs from the cell. Human cells contain only one gene encoding such a protein, the *MDR1* gene, whereas mice have two genes for drug pumping P-gps, *mdr*1a and *mdr*1b (also known as *mdr*3 and *mdr*1, respectively) (reviewed in [1–3]).

In addition to these drug-transporting P-gps, mammals also contain a P-gp that appears unable to transport drugs [4,5]. This P-gp is mainly found in the canalicular membrane of the liver [6,7] and is encoded by the *MDR3* (also called *MDR2*) gene in humans [8,9] and the highly homologous *mdr*2 gene in mice [4].

To find the physiological function of this P-gp, we have generated mice with a disruption of the *mdr*2 gene [10]. These mice are unable to secrete phospholipid into their bile. As biliary phospholipid is nearly exclusively PC, this suggested that the mdr2 P-gp is required for the transport of PC through the canalicular membrane of the hepatocyte [10].

To get more information on the mechanism of PC transport by the mdr2/MDR3 P-gp, we have analyzed the transport of newly synthesized PC in mouse fibroblasts expressing the human *MDR3* gene at a high level. Normal mouse fibroblasts do not express the *mdr*2 gene and these were used as control for PC transport in the absence of P-gp.

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2. Materials and methods

2.1. Cells and cell culture

Primary *MDR3* transgenic murine ear fibroblast cells were isolated from transgenic FVB mice expressing a *MDR3* mini gene behind a vimentin promoter-enhancer expression cassette. *MDR3* transgenic cell line V01V01 was derived from these cells after immortalization by infection with SV40 virus. Control cell line FVB#c was similarly made from wild type mice, as described earlier [6]. The cells were cultured in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 4 mM L-glutamine, penicillin (50 units/ml), streptomycin (50 μg/ml) and 10% fetal bovine serum in the presence of 5% CO_2. The cells were subcultured every 3 to 4 days.

2.2. Materials

[methyl-^{14}C]choline chloride (55 mCi/mmol) was purchased from Amersham (Buckinghamshire, UK). All tissue culture chemicals were obtained from Gibco BRL (Bethesda, USA). PC transfer protein (PC-TP) was purified from bovine liver according to Kamp and Wirtz [11].

2.3. Preparation of acceptor liposomes

Liposomes were prepared from egg PC, cholesterol and phosphatidic acid (molar ratio = 10:10:1). The lipids were dried from a chloroform/methanol 2:1 (v/v) solution and dispersed in buffer A (140 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 15 mM glucose, 10 mM HEPES, pH 7.4) at a final concentration of 2 mM PC. Liposomes were obtained by sonication of the lipid suspension with a Branson sonifier at 65 W for 30 min at 0°C.

2.4. PC transport assay

The cells were trypsinized and replated in six-wells plates (Costar, 9 cm^2 per well) at a density of 5×10^5 cells per well and cultured for 20 h as described above. The medium was removed and the cells were washed once with phosphate buffered saline (PBS). 2 ml inositol free and choline low Hanks Minimal Essential Medium (HMEM) was added, supplemented with 0.4 μCi [methyl-^{14}C]choline chloride (55 Mci/mmol). The cells were metabolically labeled for 3 h under standard growth conditions. Afterwards the medium was removed and the monolayer was washed twice with buffer A. To the cells 1 ml of buffer A with various amounts of PC-TP and with a liposome suspension containing 200 nmol PC was added, this is a 15-fold excess compared to the estimated amount of PC present in the cells. The cells were incu-

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bated at 37°C, while shaking gently (approx. 50 rpm). At various time points the buffer was removed and the cells were washed once with PBS and trypsinized; the PBS used in this washing step did not contain a significant amount of radioactivity. Lipids were extracted from both the cells and the medium according to Bligh and Dyer [12]. A fraction (500 μl) of the chloroform phase (1.9 ml) was dried down and the radioactivity was determined by liquid scintillation counting, using a Packard Tri-Carb 1500 Liquid Scintillation Analyzer.

