

Review

## ABC transporters in lipid transport

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### Abstract

Since it was found that the P-glycoproteins encoded by the MDR3 (MDR2) gene in humans and the *Mdr2* gene in mice are primarily phosphatidylcholine translocators, there has been increasing interest in the possibility that other ATP binding cassette (ABC) transporters are involved in lipid transport. The evidence reviewed here shows that the MDR1 P-glycoprotein and the multidrug resistance (-associated) transporter 1 (MRP1) are able to transport lipid analogues, but probably not major natural membrane lipids. Both transporters can transport a wide range of hydrophobic drugs and may see lipid analogues as just another drug. The MDR3 gene probably arose in evolution from a drug-transporting P-glycoprotein gene. Recent work has shown that the phosphatidylcholine translocator has retained significant drug transport activity and that this transport is inhibited by inhibitors of drug-transporting P-glycoproteins. Whether the phosphatidylcholine translocator also functions as a transporter of some drugs in vivo remains to be seen. Three other ABC transporters were recently shown to be involved in lipid transport: ABCR, also called Rim protein, was shown to be defective in Stargardt's macular dystrophy; this protein probably transports a complex of retinaldehyde and phosphatidylethanolamine in the retina of the eye. ABC1 was shown to be essential for the exit of cholesterol from cells and is probably a cholesterol transporter. A third example, the ABC transporter involved in the import of long-chain fatty acids into peroxisomes, is discussed in the chapter by Hetteema and Tabak in this volume. © 2000 Elsevier Science B.V. All rights reserved.

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### 1. Introduction

In 1993, Smit et al. [1] found that a P-glycoprotein (Pgp) is essential for the secretion of phosphatidylcholine (PC) into bile. Subsequent work showed that

this Pgp, encoded by the *Mdr2* gene in mice and by the very homologous *MDR3* (also called *MDR2*) gene in humans, is a PC translocator [2–5] with ves-tigial drug transport activity [6]. This has led to an analysis of other transporters belonging, like Pgp, to the large family of ATP binding cassette transporters [7] to see whether these can also translocate lipids. This review presents an update of the results thus far, focusing on transporters also known to be involved in multidrug resistance.

ABC transporters are primary active transporters, i.e. they bind their substrate and move it through the membrane using ATP hydrolysis to pump against a

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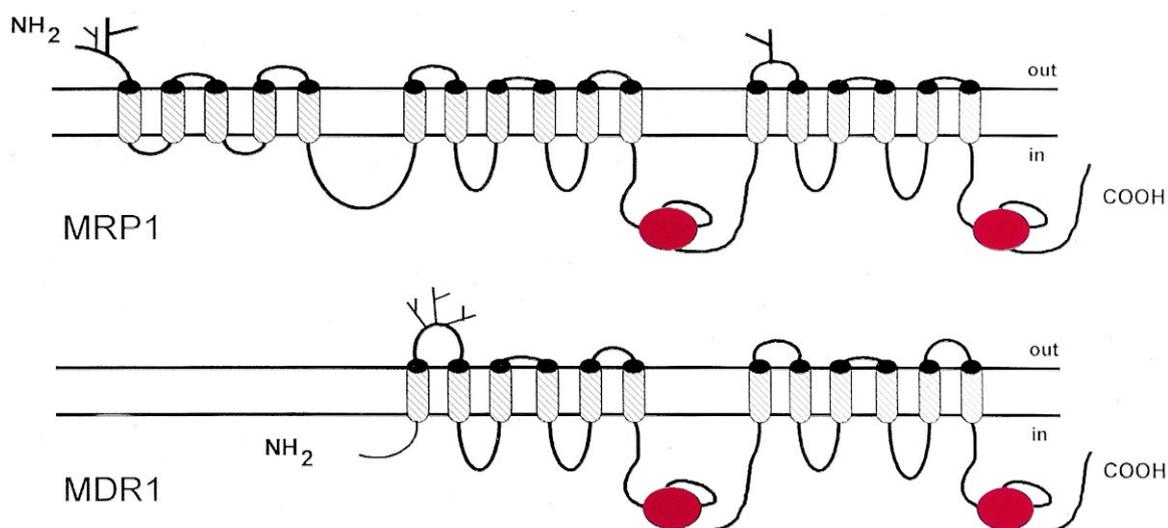


Fig. 1. Putative membrane topology of two ABC transporters involved in multidrug resistance. (Top) The multidrug resistance (-associated) protein, MRP1; (bottom) MDR1 P-glycoprotein. The position of carbohydrate side chains is indicated by little trees, the nucleotide binding domains by black eggs. (See text for background).

substrate concentration gradient. The prototype ABC transporter is a large protein with 12 transmembrane segments and two nucleotide binding sites. Some ABC transporters are assembled from half-molecules or quarter molecules (two transmembrane domains, two ATP binding domains), others are made in one piece, such as the P-glycoprotein depicted in Fig. 1.

ABC transporters mediating multidrug resistance are ancient. Hydrophobic drugs evolved as an early and successful weapon in the chemical warfare in nature. These drugs do not require active uptake systems in the target – as required for hydrophilic toxins – since they can enter cells by passive diffusion through the membrane. Hence, the defense against these drugs must either involve enzymic inactivation, or active extrusion by a drug efflux pump. Obviously, bacteria that produce these toxins must protect themselves against their own product. Indeed, the streptococcus that produces doxorubicin (adriamycin), an anthracycline, also makes an ABC transporter efficiently extruding doxorubicin [8]. Organisms targeted by these toxins have learned to survive with the help of effective drug pumps as well. The *Escherichia coli* genome contains genes for 29 putative drug pumps, belonging to four different transporter families: two of these are ABC transporters [9].

