Human Multidrug Resistance ABCB and ABCG Transporters: Participation in a Chemoimmunity Defense System

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Sarkadi, Balázs, László Homolya, Gergely Szakács, and András Váradi. Human Multidrug Resistance ABCB and ABCG Transporters: Participation in a Chemoimmunity Defense System. Physiol Rev 86: 1179–1236, 2006; doi:10.1152/physrev.00037.2005.—In this review we give an overview of the physiological functions of a group of ATP binding cassette (ABC) transporter proteins, which were discovered, and still referred to, as multidrug resistance (MDR) transporters. Although they indeed play an important role in cancer drug resistance, their major physiological function is to provide general protection against hydrophobic xenobiotics. With a highly conserved structure, membrane topology, and mechanism of action, these essential transporters are preserved throughout all living systems, from bacteria to human. We describe the general structural and mechanistic features of the human MDR-ABC transporters and introduce some of the basic methods that can be applied for the analysis of their expression, function, regulation, and modulation. We treat in detail the biochemistry, cell biology, and physiology of the ABCB1 (MDR1/P-glycoprotein) and the ABCG2 (MXR/BCRP) proteins and describe emerging information related to additional ABCB- and ABCG-type transporters with a potential role in drug and xenobiotic resistance. Throughout this review we demonstrate and emphasize the general network characteristics of the MDR-ABC transporters, functioning at the cellular and physiological tissue barriers.
addition, we suggest that multidrug transporters are essential parts of an innate defense system, the “chemo-immunity” network, which has a number of features reminiscent of classical immunology.

I. INTRODUCTION: MULTIDRUG/XENOBIOTIC RESISTANCE AND ABC TRANSPORTERS

The so-called “multidrug resistance” proteins were discovered as membrane transporters producing chemotheraphy resistance in cancer. In the mid to late 1970s, clinical oncologists realized that certain tumors show an inherent resistance pattern, while others develop resistance during the course of the treatment against a number of otherwise highly efficient chemotherapeutic compounds. Moreover, when tumor resistance developed against a single particular chemotherapeutic agent, in many cases the resulting phenotype involved a wide-range (or multi-)drug resistance pattern. Biochemists, molecular biologists, and cell biologists in the last 30 years clearly established that this phenomenon is due to the expression of plasma membrane “pumps,” which actively extrude various cytotoxic agents from cancer cells. Upregulation of pump expression and/or selection of resistant cancer cells thus result in cancer multidrug resistance, which extends to the diverse substrates of these transporters (for the first description of cancer “drug pumps,” see Refs. 71 and 164).

Multidrug resistance transporters belong to the evolutionarily conserved family of the ATP binding cassette (ABC) proteins, present in practically all living organisms from prokaryotes to mammals. ABC transporters are large, membrane-bound proteins, built from a combination of characteristic domains, including membrane-spanning regions and cytoplasmic ATP-binding domains (see sect. ii).

In humans, the three major types of multidrug resistance (MDR) proteins include members of the ABCB (ABCB1/MDR1/P-glycoprotein), the ABCC (ABCC1/MDR2, ABCC2/MDR2, probably also ABCC3–6, and ABCC10–11), and the ABCCG (ABCCG2/MDR5/BCRP) subfamily. On the basis of a great deal of clinical and experimental work, it has been established that these pumps recognize a very wide range of drug substrates. Although recognized substrates are mostly hydrophobic compounds, MDR pumps are also capable to extrude a variety of amphipathic anions and cations. As discussed in detail below, ABCB1 preferentially extrudes large hydrophobic molecules, while ABCC1 and ABCCG2 can transport both hydrophobic drugs and large anionic compounds, e.g., drug conjugates (Fig. 1). This “promiscuous” character, and the additional overlapping substrate recognition by the three major classes of the MDR-ABC transporters, provide an amazing network of drug resistance capacity in cancer cells.

Of course, these multidrug transporters did not evolve to protect cancer cells from medical interventions. In contrast, they are only co-opted and “misused” by the rapidly dividing cancer cells, especially when a population of malignant cells overexpressing an MDR-ABC transporter is selected by drug treatment. With regard to their physiological role, MDR-ABC transporters most probably evolved as complex cellular defense systems, for the recognition and the energy-dependent removal of toxic agents entering the living cells or organisms from their environment. In this review we document the wide variety of cellular functions that involve multidrug transporters. In addition, we put forward a general concept to suggest that the key physiological task of the MDR-ABC transporter network is to provide general xenobiotic resistance.