2.5. Viability assay

To determine the viability of the cell lines during the exchange experiment, cells of both lines were plated in six-wells plates and grown for 20 h. The monolayer was subsequently washed with PBS and the cells were incubated with either DMEM or buffer A for 4 h. Afterwards the cells were washed with PBS, trypsinized and the number of viable (Trypan blue excluding) cells and non viable cells was determined.

2.6. Thin layer chromatography

After extraction of the lipids from the cells, a quarter of the chloroform/methanol phase was evaporated, taken up into 50 μl chloroform and applied on Silica Gel 60 plates (0.25 mm, Merck). The plates were developed twice in chloroform/methanol/ammonia (25%, w/v)/water (50:65:5:4, by volume) and visualized by primuline staining. Lipid containing spots were scraped off and counted in 4 ml of Packard Ultima Gold liquid scintillation fluid.

3. Results

In Fig. 1 the rationale of the exchange assay is schematically depicted. The cells incubated with [^{14}C]choline will synthesize labeled PC in their endoplasmic reticulum (ER). From the ER [^{14}C]PC will be transported to the plasma membrane, either by endogenous lipid transfer protein (L-TP) mediated monomeric diffusion, inserting the labeled PC into the inner leaflet only, or by vesicle flow, delivering the PC to both leaflets. Only the labeled PC in the outer leaflet can be transferred to the acceptor liposomes in the medium by the exogenous PC-TP (Fig. 1B). In cells lacking a PC transporter in the plasma membrane PC can only reach the outer leaflet by vesicular transport or by a low rate of spontaneous flipping. The presence of a translocator (also called flippase) will result in a more rapid appearance of labeled PC in the outer leaflet and hence in a more rapid labeling of added liposomes in the presence of exogenous PC-TP. A transporter mediating vesicular secretion of PC would result in labeling of liposomes in the absence of PC-TP.

To estimate the time needed for efficient labeling of the cell lines, the *MDR*3 transgenic cell line V01V01 and the wild type FVB#c cells were incubated with labeled choline. Every half hour a sample was trypsinized and lipids were extracted. In both cell lines substantial amounts of labeled PC were only synthesized after 2 h of labeling (Fig. 2). After 3 h, a sufficiently high level of PC labeling was reached to start the exchange experiment by removing the radioactive choline and adding PC-TP and acceptor liposomes. The time scale for the synthesis of [^{14}C]PC was similar for the FVB#c and V01V01 lines, but in all experiments the amount of radioactive PC produced was 40 to 50% lower in the V01V01 cells than in the FVB#c cells (Fig. 2). With one-dimensional thin layer chromatography we found that the bulk of labeled lipid was [^{14}C]PC; only a minor fraction was sphingomyelin and no other labeled lipids were detected (results not shown).

Determination of the viability of the cells in buffer A showed that the total number of cells and the percentage of non-viable cells did not differ significantly between the two cell lines, nor between the two incubation media. The percentage of non-viable cells did not exceed 3% in any of the groups (results not shown).

To determine the amount of PC-TP required to exchange PC between the cells and the liposomes, [^{14}C]PC labeled cells were incubated with various amounts of PC-TP for one hour. Figure 3 shows the percentage of radiolabeled cellular PC transported to the liposomes. In the absence of PC-TP, only 2% of the labeled PC appears in the liposome fraction and this value was not affected by the presence of MDR3 P-glycoprotein in the plasma membrane. We attribute this background to vesiculation of intact membrane or fragmentation of membranes from cells in poor condition. The background transfer varied from experiment to experiment and dropped to less than 1% in later experiments (c.f. Fig. 4).

Addition of 0.2 mg PC-TP per ml incubation medium resulted in a substantial increase in the labeled PC appearing in the liposome fraction, but this only occurred in the *MDR*3 transgenic V01V01 cells, not in the FVB#c cells (Fig. 3). Increasing the PC-TP concentration to 0.7 mg/ml gave a small further increase of label transfer (Fig. 3). Hence, we settled for a concentration of 0.2 mg/ml. Note that the acceptor liposomes are in large excess in these experiments. The rate of back exchange of labeled PC to the cells is therefore negligible.