## 2. The transport mechanism

The bacterial drug transporter studied most intensively is the LMR-A transporter of *Lactococcus* [10,11]. The LMR-A gene encodes a half-transporter that forms catalytically active homodimers in the bacterial membrane. It is relatively easy to produce large amounts of bacterial membrane vesicles containing high concentrations of LMR-A and these vesicles have been used to study the mechanism of drug transport by LMR-A. The experimental results support the model presented in Fig. 2. Drug reaching the cell rapidly inserts into the outer leaflet of the

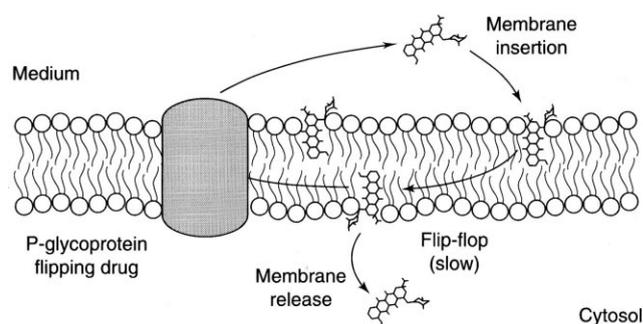


Fig. 2. A drug-transporting Pgp in action, showing extrusion of an anthracycline molecule from a multidrug-resistant cell. In reality the positively charged part of the drug interacts more with the head groups of acidic membrane phospholipids than indicated here (adapted from [102]).

plasma membrane. Flipping of the drug to the inner membrane leaflet is relatively slow and the rate-limiting step in entry. In fact, the  $t_{1/2}$  of anthracycline flipping is estimated to be nearly 1 min [12]. LMR-A is able to transport drug from the inner leaflet back into the medium, counteracting the rate-limiting step in entry [11].

Higgins and Gottesman proposed [13] that mammalian drug-transporting Pgps are flippases, i.e. that they flip drug from the inner leaflet to the outer leaflet of the plasma membrane. The biophysical studies with fluorescent drugs show, however, that LMR-A does not act as a flippase, but directly deposits drug in the medium [11]. It is possible that Pgp handles drug differently, but this seems unlikely as overproduction of bacterial LMR-A results in a markedly similar spectrum of drug resistance as overproduction of mammalian Pgp [10]. In fact, LMR-A synthesized in mammalian cells is properly inserted in the plasma membrane and results in a MDR phenotype indistinguishable from that of cells overproducing the MDR1 Pgp [14]. Hence, it looks as if mammals and streptococci encounter a similar spectrum of hydrophobic toxins in their environment and developed an ABC transporter with similar properties to deal with this threat. The simplest hypothesis is therefore that LMR-A and Pgp use the same mechanism to translocate drugs.

How ABC transporters, such as Pgp, recognize and translocate substrate is still unclear. There is some information on the 3-D structure of the ATP binding part of ABC transporters, and electron microscopy of Pgp arrays has suggested that the protein contains a central pore closed at the exoplasmic site [15,16]. Whether drugs pass through this pore remains unknown. Translocation involves alternate action of the two ATP binding sites and both must be functional to allow sustained drug transport. Pgp appears to have two drug binding sites that interact, but the protein parts involved in these sites are still not precisely defined [17–20].

### 3. The MRP family

Besides the Pgps, mammalian cells contain a second family of ABC transporters able to transport anti-cancer drugs, the MRPs. The multidrug resis-

tance (-associated) protein MRP1 was discovered in 1992 [21,22] and five additional family members, MRP2–6, have been described since [23–25]. MRP1, MRP2, MRP3 and MRP5 have been shown to transport drugs. We shall limit the discussion here to MRP1, the only family member known to transport lipid analogues thus far. As MRP2 [26], 3 [27–29] and 6 [25] are rather similar to MRP1, however, it would not be surprising if these transporters would translocate lipid analogues (or lipids) as well.

The current topology model for MRP1 is presented in Fig. 1. Besides a Pgp-like core structure, MRP1 contains an additional domain, TMD<sub>0</sub>, with five putative transmembrane segments, fused by means of a long internal loop, L<sub>0</sub>, to the core domain [30–32]. Interestingly, drug transport and protein routing to the cell surface do not require TMD<sub>0</sub>, but without L<sub>0</sub> the protein is inactive and becomes trapped in the endoplasmic reticulum [33]. The function of TMD<sub>0</sub> remains to be determined.

MRP1 differs from Pgp in other respects: the most important difference is that MRP1 is an organic anion transporter with high activity towards compounds conjugated to glutathione (GSH), glucuronide or sulfate [34–38]. Other organic anions, such as calcein and other negatively charged dyes, are also transported by MRP1 [39–41]. In contrast, Pgp preferentially transports neutral or basic organic compounds.

Because of its ability to transport glutathione-conjugated drugs, MRP1 has been referred to as a GS-X pump [42,43]. Interestingly, MRP1 can even transport complexes of heavy metals and GSH, such as arsenite or antimonite complexed with three GSH molecules [44]. More remarkable is the ability of this organic anion pump to transport neutral or basic drugs not known to be conjugated to acidic ligands in the cell. Examples are the vinca alkaloids and the anthracyclines [44]. There is considerable evidence that these drugs are co-transported with GSH, even though the stoichiometry between drug and GSH transported is still not known [45–48]. Cells transfected with *MRP1* (or *MRP2*) gene constructs secrete GSH [26]. Most investigators think that GSH is a low-affinity substrate for MRP1 [49], but because the affinity is so low it is technically tough to demonstrate that GSH is a substrate in vesicular uptake experiments. It is still not clear either whether cells

overproducing MRP1, secrete GSH alone or together with an endogenous (unidentified) ligand.

MRP1 also differs from Pgp in its cellular location. In normal cells Pgp is located in the plasma membrane, whereas MRP1 is mainly located in an unidentified intracellular vesicular fraction [50]. Only in cancer cells that overproduce the protein it is mainly in the plasma membrane [51]. In polarized cells Pgp routes to the apical membrane and MRP1 to the basolateral membrane [52].

Finally, no clear function has been found for drug-transporting Pgps in normal cellular physiology. These Pgps only seem to protect mammals against xenotoxins [53]. In contrast, MRP1 has at least one important physiological function: it is the main transporter for the cellular excretion of the lipid metabolite leukotriene C<sub>4</sub> (LTC<sub>4</sub>) [54]. In addition, MRP1 contributes to the protection of some tissues against xenotoxins [54,55], notably the oropharyngeal epithelium, the testis, the kidney, and the cerebrospinal fluid [54].

