P-glycoproteins (P-gps) are plasma membrane glycoproteins of about 170 kDa that belong to the superfamily of ATP-binding cassette (ABC) transporters, also called traffic ATPases (Refs 1, 2). They were discovered by Juliano and Ling2 in multidrug-resistant (MDR) cancer cells and they cause resistance by the active extrusion of a wide range of amphipathic (natural-product) drugs used to treat cancer.

Genetic approaches have contributed to our understanding of P-gp function in at least three ways: (1) transfection studies initially established those P-gps that could transport drugs and those that could not; (2) reverse genetics has located residues involved in substrate specificity and ATP hydrolysis, and provided information on the transmembrane topology of these proteins; (3) gene disruptions in mice (knockout mice) have helped to define the physiological role of mammalian P-gps and to eliminate some of the more colourful functions attributed to these transporters by imaginative scientists. Topics (1) and (2) have been amply reviewed in Refs 4–7. Here we concentrate mainly on topic (3).

Structure, modification and nomenclature of mammalian P-gps

Humans have two known P-gp genes, MDR1 and MDR3 (also known as MDR2). The genes are adjacent on chromosome 7 and their intron–exon structure is known7,8. MDR1 encodes a drug-transporting P-gp, whereas MDR3 encodes a P-gp that is highly specific for the translocation of phosphatidylcholine10–14. The other mammals studied also have a single phosphatidylcholine translocator P-gp, but they have multiple drug-transporting P-gps (Table 1): two in rodents and probably more in pigs. In rodents, the P-gp genes are also linked, but the structure of the locus is not yet known in detail.

The transmembrane topology of P-gp (Fig. 1) was initially deduced from hydrophathy plots. More recently, several techniques have been used to verify the predicted structure. There is agreement that the N- and C-terminus, the ATP-binding sites and the linker region are located intracellularly, but the exact number of transmembrane segments remains controversial, especially in the C-terminal half of P-gp. Most studies support the structure shown in Fig. 1, although some suggest fewer or more transmembrane segments (reviewed in Ref. 7). The two halves of the molecule must be closely associated in the membrane to allow a single MRK16 antibody molecule to interact with determinants present in both halves of P-gp (Ref. 15).

P-gps undergo considerable post-translational modification. In the MDR1 P-gp there are three carbohydrate side chains, all located in the first extracellular loop (Fig. 1). Removal of these N-glycosylation sites does not affect the function or the substrate specificity of the MDR1 P-gp, but reduces the effectiveness of P-gp as a mediator of MDR, possibly because of lowered stability, or less effective routing of the protein to the plasma membrane10. Other studies agree with this interpretation7. P-gps are also phosphorylated at many serines and threonines, and indirect evidence has raised the possibility that phosphorylation regulates drug transport by P-gps. However, systematic replacement of Ser/Thr by Ala (which cannot be phosphorylated), or Asp (which mimics the negative charge of a phosphorylated Ser/Thr), has not yielded P-gps with altered drug transport activity17,18.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Drug transporters</th>
<th>Phosphatidylcholine translocatora</th>
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<tbody>
<tr>
<td>Humans</td>
<td>Mdr1a (mdr3)</td>
<td>Mdr1b (mdr1)</td>
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<tr>
<td></td>
<td>MDR1</td>
<td>MDR3 (MDR2)</td>
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<td>Mice</td>
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<tr>
<td>Rats</td>
<td>Mdr1a</td>
<td>Mdr1b</td>
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<tr>
<td>Hamsters</td>
<td>Pgp1</td>
<td>Pgp2</td>
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a The P-glycoproteins (P-gps) encoded by the human MDR3 and the murine Mdr2 genes encode phosphatidylcholine translocators8. In view of the high degree of sequence identity of these two P-gps with the P-gps encoded by the Pgp3 genes of rats and hamsters, it is likely that these are also phosphatidylcholine translocators, but this has not been experimentally verified.

b Evidence for sequences corresponding to a second P-gp gene in humans, called MDR2, was first obtained by Roninson et al48. A functional and expressed gene was later cloned by Van der Bliek et al49. It is not known if this P-gp gene corresponds to the murine Mdr2 gene.

c The first two murine P-gp genes were discovered and cloned by Ruetz and Gros6, called mdr1 and mdr2. To avoid confusion, Hsu, Lothstein and Horwitz later introduced the Mdr1a/Mdr1b nomenclature used here (Ref. 46).
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Figure 1. Model of the human multidrug resistance gene product P-glycoprotein (P-gp) and its functional domains. Two putative ATP-binding sites are circled in grey and putative N-linked carbohydrates are represented as wiggly lines. Amino acid residues that, when substituted, alter the substrate specificity of the multidrug transporter are indicated in black. Phosphorylation sites are shown as a circled P. Bars indicate the general region that appears to be involved in determining the substrate specificity of P-gp and the photoaffinity labelling sites. (Reprinted by permission from Ref. 42 and updated in Ref. 7.)