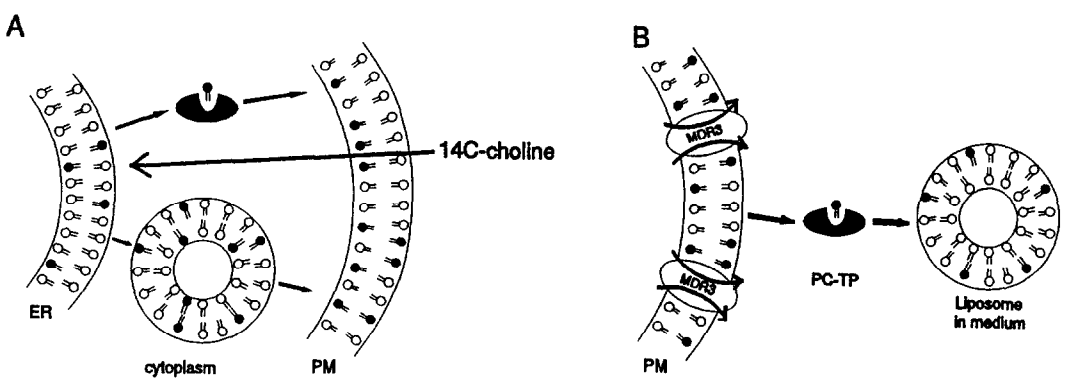

Fig. 1. Schematic representation of the [^{14}C]PC exchange assay that was used and the hypothetical role of MDR3 in this process. A, incorporation of [^{14}C]choline labels PC (black symbols) in the endoplasmic reticulum (ER) and transport of labeled PC from the ER to the plasma membrane (PM) either by monomeric diffusion (top), e.g. by PC-TP, inserting labeled PC in the inner leaflet only, or by vesicle flow (bottom), delivering PC to both leaflets. B, flipping of labeled PC to the outer leaflet in the presence of MDR3 and exchange of labeled PC by PC-TP from the outer leaflet to liposomes in the medium. In the absence of MDR3 only the labeled PC inserted directly into the outer leaflet by vesicle flow can be transferred to the liposomes.

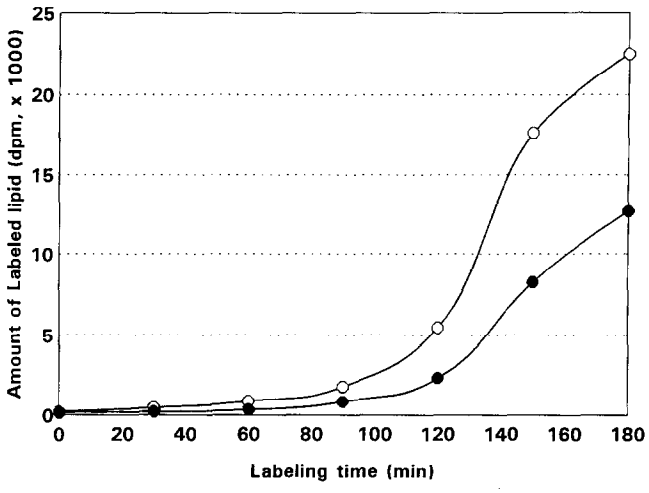

Fig. 2. Time course of the synthesis of radiolabeled PC from [methyl-^{14}C]choline in the murine fibroblast lines FVB#c (open circles) and V01V01 (closed circles).