#### 4. The phosphatidylcholine translocator encoded by the MDR3/Mdr2 Pgp genes

Humans contain 2 Pgp genes, the *MDR1* and the *MDR3* (also known as *MDR2*) genes (Fig. 3). The *MDR1* gene encodes a drug transporter. The function of *MDR3* and its close murine homologue *Mdr2* remained a mystery until Smit et al. [1] generated a mouse homozygous for a disrupted *Mdr2* allele (a *Mdr2*  $-/-$  or *Mdr2* null mouse) and found that this mouse is completely unable to secrete phosphatidylcholine into bile. The absence of PC in bile leads to a mild liver disease because bile salt secretion is normal in these mice and the high bile salt concentrations damage the canalicular membrane of the hepatocyte and the small bile ducts, resulting in extensive bile duct proliferation and some hepatocyte damage [56]. Smit et al. [1] already predicted that the absence of the human MDR3 Pgp would result in more serious liver disease than lack of the murine *Mdr2* Pgp. Human bile salts are more hydrophobic than their murine counterparts and feeding *Mdr2* ( $-/-$ ) mice hydrophobic bile salts aggravates their liver disease [57]. Indeed, the recently identified in-born human MDR3 Pgp deficiency characterized as

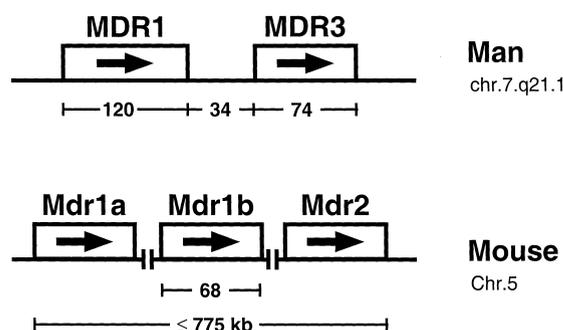


Fig. 3. The human [103] and murine [104] P-glycoprotein genes (adapted from [105]). All sizes are in kb.

progressive familial intrahepatic cholestasis (PFIC) with high  $\gamma$ -glutamyl transpeptidase (GT) causes serious liver disease requiring transplantation [58]. Even heterozygotes for this PFIC variant appear susceptible to cholestasis under stress, e.g. in pregnancy [59].

Our present ideas about PC secretion from hepatocyte into bile are summarized in Fig. 4. PC secretion depends both on bile salts and on the *Mdr2* Pgp. If either is lacking no PC secretion is detectable. If *Mdr2* Pgp is present in the hepatocyte canalicular membrane, the rate of PC secretion is curvilinearly dependent on the bile salt concentration as shown in Fig. 5. Interestingly, at all bile salt concentrations PC secretion is dependent on the *Mdr2* Pgp concentration. PC secretion is higher in wild-type *Mdr2* ( $+/+$ ) mice than in *Mdr2* ( $+/-$ ) heterozygotes. Secretion is highest in mice transgenic for the *MDR3* gene, which have a supra-physiological PC translocator concentration in their livers [60] and very low in the A1 *Mdr2* ( $-/-$ ) with low MDR3 transgene expression.

The source of the PC used by the *Mdr2* Pgp for secretion has been a matter of speculation [56]. PC is synthesized in the endoplasmic reticulum. If it would be transported by vesicular transport directly to the canalicular membrane half of the cargo of these vesicles would end up in the outer leaflet of the canalicular membrane. Presumably, this half would be directly available for bile formation, making PC secretion partially independent of *Mdr2* Pgp. This was not observed in practice (Fig. 5). Both Cohen and coworkers [61] and Smit et al. [1] therefore proposed that PC is transported from the endoplasmic reticulum to the inner leaflet of the canalicular membrane by the PC transfer protein (Pc-tp). We critically tested this proposal by generating a Pc-tp ( $-/-$ )

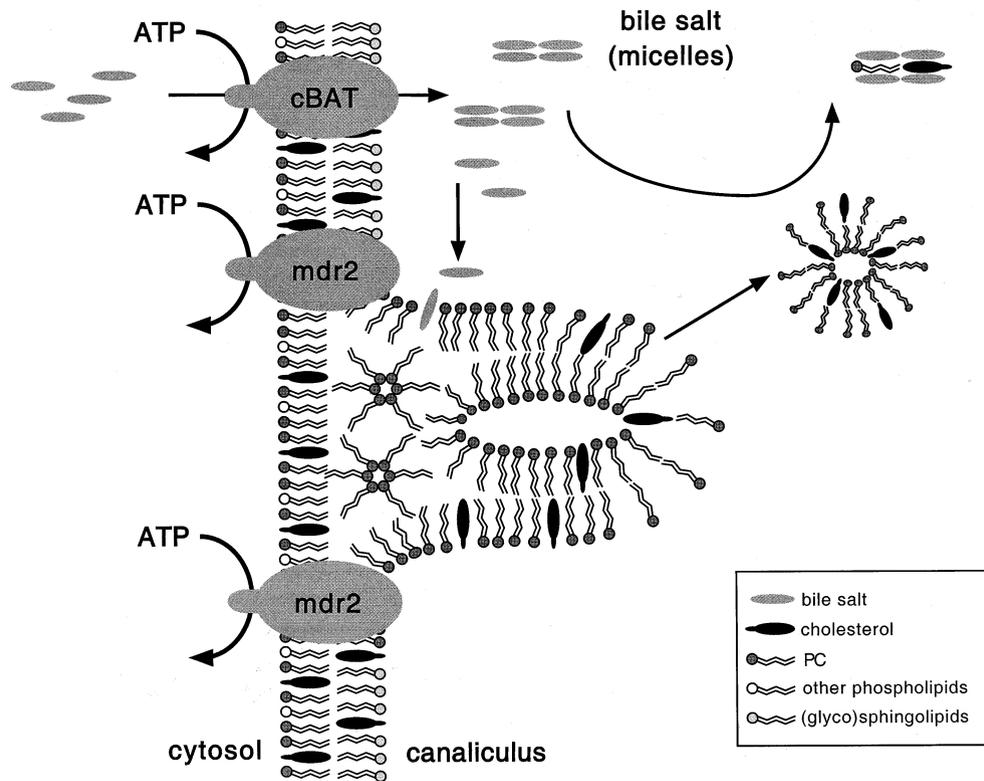


Fig. 4. The role of Mdr2/MDR3 Pgp in bile formation (adapted from [56]). cBAT is the canalicular bile acid transporter. Phosphatidylcholine from the inner leaflet of the canalicular membrane is flipped by Mdr2/MDR3 Pgp to the outer leaflet where it is accessible to extraction by bile salts.

mouse. PC secretion into bile is normal in this mouse [62]. As we did not observe a compensatory increase in non-specific lipid transfer proteins, it seems highly unlikely now that PC arrives at the canalicular membrane by monomeric transport.