We also describe the general structural and mechanistic features of the MDR-ABC transporters and introduce some of the basic methods that can be applied for the analysis of their expression, function, regulation, and modulation. We treat in detail the biochemistry, cell biology, and physiological aspects of the ABCB1 (MDR1/P-glycoprotein) and the ABCG2 (MXR/BCRP) proteins, while a detailed description of the ABCB (MRP) group of transporters is provided by the review of Deeley et al. (73). We also mention here emerging information related to additional ABC transporters with a potential role in drug and xenobiotic resistance and provide a general picture about key aspects of their cellular regulation.

Throughout this review, although biased in focusing on selected human ABC proteins, we demonstrate and emphasize the general network characteristics of the multidrug transporters, functioning at cellular and physiological tissue barriers. We try to provide a new framework for the appreciation of their role in physiological defense against chemicals, by suggesting that multidrug transporters are essential parts of an immune-like defense system. This cellular antitoxic network provides a “chemoimmunity,” having a number of features reminiscent of innate immunology.

Thus physiology, biochemistry, pharmacology, and toxicology aspects inherently overlap in the present review. We certainly hope that, in addition to a detailed characterization of these transport systems, we will be able to convince the readers about the validity of a general concept that we hope will further our understanding of these multidrug transporter proteins of outstanding medical importance.
II. GENERAL STRUCTURE AND MECHANISM OF ACTION OF MDR-ABC TRANSPORTERS

A. Basic Features of the ABC Transporters

1. Conserved domains, structural motifs, and catalytic mechanism

ABC proteins have been identified in each genome sequenced, and they typically form large families with 30–100 members in various organisms. ABC proteins are named after a conserved, specific ABC domain (140), a 200- to 250-amino acid globular protein unit, which can bind and hydrolyze ATP. The ABC unit (also called nucleotide binding domain or NBD) harbors several conserved sequence motifs. From NH$_2$ to COOH terminal, these are the Walker A (P-loop), a glycine-rich sequence; a conserved glutamine (Q-loop), the family-specific ABC-signature (LSGGQ) motif (also called the C-loop), the Walker B motif, and a conserved His (His-switch). The ABC-signature motif is diagnostic for the family as it is present only in ABC proteins, while Walker A and B motifs are found in many other ATP-utilizing proteins (396).

ABC transporters also contain transmembrane domains (TMD), composed in most cases of six membrane-spanning helices. In archa and in prokaryotes, the ABC and the transmembrane domains are often encoded by separate genes within the same operon, while in some cases a single gene contains the TMD fused to an ABC unit. In bacteria these proteins either function as importers of essential compounds, or they export materials from the cell or lipids into the outer leaflet of the membrane. In eukaryotes, most active ABC transporters export compounds from the cytosol to the outside of the cell, or move molecules into intracellular organelles, like the endoplasmic reticulum, or the peroxisome. The human (mammalian) xenobiotic transporters discussed in this review are all export pumps, predominantly residing in the plasma membrane.

The multidrug/xenobiotic resistance (MDR) ABC proteins are primary active transporters, since they utilize the energy of cellular ATP for the promotion of vectorial, transmembrane movement of drugs or xenobiotics. These ATP hydrolytic enzymes (ATPases) interact with two different types of substrates. The energy donor substrate is the intracellular MgATP complex, and the chemical energy for the active transport of substrates is provided by binding and hydrolysis of ATP within the ABC units. In contrast to P-type ATPases, in MDR-ABC proteins ATP hydrolysis does not involve covalent phosphorylation. The end products of the hydrolysis are intracellular ADP and inorganic phosphate (for recent reviews, see Refs. 13, 34, 48, 102, 210, 213, 300, 331, 389).

Based on this molecular mechanism of action, the catalytic and transport properties of MDR-ABC transporters are significantly different from those of the P-type ATPases. Since these data are best provided for the MDR1/P-glycoprotein (Pgp) transporter, we discuss these issues in section IV.