Substrate binding and translocation domains of P-gp

Attempts to define these domains have followed three routes: (1) analysis of amino acid substitutions in P-gps with altered substrate specificity; (2) systematic replacement of amino acids, or protein segments, and evaluation of the effects on P-gp function; (3) analysis of P-gp peptides labelled by substrate analogues that can be covalently linked to P-gp. The results of these analyses are summarized in Fig. 1, taken from a review by Germann in which all available experimental data are critically reviewed. Mutations that affect the conserved residues of the nucleotide-binding sites of P-gp usually abolish drug transport, even if ATP binding is not affected. ATP hydrolysis is required for transport and, interestingly, both ATP-binding sites are essential. On the basis of vanadate-trapping experiments, Senior et al. proposed that the sites alternate in catalysis explaining why both are required.

Experiments designed to define the parts of P-gp that interact with a drug have given complex results and suggest that there is no simple single drug-binding site or drug pore in P-gp. Drug analogues are primarily cross-linked to protein segments containing the transmembrane segments 6 and 12, suggesting that these play a special role in drug handling. However, amino acid substitutions in, or near, most of the transmembrane segments affect substrate specificity or transport efficiency. Although this is compatible with an important role of the transmembrane segments in drug binding and/or transport, it should be realized that the cytoplasmic loops have not been systematically checked for the effect of amino acid substitutions on drug transport. Indeed, substitution of several amino acids in the poorly conserved ‘linker’ region, which links the two halves of the protein, had major effects on substrate specificity and transport efficiency.

More direct tests of drug binding and of transport kinetics are required to determine whether such mutations affect the conformation of the protein or a drug-binding site.

The physiological functions of drug-transporting P-gps: studies with knockout mice

Life is a competitive business. Scientists might feel the pressure at times, but in the soil around the institute competition is considerably fiercer. A multitude of organisms compete for food, and in these battles a variety of toxins are used to kill or repel competitors or predators. Defense against toxins is, therefore, a major preoccupation of even the simplest microorganism, and drug-transporting ATPases are essential components of this defense. Bacteria, fungi, protozoa and simple metazoans, such as Caenorhabditis elegans, contain multiple transporters of this class allowing them to survive drugs, toxins or heavy metals. Some of these transporters confer MDR with a resistance profile remarkably similar to that of mammalian MDR cells. Examples are the transporters encoded by the \textit{LmrA} gene of \textit{Lactococcus} and by the \textit{ldm2r} gene of the ancient unicellular eukaryote \textit{Leishmania} (reviewed in Ref. 22).

It is, therefore, reasonable to expect that mammalian P-gps also play a role in defense against xenotoxins; they are well placed for this role in the body. P-gps are present in the apical membranes of epithelia in contact with food, in the canalicular membrane of the liver cells and in the kidney tubules where they might help in drug excretion, and at blood-tissue barriers, such as the blood-brain barrier, where they might help to protect vital structures against amphipathic toxins. Nevertheless, many additional functions have been proposed for the drug-transporting P-gps. These range from transport of steroid hormones to secretion of cytokines, from a role...
in cytotoxicity of natural killer cells to intracellular transport of cholesterol (reviewed in Ref. 23).

To analyse the physiological functions of P-gps, we have generated knockout mice with disruptions of the \textit{Mdrla} gene, of the \textit{Mdrlb} gene, and of both genes, \textit{Mdrla/b} (-/-) or double knockout mice. Details of these mice have been published\cite{24,25}. In each case, the available evidence indicates that the disrupted allele is a null allele and does not give rise to functional fragments of protein.