Several alternative routes have been considered. We favor the following and speculative route: the plasma proteins secreted into the blood reach the basolateral membrane of the hepatocyte by vesicular transport. This entails a high rate of PC transport from endoplasmic reticulum to plasma membrane. PC deposited in the outer leaflet of the plasma membrane cannot reach the canalicular membrane by lateral diffusion as this is blocked by the tight junctions between hepatocytes. In contrast, lateral diffusion can occur unimpeded in the inner leaflet, allowing PC inserted in the basolateral membrane to reach the canalicular membrane and be translocated by the PC translocator. The substrate selectivity of the Mdr2 Pgp might be responsible for the fatty acid

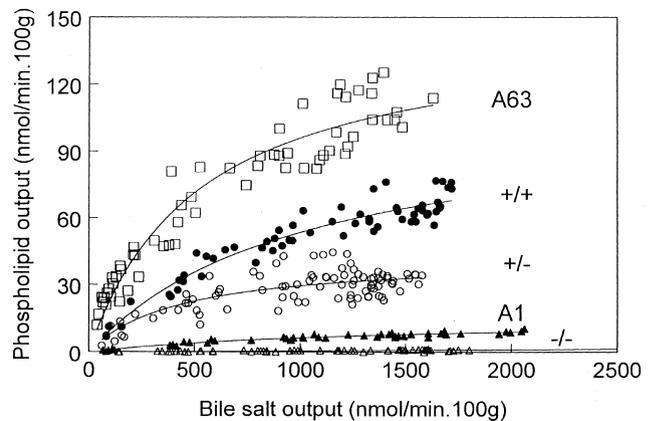


Fig. 5. The dependence of PC secretion into bile on bile salt and Mdr2 Pgp concentration (from [64]). +/+, wild-type mice; +/- and -/-, mice heterozygous or homozygous for a Mdr2 null allele; A63, a wild-type mouse transgenic for a human MDR3 gene construct highly expressed in the liver; A1, a -/- mouse transgenic for a human MDR3 gene constructs expressed at low level in the liver.

composition of the bile PC, which differs from that of the average liver PC molecule.

Crawford and coworkers [63] have shown by electron microscopy that monolamellar vesicles seem to detach from the canalicular membrane during PC synthesis. These vesicles require the presence of functional Mdr2 Pgp. Interestingly, the appearance of lipoprotein X (LpX) in the blood of cholestatic mice is also completely dependent on functional Mdr2 Pgp [64]. LpX consists of 40–100 nm vesicles consisting of phospholipid and cholesterol with an aqueous lumen. They appear shortly after bile duct ligation in wild-type mice, but not in Mdr2 (–/–) mice. How LpX reaches the blood is not known. Oude Elferink et al. [64] favor a model in which biliary vesicles continue to be formed at the canalicular membrane after ligation and that these LpX vesicles reach the blood by transcytosis through the hepatocyte. Another possibility is that the increased pressure in the biliary compartment after ligation is released from time to time by opening of the tight junctions between hepatocytes and a paracellular flux of bile into the blood.

## 5. The substrate specificity of the PC translocator (Mdr2 and MDR3 Pgps)

To study the transport of lipids and lipid analogues by the PC translocator three different test systems have been used. We shall discuss each of these and illustrate the usefulness and limitations of each system on the basis of results obtained with the Mdr2/MDR3 Pgp.

### 5.1. Vesicular transport with isolated yeast inside-out vesicles

Ruetz and Gros [2] used a yeast mutant transfected with an Mdr2 cDNA construct. This temperature-sensitive mutant is defective in vesicular transport and under restrictive conditions it accumulates vesicles en route to the surface. These vesicles can be isolated and represent a pure population of inside-out vesicles, i.e. the cytoplasmic face of Pgp is on the outside and they can be used to study drug transport into the vesicles. Ruetz and Gros made three important observations with this system. First, their experi-

ments were the first to show direct translocation of a PC analogue by the Mdr2 Pgp in a defined system. Although the Mdr2 (–/–) null mouse of Smit et al. [1] had shown that this Pgp was essential for biliary PC secretion, the mouse results did not rule out the (unlikely) possibility that PC transport was a secondary effect of Pgp action. Secondly, Ruetz and Gros showed that the Mdr2 Pgp is highly specific for phospholipid (PL) analogues with a PC head group. Finally, they showed that translocation was inhibited by an inhibitor of drug-transporting Pgps, verapamil. We return to this remarkable observation below.

The yeast vesicular transport system has not been adopted in other labs. The transport assay is complex and involves measurement of fluorescence quenching against a high background. The yeast mutant has also been difficult to work with and we have never been able to get a satisfactory yield of vesicles.

### 5.2. Measurement of long-chain PC transport using purified Pc-tp

The principle of this assay is depicted in Fig. 6. The strength of this assay is that it measures the translocation of long-chain labeled PC that is exchanged for cold PC by Pc-tp shuttling between the outer leaflet of the plasma membrane and acceptor liposomes. Although this assay has been used to demonstrate that the MDR3 Pgp actually translocates PC [3], its main drawback is that it requires purified Pc-tp in rather large amounts. Another drawback is that the assay has a substantial background coming from PC carried directly by vesicular transport to the outer leaflet of the plasma membrane without the intervention of the PC translocator.

### 5.3. MDR3 Pgp transport activity in transfected polarized kidney cells

Some immortalized kidney cell lines are able to grow as monolayers on porous filters. Paracellular flow is minimal through these layers, because of the tight junctions between the cells. The intracellular routing of ABC transporters is normal in these cells. Transporters that go to the apical membrane in vivo, such as the MDR1, MDR3, Mdr1a and Mdr1b Pgps also do this in the kidney cell layer. MRP1, a baso-

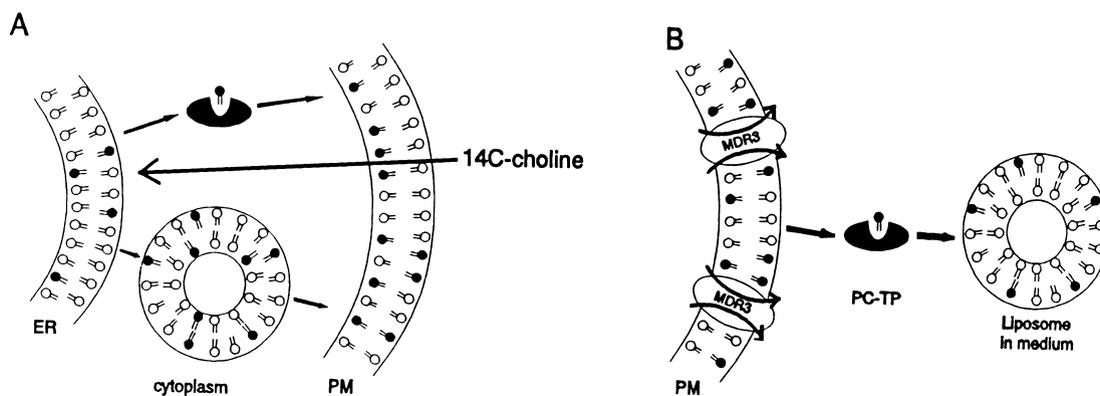


Fig. 6. Assay used to measure translocation of long-chain PC by MDR3 Pgp through the plasma membrane of intact cells. (A) Incorporation of [ $^{14}\text{C}$ ]choline labels PC (black symbols) in the endoplasmic reticulum (ER) and transport of labeled PC from the ER to the plasma membrane (PM) either by monomeric diffusion (top), 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 Pgp and exchange of labeled PC by PC-TP from the outer leaflet to liposomes in the medium. In the absence of MDR3 Pgp only the labeled PC inserted directly into the outer leaflet by vesicle flow can be transferred to the liposomes (from [3]).

lateral transporter *in vivo*, also routes to the basolateral membrane in the kidney cells.