In all MDR-ABC transporters, the sites interacting with the transported substrates are most probably located within the TMDs. It seems likely that a minimum of 12 membrane-spanning helices are required to ensure the complex reaction with the transported substrates. In a phenomenological sense, the transported substrates are bound to intracellular (or in some cases probably intramembrane), high-affinity “on” sites and are unloaded at extracellular, low-affinity “off” sites. However, all recent
structural studies indicate a relatively large drug binding pocket within the transmembrane regions of the MDR-ABC proteins (see Refs. 48, 50, 156, 213). The molecular link, transmitting intramolecular signals between the TMDs and the ABCs, that is the substrate binding area and the catalytic machinery, respectively, is still unidentified.

These basic catalytic and active transport features of MDR-ABC transporters have been documented in numerous expression and isolation/reconstitution systems, although the exact binding sites, as well as the energetics and thermodynamics (“uphill” or “downhill” nature) of the transport processes are difficult to estimate. As detailed in this review, many of the transported substrates are hydrophobic molecules, which are concentrated in the membranes, while they have only minimum solubility either in the cytoplasmic or extracellular water phase. Therefore, the classical solute concentration ratios or electrochemical potential gradients cannot be fully appreciated, and even the stoichiometry of the transport and ATP cleavage is difficult to determine. Moreover, it is often questionable if a given molecule is a transported substrate, an inhibitor, or a transport modulator (see sect. iv). In the respective sections we discuss some of the details of the energetics and substrate recognition of individual MDR-ABC transporters.

The main subjects of this review are the human ATP-driven ABC transporter proteins, which can act as xenobiotic exporters. However, there are several membrane-associated human ABC proteins with predominant channel- or even receptor-type functions, which share common structural and regulatory features with the active drug transporters. While active ABC transporters hydrolyze ATP in close coupling with the transmembrane movement of a substrate molecule, channels and receptors use the ATP binding domains mostly for the regulation of opening and/or closing pathways, allowing the passage of ions or conveying information through the membrane. Among the human ABC transporters, well-characterized proteins carrying out such functions are ABCC7/CFTR, ABCC8/SUR1, and ABCC9/SUR2.

The ABCC7/CFTR protein forms a chloride ion channel, in which opening and closing is regulated by the binding of ATP, and by a subsequent, relatively slow ATP hydrolysis. Here the driving force of the chloride ion movement is the electrochemical potential gradient, and the ion movement has no stoichiometric relationship with ATP hydrolysis by the CFTR (for recent reviews, see Refs. 99, 142, 286, 391). The sulfonylurea receptors (ABCC8 and -9 or SUR1 and SUR2, respectively) work as regulatory subunits of ATP-dependent potassium channels in the insulin-producing beta cells of the pancreas, and in the heart, respectively. The SUR/KATP channel tetramer, formed by ABCC protein/Kir6.x heterodimer units, is activated by ADP and inhibited by ATP; therefore, the SUR subunit serves as an ADP/ATP sensor that “translates” cellular metabolic changes into alterations of the membrane potential. Again, ATP hydrolysis is very slow in this protein complex (38, 233, 324).

2. Composition and membrane topology of human MDR-ABC transporters

According to a general consensus, all functionally active ABC transporters contain a minimum of two ABC units and two TMDs. These four elements in many cases are present in one single polypeptide chain, called “full transporters,” like the MDR1/Pgp/ABCB1 protein. In contrast, “half-transporters,” such as the members of the ABCG family, possess only a single ABC and a single TMD. Half-transporters must form homodimers or heterodimers to generate a functional ABC transporter.

The human genome encodes 48 (according to some databases, 49) ABC proteins. Their amino acid sequence alignments revealed that these proteins can be grouped into seven subfamilies, from A to G. The proteins relevant in multidrug transport are depicted in Figure 2A. With the comparison of the individual members, sequence identity/similarity of the ABC units is generally higher than that of the TMDs. However, each subfamily is characterized by typical and somewhat different membrane topology patterns. There are no high-resolution structural data available for any of the eukaryotic ABC transporters; therefore; combination of computer-assisted prediction methods, biochemical experimental data, and model building has been used to establish the position and orientation of the transmembrane segments within the polypeptide chain.

To elucidate the structural properties of ABC transporters, the biochemical arsenal includes epitope insertion, localization of N-glycosylation sites, generation of new N-glycosylation sites, limited proteolysis, and chemical cross-linking experiments. These experimental data are detailed in the relevant sections, discussing the human MDR-ABC transporters.