All three knockout mice appear to be completely normal as long as they are not challenged with drugs. The oldest \textit{Mdrla/b} (-/-) mice are now 12 months old and they are as healthy and fertile as their (+/-) litter mates. Hence, drug-transporting P-gps appear to have no essential physiological functions required for living in the protected, congenial environment of the animal house of The Netherlands Cancer Institute. We add two caveats to this conclusion: (1) the analysis of the double knockout mouse is not yet complete, and subtle defects might still emerge as the analysis progresses; and (2) the absence of P-gps \textit{ab initio} might allow the development of compensatory mechanisms that obfuscate the detection of essential P-gp functions. For instance, the expression of \textit{Mdrlb} is upregulated in the liver and kidney (but not in other tissues) of \textit{Mdrla} (-/-) mice\cite{26}. We have looked at possible compensatory upregulation of genes for other transporters, such as \textit{Mrp}, \textit{Sister of P-gp}, \textit{Oct1} and \textit{Cfr}, in the \textit{Mdrla/b} (-/-) mice and found none\cite{27}, but this is obviously only a very small fraction of the genes that might compensate for the absence of P-gp.

**Mice lacking one or both drug-transporting P-gps are hypersensitive to amphipathic drugs**

Mice that lack one or both drug-transporting P-gps have problems in handling drugs transported by P-gp. These problems are illustrated by the oversimplified drug mouse in Fig. 2a. Many amphipathic drugs are taken up from the bloodstream by the liver and secreted into the bile, often unmodified. It is thought that P-gp in the canalicular membrane plays a major role in their excretion. On the long journey towards faeces and the outside world, these drugs are in contact with ductal and gut membranes, and they would passively diffuse back into epithelial cells if these membranes were not protected by P-gp. In fact, they are normally protected, as indicated in Fig. 2b.

So far, drug handling has only been studied in detail in the \textit{Mdrla} (-/-) mouse. The MDR1A P-gp is the major mouse P-gp, and the only P-gp in brain capillaries and in the gut epithelium. It is also present in substantial concentrations in other major organs, such as heart and lung\cite{28}. The absence of this P-gp should, therefore, lead to a decreased elimination of amphipathic drugs from the body, unless the drug is rapidly metabolized into products not transported by P-gp. Decreased elimination has, indeed, been found for drugs such as the anticancer drug vinblastine\cite{29,30} and the heart drug digoxin\cite{31}. A more detailed evaluation of the contribution of P-gp to drug elimination will have to come from further studies in the \textit{Mdrla/b} (-/-) mice, in which the MDR1B P-gp present in kidney and liver is also eliminated.

The complete loss of P-gp from the gut and the blood-brain barrier has profound effects on drug distribution.

The elimination of the MDR1A P-gp from the apical membranes of brain capillaries dramatically increases the penetration of some drugs into the brain. The accumulation of the acaricide ivermectin\cite{32}, of vinblastine\cite{33,34} and of digoxin\cite{35} in the brain increases by two orders of magnitude. Relatively innocuous drugs with a wide margin of safety in wild-type mice, such as ivermectin, are lethal to \textit{Mdrla} (-/-) mice\cite{36}. A substantial increase in brain penetration was also found for several other drugs normally transported by P-gp (Ref. 29). Obviously, P-gp is an important component of the blood-brain barrier and is essential to keep amphipathic toxic compounds out\cite{37}.

We have also established an important role for intestinal P-gp in the excretion of drugs into the intestine and in limiting the uptake of drugs from the intestine. In
wild-type mice, digoxin is mainly excreted in the faeces, even after intravenous administration. This is, in part, due to a direct excretion of digoxin into the gut lumen and this excretion is abolished in the Mdr1a (−/−) mice. P-gp in the brush border of the intestinal cells also hinders drug uptake from the gut, as shown by experiments with paclitaxel (Taxol). The amount of drug reaching the blood after oral administration (the oral availability) is threefold higher in Mdr1a (−/−) compared with (+/+) mice. As it is now possible to block intestinal P-gp effectively with relatively specific P-gp inhibitors (discussed in Ref. 23).

**How do P-glycoproteins work?**

The elucidation of the primary structure of P-gp and its deduced transmembrane topology initially led to the idea that P-gps can literally function as pumps. According to this hypothesis, the 12 transmembrane segments come together to form a drug pore and drugs are transported through this pore with the help of energy generated by the hydrolysis of ATP. How ATP hydrolysis is coupled to vectorial transport is not clear and this remains a weak point of all versions of this model proposed so far. Several modifications of the drug pore model have been proposed since 1986, an important one being that drugs can also enter the putative central pore of P-gp from the membrane as well as from the cytoplasm. Raviv et al. even suggested that drugs enter P-gp preferentially from the membrane, and that P-gp is a membrane vacuum cleaner recognizing molecules that do not belong in the membrane and removing them.