Cells stably transfected with MDR1, MDR3 or MRP1 cDNA constructs have been used intensively to study drug transport. The principle is illustrated in Fig. 7A. Drug transport through the monolayer can be tested quantitatively and in a sensitive fashion in both directions. Cell lines used thus far are the pig LLC-PK1 cells and the canine MDCKII cells. Although this system has yielded valuable information, it has pitfalls.

(a) Drug transport can only be observed if drugs enter the cell at sufficient rate. This is not trivial as hydrophilic substrates usually do not enter, whereas also some classical MDR drugs, such as vincristine, penetrate too poorly.

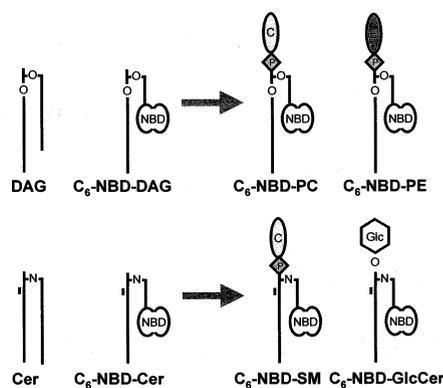
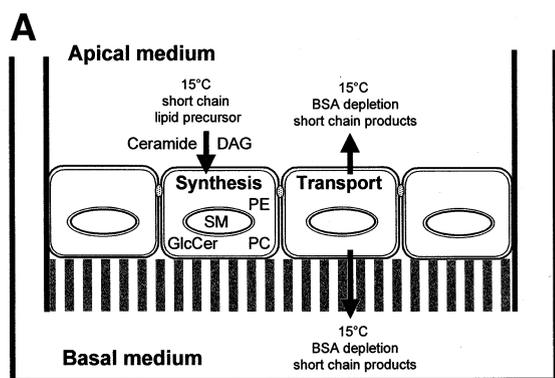
(b) There is significant background activity in these cells due to endogenous pig/canine transporters. As the activity of these transporters may vary somewhat in sub-lines, transport activity in transfected cells that is not substantially above the background transport seen in controls does not mean much. To circumvent this problem A.H. Schinkel (pers. commun.) has tried to develop a suitable kidney cell line from triple null mice lacking *Mdr1a* and *b* Pgps and *Mrp1*, without success thus far. Using LLC-PK1 pig cells transfected with an MDR3 cDNA construct, Van Helvoort et al. [5] studied translocation of lipid analogues of membrane lipids

by the method summarized in Fig. 7A. As in this method the translocated lipid is extracted from the outer leaflet of the plasma membrane with albumin, it is only suitable for lipids that have at least one short-chain fatty acid. Normal long-chain PC is too tightly wedged in the lipid bilayer to be extracted.

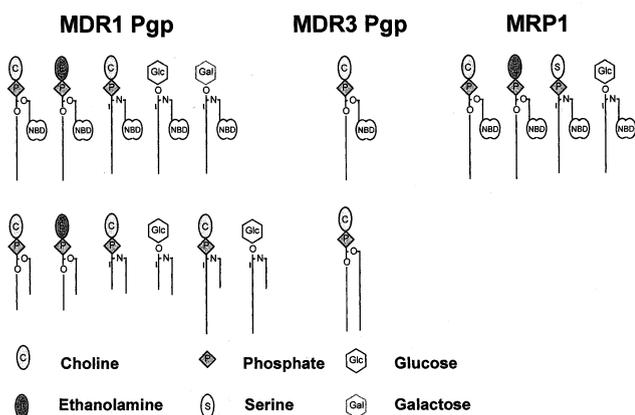
Using this system, Van Helvoort confirmed the high specificity of MDR3 Pgp for lipids with a choline head group. Interestingly, this Pgp is also rather selective towards the fatty moieties. It was able to translocate  $\text{C}_6$ -NBD-PC, a lipid analogue with a  $\text{C}_{16}$  fatty acid at the *sn*-1 position and a fluorescent  $\text{C}_6$  fatty acid at the *sn*-2 position, but not a PC analogue with two  $\text{C}_8$  fatty acids or  $\text{C}_6$ -NBD-SM, also an analogue with a phosphocholine head group but with a ceramide (Cer) backbone. Translocation in this system was inhibited by verapamil, confirming the results of Ruetz and Gros [2].

## 6. The MDR3 Pgp can transport drugs

Initial experiments with cells transfected with either *Mdr2* or MDR3 gene constructs failed to result in resistance to any of a large panel of drugs tested. The *Mdr2* Pgp also showed considerably reduced binding of the photoactivatable drug analogue iodoarylazidoprazosin, compared to *Mdr1* Pgp [65]. It was therefore surprising that transport of  $\text{C}_6$ -



**B**  
Lipid translocation by multidrug transporters



NBD-PC by both Pgps is inhibited by verapamil. Verapamil is the prototype MDR reversal agent and it is also a good substrate for drug-transporting Pgps. It is therefore difficult to escape the conclusion that the PC translocator can also transport drugs, even though it has a high specificity for choline containing PLs. Since the PC translocator gene obviously arose from the duplication of a gene for a

Fig. 7. (A) The use of kidney cell monolayers to study drug transport and translocation of the lipid analogues indicated in the lower part of the figure. Cells are cultured in the presence of a short-chain lipid precursor which is taken up and converted into the corresponding short-chain lipid analogue. The short-chain lipid product can be extracted from the outer leaflet of the cell membrane by BSA if it is actively transported across it. For an extensive description of the methodology see [5]. BSA, bovine serum albumin; Cer, ceramide; DAG, diacylglycerol; GlcCer, glucosylceramide; NBD, N-6 (7-nitro-2,1,3-benzoxadiazol-4-yl); PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM sphingomyelin. (B) Membrane lipid analogues transported by MDR1 Pgp, MDR3 Pgp, and MRP1.

drug-transporting Pgp early in vertebrate evolution, and since the MDR1 and MDR3 Pgps are 76% identical in amino acid sequence, it is not implausible that the PC translocator would have retained drug-transporting activity.