As a short summary, members of the ABCA subfamily are “full transporters” with the domain arrangement of TMD1-ABC1-TMD2-ABC2. The TMDs in this family contain very large extracellular loops with numerous glycosylation sites. The ABCB subfamily consists of three full transporters (including MDR1/Pgp/ABCB1) with the domain arrangement of TMD1-ABC1-TMD2-ABC2, and seven TMD-ABC type half-transporters. The membrane topology of the 12 members of the ABCB subfamily, containing an NH2-terminal extension, is discussed in detail in the review by Deeley et al. (73). The ABCD subfamily includes four half-transporters, with a TMD-ABC type arrangement. Members of the ABCE and ABCF family are not involved in membrane transport processes and lack
transmembrane domains. The five half-transporters in the ABCG subfamily show a reverse domain arrangement (ABC-TMD).

Of the 48 ABC transporters, MDR1/Pgp, several MRPs, and the ABCG2 protein certainly qualify for the MDR-ABC protein status. MDR3 (ABCB4), a closely related protein to MDR1/Pgp, and the relatives of ABCG2, the heterodimer ABCG5/ABCG8, are also ATP-dependent active transporters, and their involvement in drug and/or xenobiotic transport has also been documented. Figure 2 demonstrates the schematic membrane topology arrangements for the relevant ABCB and ABCG transporters.

Emerging information may suggest a similar, active drug transporter role for ABCG1 and/or ABCG4, as well as for ABCB5 and some ABCA type proteins, but we still have relatively little knowledge about these transporters. There are many other ABC transporters that most probably carry out substrate translocation by using the energy of cellular ATP [ABCB11 (BSEP/sister-Pgp), ABCB2/ABCB3 (TAP1/TAP2), ABCA4, members of the ABCD subfamily], but these have been not implicated in drug or xenobiotic transport and therefore are not discussed in this review.

B. Structural Basis of the Molecular Mechanism of Action in ABC Transporters

As of today, high-resolution structures are only available for bacterial ABC transporters. Therefore, for the discussion of human MDR-ABC proteins we have to rely on these data and models, which indicate a well-conserved structure and suggest a common basic mechanism of action. Unfortunately, the models reveal relatively little information about the substrate recognition or the intramolecular regulation of the individual mammalian homologs. In this section we discuss only general molecular aspects and provide further mechanistic details in the sections dealing with individual transporters.

1. The ABC fold

In several bacterial ABC proteins, the ABC units are expressed as separate proteins, and the first structural information was obtained in such systems. The first high-resolution structures of an ABC unit, that of RbsA (16) and HisP (139), were published in 1998. The HisP structure was solved with a resolution of 1.5 Å and represents an ABC monomer with the typical L-shaped (two lobe) ABC fold. Since then, the structure of several ABC units has been determined (see Fig. 3).

From these studies it became clear that the ABC domain has a unique fold, as the particular assembly of all the characteristic secondary structure elements is found only in ABC-ATPases. The ABC structure can be divided into three subdomains. The “F1-type ATP-binding core” is similar to the core of F1-ATPases and consists of six (5 parallel and 1 antiparallel) β-sheets. This subdomain contains the Walker A motif (P-loop), the Q-loop (γ-phosphate linker), the Walker B motif, and the “His-switch” (see Fig. 3A). The ABC-specific α-subdomain is built of four α-helices and contains the family-specific ABC signature (LSGGQ) motif, while the antiparallel β-subdomain contains elements responsible for the ribose/adenine orientation and interaction. The F1-type core and the ABC-specific β-subdomain form the larger lobe (the longer arm of the L-shaped structure), while the α-subdomain forms the smaller lobe (the shorter arm of the L).
In the first publications, the ABC structures were obtained for monomers, and the authors suggested a dimer, in which the two ABC units were positioned in a back-to-back orientation (139) (see Fig. 3B, I). However, this assembly represents an energetically unfavorable interaction of the two monomers and suggests two highly exposed nucleotide binding/catalytic sites, which is difficult to reconcile with the regulated function of the active sites. By now it has been convincingly documented that the two functionally interacting ABC subunits dimerize in a “head-to-tail” orientation. The two ABC domains complement each other’s active sites, forming two composite catalytic centers. The Walker A sequence of one subunit and the ABC signature motif of the opposite subunit are involved in the formation of each of the two composite ATP-binding/catalytic sites (Fig. 3B, II). This orientation was first suggested in studies describing the Rad50cd structure (a nontransporter bacterial ABC-ATPase) (130) and in models built using the HisP monomers (160). Later, similar arrangements were found in the ABC domains of various bacterial transporters, including that of MJ0796 (348), HlyB (417), and MalK (54). The orientation of the two ABC units in the dimer and the positions of the conserved sequence motifs are illustrated in Figure 3A, based on the high-resolution structure of the HlyB-ABC-ABC dimer. The regulated formation of these composite active sites is in harmony with many mechanistic studies, indicating the direct involvement of both the Walker A and the ABC signature regions in ATP binding and hydrolysis. Further analysis of Rad50cd crystal structures obtained in the presence or absence of nucleotide analogs revealed that during the catalytic cycle, the composite site is formed as a result of a major intramolecular rotation, which brings the contralateral Walker A/B and signature sequences within close proximity.