A second model proposes that P-gp is not a primary drug transporter, but that it alters the ionic composition of the cell and that this, secondarily, changes drug distribution between the cell and its surroundings. This model originated from experiments that were interpreted to show that the action of P-gp results in an alkalization of the cell with concomitant redistribution of drugs. Although this model still has supporters, we shall not further discuss it here because the evidence that P-gp really is a primary drug transporter, summarized by Germann, and Ruetz and Gros, is now overwhelming in our opinion.

How P-gp transports drugs is still unknown. An interesting idea is that it acts as a drug flipase. This model is based on the analogy between amphipathic drugs and the normal phospholipid constituents of membranes. Whereas the lateral mobility of phospholipids within the membrane is high, the spontaneous rate of flipping between the two leaflets of the membrane is very low, because the polar head groups of the phospholipids cannot easily pass the hydrophobic interior of the membrane made up by the hydrophobic parts of the phospholipids. Enzymes have been described that can speed up this flipping reaction and these enzymes are called flipases or phospholipid translocators (Ref. 39).
Higgins and Gottesman\textsuperscript{38} pointed out that a flippase that flips drugs from the inner leaflet of the plasma membrane towards the outer leaflet against a concentration gradient, would act as a drug exporter, as illustrated in Fig. 3. Although this model was initially only based on theoretical considerations, it received a considerable boost when Smit \textit{et al.}\textsuperscript{22} found that the murine MDR2 P-gp is essential for the normal transport of phosphatidylcholine from the hepatocyte into bile. This suggested that this P-gp is a phosphatidylcholine flippase and subsequent work has supported this interpretation\textsuperscript{11-13}. The phosphatidylcholine-translocating P-gps are about 75% identical in amino acid sequence to the drug-transporting P-gps and the topology of both P-gp classes looks the same (Table 1). If the former are flippases\textsuperscript{10-14}, the latter could be flippases as well.

Further support for this model has come from experiments with fluorescent probes and with phospholipid analogues. The prototype of the fluorescent probes is BCECF. The acetoxymethyl ester of this probe, BCECF-AM, is nonfluorescent and the compound will only show up after passing through the plasma membrane and hydrolysis of the ester bond by esterases in the cytosol. Sarkadi and co-workers demonstrated that P-gp can intercept BCECF-AM in the membrane before it reaches the cytosol, supporting the idea that P-gp acts on drugs in the membrane. This was confirmed in \textit{Lactococcus lactis}, which makes a bacterial drug transporter that resembles the mammalian drug-transporting P-gps in substrate specificity\textsuperscript{15}. Additional experiments with another probe indicated that the rate of drug expulsion was proportional to the concentration of probe in the inner (but not the outer) layer of the plasma membrane\textsuperscript{16}, in agreement with a flippase mechanism for drug transport.

Additional support for this mechanism is provided by recent experiments by Van Helvoort \textit{et al.}\textsuperscript{14}, who studied flipping of short-chain fluorescent phospholipid analogues in pig kidney cells transfected with \textit{MDR1}, \textit{MDR3} or \textit{Mdr1a} cDNA constructs. They confirmed the high specificity of the \textit{MDR3} P-gp for phosphatidylcholine analogues, but found that the \textit{MDR1} and the \textit{MDR1A} P-gp can translocate a range of short-chain phospholipids from the inner to the outer layer of the plasma membrane\textsuperscript{17}. This was highly unexpected because Ruetz and Gros\textsuperscript{12} had reported that the \textit{MDR1A} P-gp is unable to flip phospholipids when present in vesicles isolated from yeast transfectants. The reason for the discrepancy is unclear, but we think that the positive result obtained with P-gp in its normal location in the apical membrane of a polarized mammalian cell\textsuperscript{18} is the more significant one. The simplest interpretation of these recent experiments is, therefore, that drug-transporting P-gps act as flippases, just like the P-gps transporting phosphatidylcholine. It is ironic that the aminophospholipid translocase of the plasma membrane, recently cloned, is not related to P-gp, but a member of a subfamily of P-type ATPases (Ref. \textsuperscript{41}).