The first indication of drug transport by the PC translocator was obtained by Kino et al. [6]. They transfected yeast cells with an MDR3 gene construct and showed that this resulted in low-level resistance against the antifungal agent aureobasidin A. The issue was further addressed by Smith [66,67] who studied drug transport by pig kidney cell monolayers expressing the MDR3 gene. These cells showed substantial transport of digoxin, lower rates of transport of paclitaxel and vinblastine, and no significant transport of some other drugs that are transported at high rate by drug-transporting Pgps, such as cyclosporin A (CsA) and dexamethasone. Digoxin transport was efficiently inhibited by inhibitors of drug-transporting Pgps, CsA, PSC833 and verapamil.

These results are consistent with the idea that the PC translocator has low drug-transporting activity. This activity is only visible in the sensitive kidney monolayer drug transport assay, but not as drug resistance in transfected non-polarized cells. Direct binding of drugs to the MDR3 Pgp was recently demonstrated by Szabo et al. in the group of Sarkadi (pers. commun.). They expressed MDR3 in insect Sf9 cells and studied vanadate-dependent nucleotide trapping at low ATP concentrations. For the MDR1 Pgp Szabo et al. [68] have previously shown that the rate of nucleotide trapping is accelerated by low concentrations of some substrates and inhibitors of this protein and decreased at higher concentra-

tions. In the case of MDR3 Pgp drug addition only decreased trapping. The Pgp substrates paclitaxel and vinblastine and the inhibitors cyclosporin A and PSC833 were all able to decrease nucleotide trapping by 90% or more in concentrations similar to those used for inhibiting MDR1 Pgp. These results show that the MDR3 Pgp has substantial affinity for drugs that interact with MDR1 Pgp and they support the idea that the drug transport associated with this protein is actually done by the MDR3 Pgp itself and not by another transporter somehow activated by MDR3 Pgp.

## 7. A paradox

All available evidence suggests that the Mdr2/MDR3 Pgps are specialized PC translocators required for secretion of PC into bile. Although low levels of Mdr2/MDR3 RNA have been detected in some other tissues than liver, notably the B-cell compartment, heart and skeletal muscle, the corresponding protein was not detected in these tissues by immunohistochemistry or Western blots [69]. Low amounts of Mdr2 Pgp are present in mouse erythrocytes [70]. Moreover, the defects in the Mdr2 ( $-/-$ ) mice can be rescued by a MDR3 transgene driven by an albumin promoter and only expressed in liver [71].

The requirement for a new specialized PC translocator in mammals is also obvious from the results with Mdr2 ( $-/-$ ) mice. The bile of these mice is devoid of PC notwithstanding the presence of the two murine drug-transporting Pgps, Mdr1a and b in the canalicular membrane [1]. Obviously, these transporters are unable to translocate the PC from the cytosolic leaflet of the canalicular membrane toward extractable monolamellar vesicles and rescue the Mdr2 ( $-/-$ ) phenotype.

The first paradox is therefore that the PC translocator can transport drugs at all. As this Pgp has been optimized for PC transport in evolution, and as the canalicular membrane of the murine hepatocyte contains two dedicated drug-transporting Pgps, the Mdr1a and 1b Pgps, it is not obvious why retention of drug transport in the PC translocator would have any selective advantage. Three explanations for this paradox can be considered. First, it is possible that drug transport by MDR3 Pgp is an accidental side-

product of evolution. There is evidence for gene conversion between the *MDR1* and *MDR3* [72] genes and such gene conversion may have counteracted the genetic drift accompanying the evolutionary optimization of the protein as a PC transporter. Second, optimal transport of PC may require a protein that transports some drugs as an unavoidable consequence. We find that unlikely. ABC transporters have been adapted in evolution to a variety of highly specialized tasks and, in fact, the substrate specificity of the MDR3 Pgp towards membrane PLs is exquisite [5]. Third, MDR3 Pgp might be a dual-function protein, able to transport PC, but also some toxins that are especially threatening to the liver, the only organ where this transporter is present in high concentration. Both explanations 1 and 3 are compatible with the experimental evidence.

This brings us to the second paradox: How can the PC translocator transport drugs at all, if it is saturated with PC for which the transporter has a high affinity? A possible explanation could be that the PC translocator is able to locally deplete the inner leaflet of the plasma membrane of PC. In the absence of competing PC, the Pgp can bind inhibitors and transport substrates. This model [66,67] can also explain another paradoxical observation: whereas an efficient inhibitor of drug-transporting Pgps, such as PSC833, completely blocks drug transport by the MDR3 Pgp and also translocation of short-chain PC analogues [66], it has no effect whatsoever on PC secretion into bile in vivo, under conditions where digoxin transport into bile by drug-transporting Pgps is largely inhibited [73]. A possible explanation is that PSC833 can compete with low-affinity substrates, but not with the high-affinity substrate PC. This explanation cannot account, however, for the nucleotide trapping experiments of Szabó et al. [68], mentioned in the previous section. They found a high affinity of MDR3 Pgp for drugs at low ATP concentrations. Under these conditions the activity of Pgp is minimal and it certainly cannot deplete its local environment of PC.

Another explanation for the paradox should therefore be considered. This explanation postulates that MDR3 Pgp has a high affinity for drugs, higher than for PC, but a lower catalytic efficiency than MDR1 Pgp. This explains the combination of high affinity drug binding and a relatively low rate of transport,

too low to result in drug resistance. It also explains why Szabó et al. (see preceding section) were unable to find ATPase activity of MDR3 Pgp at Pgp concentrations where MDR1 Pgp ATPase activity is readily detectable. As PC is usually present at high concentrations, a low-efficiency, low-affinity transporter might be adequate for PC transport. It is conceivable that the high discriminatory power required for transport of PC, but not other membrane lipids may not be compatible with a high-affinity, high-efficiency transporter. Obviously, the explanation cannot account for the observation that PSC833 is unable to inhibit PC secretion into bile in vivo [73]. This is not a trivial objection: Fig. 5 shows that PC secretion is a sensitive measure of MDR3 Pgp activity.