The time course of the appearance of radiolabeled PC in the acceptor liposomes is shown in Fig. 4. To correct for the aspecific PC release, a sample without PC-TP was assayed for each time point and for both cell lines. The resulting curves show that PC-TP dependent PC exchange occurs in both cell lines, but in the *MDR3* transgenic cells the PC exchange starts earlier and runs at a higher rate. The unusual biphasic time course of label transfer from the *MDR3* transgenic cells can be explained by considering the two intra-cellular routes known to contribute to the labeling of the plasma membrane in other cells [13–15], i.e. fast transport by cytosolic L-TP and slow transport by intra-cellular vesicles. Transport by L-TP delivers PC into the inner leaflet of the plasma membrane. We postulate that this is the main route of PC transport in the labeling period of our cells. This labeled PC is not available for transfer to the acceptor liposomes unless it can be flipped to the outer leaflet. Hence the large difference between control cells and the *MDR3* transgenic cells after 60 min in Fig. 4. Vesicular transport is slow and delivers labeled PC to both leaflets of the plasma membrane. This makes an increasing contribution to the PC available in the outer leaflet of both the control cells and the *MDR3* transgenic cells, explaining the increased label transfer to acceptor liposomes between 120 and 240 min in Fig. 4.

4. Discussion

Our results show that the presence of MDR3 P-gp has a profound influence on the distribution of newly synthesized PC in the plasma membrane of murine fibroblasts. The redistribution is not accompanied by detectable secretion of PC. We therefore conclude that this P-gp is a PC flippase, that can flip a PC molecule from one lipid leaflet of a bilayer to the other. As this conclusion is based on indirect experiments, possible confounding artifacts should be considered.

All available evidence indicates that extra-cellular PC-TP can only interact with PC in the outer leaflet of the plasma membrane of intact cells [16]. The PC-TP dependent transfer of radioactive PC to acceptor liposomes in our experiments must therefore be derived from the outer leaflet of the fibroblast plasma membrane. It is unlikely that the plasma membrane of the *MDR3* transgenic cells is more rapidly labeled than that of the control cells. The labeling kinetics of both cell lines are similar and (for unknown reasons) the *MDR3* cells incorporate even less label than control cells (Fig. 2). Since both cell lines have the same genetic background, it is unlikely that they differ with respect to the rate of PC delivery to the plasma membrane. The only remaining stage of PC transport to the outer leaflet is the direct transport of PC over the plasma membrane. Because the MDR3 P-gp is localized in the plasma membrane in the V01V01 cells [6], we may conclude that MDR3 P-gp must promote this translocation process to account for the increased transfer of newly synthesized PC to external liposomes from the V01V01 cells.

Up to 60 min after the start of the exchange experiment all labeled PC that reaches the plasma membrane of both cell lines must have been inserted into the inner leaflet by means of intracellular L-TP, since the outer leaflet of the wild type cells does not yet contain labeled PC exchangeable by PC-TP (Fig. 4). The rate of transfer catalyzed by MDR3 P-gp is high: between 15 and 60 min exchange time (Fig. 4), approximately 3% of the total labeled PC is transferred to acceptor liposomes. The plasma membrane of murine fibroblast cell line LM contains approximately 40% of the total cellular PC [17–19]. The PC distribution in our cells probably will not be very different, thus almost 8% of the plasma membrane PC must have been flipped by the MDR3 P-gp in the period between the start of labeling and the 60 min sample in Fig. 4. Only a minimal imbalance between the leaflets of the membrane bilayers is permissible. We therefore infer that there must be considerable back-flipping of the PC from the outer leaflet to the inner leaflet in cells containing the MDR3 P-gp. This remains to be verified.

After 120 min, the first labeled PC appears in the outer leaflet of the FVB#c control cells (Fig. 4). This may be the result of vesicle mediated PC delivery to the plasma membrane, inserting labeled PC into the outer leaflet by vesicle fusion. It takes 2 to 3 hours for the postulated vesicular PC to reach the plasma