In their 1992 article, Higgins and Gottesman\textsuperscript{38} also raise the possibility that P-gp might also flip one or more normal membrane lipids and that intercalated drugs are flipped 'by mistake'. Flipping of normal lipids by \textit{MDR1}-type P-gps now seems unlikely as the \textit{MDR1A} and \textit{MDR1B} P-gps present in the hepatocyte canalicul membrane seem unable to translocate phosphatidylcholine into bile, because translocation is zero in the absence of the MDR2 P-gp (Ref. 10). It is, therefore, more likely that MDR1-type P-gp can specifically recognize 'agents which intercalate and introduce discontinuities in the bilayer, essentially cleaning out the membrane\textsuperscript{39}.'

Concluding remarks
The genetic approach has provided useful information about the structure and function of drug-transporting P-gps. Further insight into the mechanism of drug transport will probably have to come from a detailed three-dimensional structure of the protein and additional biochemical studies on purified protein reconstituted into defined lipid membranes. The knockout mice have become a rich source of information on the physiological and pharmacological roles of P-gps in mammals. As disruptions of genes for other transporters become available, it will be possible to cross these defects into the \textit{Mdr1a/b} (\textit{--/--}) mouse, and dissect transport pathways and defense systems of increasing complexity.

Acknowledgements
We thank our colleagues and collaborators for their permission to quote unpublished experimental results and for helpful comments on the manuscript. The experimental work in our laboratory is supported in part by grant NKI 92-41 of the Dutch Cancer Society.

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Programmed cell death occurs in most, if not all, organisms as a way of removing unwanted cells. These cell deaths involve a series of distinct morphological changes, which, collectively, define the process of apoptosis. Apoptotic cell deaths are typically characterized by the blebbing of cell membranes, fragmentation of the cell with retention of organelle structure, condensation of chromatin, cleavage of DNA into nucleosomal size fragments and rapid engulfment of the dying cell by macrophages. This stereotypical morphology of cell death has been conserved between invertebrates and mammals.

Apoptosis is of central importance to the normal development of an organism and plays a key role in many diseases. In vertebrates, apoptosis can be induced by a bewildering number of distinct death-inducing stimuli, including the lack of extracellular survival factors, steroid hormones, activation of membrane-bound 'death receptors', viral infection, heat shock, oxidative stress, excitotoxicity, ionizing radiation and various other cellular insults. Remarkably, for any given cell type, different death-inducing stimuli produce corpses of identical apoptotic morphology, indicating that different signaling pathways ultimately converge to activate a common death program. This stereotypical morphology of cell death has been conserved between invertebrates and mammals.

A genetic basis for programmed cell death was first reported by Horvitz and coworkers with their discovery of ced-9, ced-3, ced-4 and Grim. Additionally, Drosophila homologs of evolutionarily conserved IAPs (inhibitor of apoptosis proteins) and CED-3/ICE-like proteases have been identified and characterized. Through the combined use of genetic, molecular, biochemical and cell biological techniques in Drosophila it should now be possible to elucidate the precise mechanism by which apoptosis occurs, and how the death program is activated in response to many distinct death-inducing signals.

Facing death in the fly: genetic analysis of apoptosis in Drosophila

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Apoptosis, a gene-directed form of cell death, occurs normally during development and plays a major role in many diseases, including cancer and neurodegenerative disorders. Molecular genetic studies in Drosophila have revealed the existence of three novel apoptotic activators, reaper, head involution defective and grim. Additionally, Drosophila homologs of evolutionarily conserved IAPs (inhibitor of apoptosis proteins) and CED-3/ICE-like proteases have been identified and characterized. Through the combined use of genetic, molecular, biochemical and cell biological techniques in Drosophila it should now be possible to elucidate the precise mechanism by which apoptosis occurs.

occuring cell deaths. This block in cell death is caused by loss-of-function for ced-3 and ced-4, but by gain-of-function for ced-9. Loss-of-function for ced-9 causes ectopic cell death, suggesting that the normal function of ced-9 is to prevent cell death. Epistasis analysis places ced-3 and ced-4 downstream of ced-9, and overexpression experiments suggest that ced-3 can act downstream of ced-4 (Refs 8, 9).

While ced-4 homologs have not yet been found, ced-3 and ced-9 are both homologous to growing families of mammalian genes (Table 1). ced-9 shares homology with BCL2, a human gene that is over-expressed...