To resolve this paradox more experiments need to be done. The drug affinity of the PC translocator should be studied in PC-free liposomes. It will also be important to verify whether MDR reversal agents, such as PSC833, are unable to inhibit translocation of long-chain PC in systems where they have been shown to block translocation of short-chain PC or drug transport by the PC translocator. The importance of defining drug effects on the PC translocator is underscored by the recent observation that women heterozygous for a MDR3 null allele are at risk for developing intrahepatic cholestasis of pregnancy [59]. It is therefore conceivable that the type of drug interactions with MDR3 Pgp described here could result in disease of heterozygotes under conditions of stress requiring maximal phospholipid secretion into bile.

## 8. MDR3-MDR1 chimeras

Attempts have been made to define what makes the MDR1 Pgp a drug transporter and the MDR3 Pgp (preferentially) a PC translocator. Buschman and Gros [74] showed that the ATP binding domains of the murine Mdr2 Pgp could replace their counterparts in a Mdr1-type Pgp without loss of drug transport. Replacement of as little as two transmembrane segments eliminated drug transport, however. Analogous results were obtained by Zhang et al. [75] who focused on the C-terminal part of Pgp. Replacement of the entire end of the MDR1 Pgp by MDR3 start-

ing at transmembrane segment 10 resulted in a non-functional protein. Replacements limited to transmembrane segment 12 led to a partial loss of transport. Further replacement attempts were made by Gottesman and coworkers who systematically replaced segments of the N-terminal half of the MDR1 Pgp gene by corresponding segments of the MDR3 Pgp gene. Construction of a chimeric protein in which the first intracytoplasmic loops of and the third and fourth transmembrane segments of MDR1 Pgp were substituted, resulted in a protein binding azidopine, but unable to transport drugs. When four loop residues (165, 166, 168, 169) in the chimera were changed back to their MDR1 equivalent, full activity was retained. Conversely, substitution of these four residues in MDR1 Pgp abolished surface expression of the mutant protein making it impossible to measure activity [76].

Subsequent work showed that MDR3 residues 165, 166, 168 and 169 only prevent surface expression in the context of a complete MDR1 Pgp, but are acceptable if present in a suitable chimera. Very recently Zhou et al. [77] were able to produce a functional drug transporter by replacing residues 1–394 (which contains the first six putative transmembrane segments; see Fig. 1) of MDR1 by the corresponding part of MDR3, provided that the critical MDR1 residues 330–332 in transmembrane segment 6 were retained. This confirms the high homology (76% identity) between MDR1 and 3 and shows that the ability to transport PC may depend on a limited number of residues. The analysis of these residues is complicated, however, by the inability of some chimeras to reach the plasma membrane. In fact, in the vaccinia viral system transiently expressed in HeLa cells, used by Zhou et al. [77], the MDR3 Pgp itself is not sufficiently expressed to measure drug binding or transport. Some of the results reported by Zhou et al. [77] may therefore reflect the intrinsic drug transport activity of MDR3 Pgp.

## 9. Transport of lipid analogues of membrane lipids by drug-transporting Pgps and MRP1

Using the polarized kidney cell monolayers transfected with human *MDR1* gene constructs, the group of Van Meer discovered that drug-transporting Pgps

are also able to translocate short-chain lipid analogues from the inner to the outer leaflet of the plasma membrane [5,78]. The range of membrane lipid analogues translocated is markedly large: not only C<sub>6</sub>-NBD-PC, but also C<sub>6</sub>-NBD-phosphatidylethanolamine (PE) is transported. Transport requirements are in fact very relaxed compared to MDR3 Pgp. A long-chain fatty acid is not required as C<sub>8</sub>C<sub>8</sub>-PC and -PE are also transported. The MDR1 Pgp is able to transport C<sub>6</sub>-NBD-SM, C<sub>6</sub>-NBD-GlcCer, C<sub>6</sub>-GlcCer and C<sub>8</sub>C<sub>8</sub>-GlcCer. A summary of all the lipid analogues transported is presented in Fig. 7B. Transport is inhibited by inhibitors of drug-transporting Pgps, such as verapamil and PSC833, and by energy depletion of the kidney cells.

The results of Van Helvoort et al. [5] were unexpected, because Ruetz and Gros [2] had found no C<sub>6</sub>-NBD-PC translocation by the murine Mdr1a Pgp in their yeast vesicular system. With hindsight these negative results may have been due to low expression of the Mdr1a gene in yeast, as Van Helvoort et al. [5] obtained the same results with Mdr1a as with MDR1 Pgp.

An important question raised by the work of Van Helvoort et al. [5] is whether drug-transporting Pgps can transport natural membrane lipids. One could argue that the NBD lipids and even the C<sub>8</sub>C<sub>8</sub> and C<sub>6</sub> lipids might be recognized by Pgp as an abnormal membrane constituent, 'a drug', and therefore removed from the inner leaflet of the plasma membrane. Indeed, there are strong arguments to support the idea that drug-transporting Pgps do not translocate long-chain PC at a significant rate. As pointed out by Van Helvoort et al. [5], Mdr2 (–/–) mice do not secrete any long-chain PL into their bile [1], even though there are substantial amounts of Mdr1a and Mdr1b Pgp present in the canalicular membrane of the hepatocyte [53]. Conversely, the Mdr1a/1b (–/–) mouse has no detectable abnormality in lipid metabolism [53]. If MDR1 Pgp would be able to transport long-chain PE and phosphatidylserine (PS), it would counteract the action of the aminophospholipid translocase that sees to it that PE and PS remain in the inner leaflet of the plasma membrane.

It remains possible, however, that Pgp recognizes unusual lipids, that arise in normal metabolism, e.g. platelet-activating factor (PAF), a C<sub>2</sub>-PC, or oxidatively fragmented PLs. Other possible natural sub-

strates could be lysoPLs or short-chain glycosphingolipids [5]. All these speculations remain to be tested.