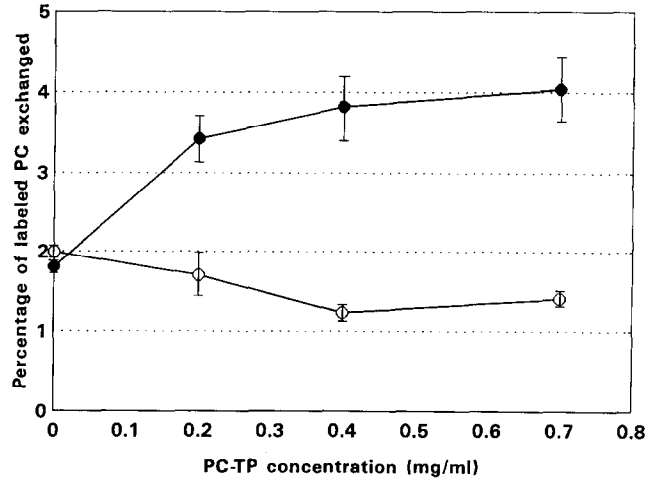

Fig. 3. Percentage of labeled PC that can be exchanged from the outer leaflet of the membrane to the liposomes in the medium by various amounts of PC-TP. After labeling with [^{14}C]choline for three hours, the cells were incubated with PC-TP for one hour. Wild type FVB#c cells, open circles; *MDR3* transgenic V01V01 cells, closed circles. All values are based on two independent experiments. The range is shown by a vertical bar.

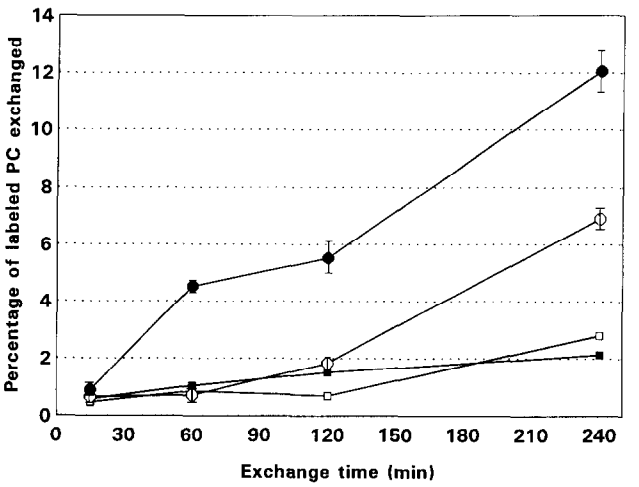

Fig. 4. Time course of the transfer of labeled PC to liposomes with or without PC-TP. Wild type FVB#c cells, open symbols; *MDR*3 transgenic V01V01 cells, closed symbols. No PC-TP added, squares; 0.2 mg/ml PC-TP added, circles. All values are based on two independent measurements. The range is shown by a vertical bar.

membrane in our fibroblasts. This is longer than the 30–60 min observed in other cell types [13–15]. This could be due to differences in metabolism or handling of the cells.

The PC-TP mediated transport from V01V01 cells to liposomes reaches a plateau between 60 and 120 minutes of exchange (Fig. 4), suggesting a decrease in the rate of delivery of labeled PC to the plasma membrane. This is not unreasonable if intracellular transport by L-TP occurs shortly after PC synthesis. The high extent of exchange that is observed between $t = 0$ and $t = 60$ min is not only the result of [^{14}C]PC delivered in this period, but also of labeled PC that accumulated in the outer leaflet between $t = -60$ and $t = 0$.

After this work was completed, Ruetz and Gros [20] reported studies on the activity of the murine mdr2 Pgp in yeast inside-out secretory vesicles. They found that these vesicles accumulate a relatively water-soluble, fluorescent PC analog and that the accumulation was dependent on ATP hydrolysis. Although PC analogs and natural PC may behave very differently under some conditions [21], the results of Ruetz and Gros [18] support the idea that the MDR3/mdr2 P-gp is a PC translocator. They have shown that this P-gp can take its PC analog from the cytoplasmic leaflet [20]. We find that it donates its PC to the ectoplasmic leaflet. In combination these results are consistent with the hypothesis that human MDR3 P-gp and its murine homologue mdr2 P-gp are flippases that transport PC from the inner to the outer leaflet of the membrane they are residing in.

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