If drug-transporting Pgps transport lipid analogues, other multidrug transporters might do the same. This was tested for MRP1 by Raggars et al. [79] in transfected kidney cell monolayers. They found that MRP1 transported C<sub>6</sub>-NBD-GlcCer and C<sub>6</sub>-NBD-SM, and that transport was inhibited by the MRP1 inhibitors sulfinpyrazone and indomethacin, but not by PSC833, one of the best inhibitors of Pgp. Transport by MRP1 was also strongly reduced by depletion of cellular GSH, indicating that GSH is required for transport of lipid analogues, as it is for transport of many drugs. GSH depletion had no effect on lipid transport by Pgp. As the *Mrp1* (–/–) mouse has no clear abnormalities [54,55], it seems unlikely that MRP1 has a significant role in lipid metabolism or transport beyond its role in leukotriene C<sub>4</sub> excretion. In experiments with erythrocytes it was shown that the transport activity of MRP1 is not limited to C<sub>6</sub>-NBD-GlcCer and C<sub>6</sub>-NBD-SM, as observed by Raggars et al. [79] in kidney cells. Kamp and Haest [80] found that C<sub>6</sub>-NBD-PS was recognized by an MRP-like activity and Dekkers et al. [81] showed that MRP1 could translocate C<sub>6</sub>-NBD-PS and C<sub>6</sub>-NBD-PC. They concluded that the fluorescent lipid analogues were recognized as xenobiotics, as the distribution of natural PS is not altered in *Mrp1* (–/–) erythrocytes. On the basis of these results we expect that all ABC transporters that can transport hydrophobic drugs will be able to transport analogues of membrane lipids. Indeed, Decotignies et al. [82] have reported that the yeast ABC transporters Yor1P and Pdr5p can extrude a fluorescent, short-chain NBD analogue of PE from the cell.

## 10. The Rim protein defective in Stargardt's disease

An ABC transporter, called ABCR or Rim protein (RmP), is defective in Stargardt's macular dystrophy [83,84] and in age-related macular degeneration [85]. ABCR is a distant relative of Pgp and it has the same putative membrane topology with 12 transmembrane segments and two ATP binding sites. ABCR is located in the rims of outer segment discs, which are the sites of photon capture in the retina. Recently,

Weng et al. [86] characterized the ocular defects in *abcr* null mice. These mice have elevated levels of all-*trans*-retinaldehyde (all-*trans*-RAL) in the retina following light exposure, elevated PE in outer segments, and accumulation of the protonated Schiff base complex of all-*trans*-RAL and PE (*N*-retinylidene-PE). The authors conclude that ABCR functions as an outwardly directed flippase for *N*-retinylidene-PE. Whether ABCR can also transport other complex lipids, or is a highly specialized transporter dedicated to a single substrate remains to be determined.

### 11. ABC1, a cholesterol transporter?

A recent series of papers [87–89] in *Nature Genetics* has moved the modest contribution of ABC transporters to lipid transport into the limelight, because they link *ABC1* to atherosclerosis [90]. *ABC1* is a distant relative of P-glycoprotein and with its 2201 amino acids one of the largest ABC transporters known. Like CFTR, it has a very large hinge region, but the hinge of *ABC1* includes a highly hydrophobic domain that may also be inserted in the membrane, in addition to the conventional 12 transmembrane segments. *ABC1* is 62% identical in amino acid sequence to ABCR (the Rim protein) [91].

*ABC1* was cloned in 1994 in the laboratory of Giovanna Chimini [92] and initial attempts to find a function for this ubiquitous ABC transporter gave equivocal results [93–95]. The recent work [87–89] now shows that mutations in the *ABC1* gene in humans result in familial high density lipoprotein deficiency, whereas the complete absence of functional *ABC1* results in Tangier disease, in which plasma high density lipoprotein (HDL) is nearly absent due to rapid degradation of HDL precursors. It had been known for some time that fibroblasts from patients with Tangier disease are defective in cholesterol efflux. The recent data indicate that *ABC1* is responsible for cholesterol efflux from the cell and that the absence of HDL from the plasma of Tangier disease patients is due to the inability of HDL precursors to acquire cholesterol from cells. The fact that *ABC1* expression is upregulated when macrophages are incubated with HDL [91] is in good agreement with the proposed function of *ABC1*. Hamon et al. [96] have reported that a fusion protein of *ABC1* with green

fluorescent protein attached at its C-terminus is routed to the plasma membrane in HeLa cells, suggesting that *ABC1* is a plasma membrane cholesterol transporter doing for cholesterol what the MDR3 Pgp does for PC. Part of the *ABC1* fusion protein also ended up in lysosomes, but this could be an artifact of overproducing the protein. Overproduced ABC transporters are often dumped in lysosomes.

Other results from the Chimini lab [94,95] on *ABC1* are not easily reconciled with its function as a cholesterol transporter. *ABC1* was reported to be an electroneutral anion transporter, required for interleukin-1 $\beta$  secretion, and involved in the engulfment of cells dying by apoptosis. *ABC1* was also reported to increase the transbilayer movement of phosphatidylserine. It is possible that the effects of *ABC1* on PS and apoptosis are related and secondary to an abnormal distribution/movement of cholesterol. The anion transport is more puzzling, but this was measured by iodide efflux and it is conceivable that this efflux might also be non-specifically affected by cholesterol movements.

Interestingly, Schmitz and coworkers [97] have observed a tremendous upregulation of *ABC8* if macrophages are incubated with acetylated low density lipoprotein. There may therefore be other ABC transporters linked to lipid transport and atherosclerosis.

### 12. Other ABC transporters involved in lipid transport

In view of the ability of drug-transporting Pgps to transport hydrophobic compounds that insert in membranes, it would not be surprising if some of these Pgps had evolved to become specific lipid transporters, other than the PC translocator. An obvious candidate was the aminophospholipid translocase, but this transporter now appears to belong to the P-type ATPase family. Tang et al. [98] cloned a Mg<sup>2+</sup> dependent ATPase from bovine chromaffin granules which belongs to a new subfamily of P-type ATPases which includes among others the *FIC-1* gene in humans, and the *DRS2* gene in yeast. A *drs2* null yeast mutant shows defects in PS internalization. However, other studies [99] have failed to obtain similar results with *drs2* mutants. A definite proof of the proteins' functions awaits the demon-

stration that expression of either of these proteins can restore aminophospholipid translocation in aminophospholipid translocase defective mutants.

In contrast, there is now considerable evidence that an ABC transporter is involved in the metabolism of long-chain fatty acids by peroxisomes. As this topic is discussed in detail by Hetteema and Tabak [100] in this volume, we only mention here that a heterodimeric ABC transporter, consisting of two Pgp-type half-molecules, is essential for import of long-chain fatty acids into peroxisomes, probably because it acts as a transporter of the acyl-CoA made in the cytosol into peroxisomes. The inability to make one of the subunits of this putative acyl-CoA translocator, called PMP<sub>70</sub>, can cause a severe inborn error in humans, the Zellweger syndrome.

It is probable that additional ABC transporters involved in lipid metabolism will turn up in the coming years. A recent paper by Piper et al. [101] has shown, for instance, that the resistance of yeast to weak acids is due to the ABC transporter Pdr12. This can extrude benzoate and acetate from the cells as well as a range of organic acids used for food preservation. We expect that there is more to come.

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