By now we also have detailed structural information regarding the amino acid residues involved in nucleotide binding and the possible catalytic steps of ATP hydrolysis within the ABC dimers. Figure 4 demonstrates the nucleotide contacts within the composite active site, based on a bacterial ABC structure (MJ0796 ABC; see Ref. 416) and sequence comparisons with human ABC transporters. Based on these structures, a detailed molecular mechanism of ATP hydrolysis by ABC transporters was suggested (see Refs. 171, 416). During ATP binding, the residues of the Walker A segment in ABC unit I coordinate the three phosphate groups of ATP, while the adenine ring is oriented by interactions with a neighboring bulky resi-
due. In the same ABC unit, the Q-loop and the Walker B glutamate alternately interact with a water molecule involved in ATP hydrolysis. From ABC unit II, residues of the signature region are also involved in the coordination of the phosphate groups and the ribose part of ATP, while an additional alanine interacts with the catalytic water molecule. During ATP hydrolysis, that is in a change from a “prehydrolytic” state to a Mg-ADP-bound, “posthydrolytic” structure, together with several minor intramolecular alterations, the Q-loop and the signature regions perform major movements. The movement of the signature (LSGGQ) segment is triggered by a rotation of the ABC-specific α-subdomain. Interactions between the signature motif and the γ-phosphate give additional cooperative stabilization to the nucleotide (“ATP-bound”) sandwich dimer. ATP can be considered a “molecular glue” with the γ-phosphate coupling the Walker A and B motifs and the Q-loop of one of the ABCs with the LSGGQ signature motif of the opposite ABC unit. After hydrolysis, ADP remains bound to the Walker A motif, while the cleaved phosphate anion remains bound to the signature region.

2. Communication between the ABC units and the membrane-bound domains

The high-resolution structures of various ABC-ABC dimers do not answer several questions concerning the structural basis of the ABC-TMD interactions that ensure the transmission of signals from the substrate binding sites to the catalytic machinery. It is also unknown how ATP binding and hydrolysis serve as the “power stroke” of transmembrane transport. Furthermore, in the separate ABC-ABC dimers, the molecular interactions are not influenced by the presence of the TMDs, and thus the ABC units may enjoy more freedom than in the full transporter complexes. In the case of eukaryote ABC transporters, only low-resolution studies, e.g., electron microscopy (EM) of single particles combined with image analysis (289) and EM analysis of two-dimensional crystals of MDRI/Pgp (203, 291, 293), are available. Typically, the resolution of these structures is 12–25 Å, allowing only the detection of major conformational changes and the relative position of the major structural elements. These data are included in the presentation of the individual MDR-ABC transporters in the following sections.

At the time when this review was compiled, several structures of complete bacterial ABC transporters were already obtained at an atomic resolution. These include the dimeric forms of the homodimer BtuCD (213) and the MsbA “half-transporters,” the latter being a close homolog of human MDR1/Pgp, for which crystal structures from various bacterial strains and in various catalytic states were solved with high resolution (49, 50, 285).

Importantly, the composite nature of the active/catalytic centers within the ABC domains, discussed above, is reflected in each of these structures. Another key piece of information provided by these structures seems to be trivial, that is, the transmembrane regions are indeed composed of α-helices, spanning the membranes with the predicted numbers. Although fully expected, this evidence settles the issue for a number of proposed alternative transmembrane domain arrangements (158, 159). Another major finding of these structures is that an intracellular domain (ICD) forms a bridge between the TMDs and the ABCs. The ICD is built up from three subdomains, formed from elements of the intracellular loops between transmembrane (TM) helices and between the TMD and ABC.

In the first published full MsbA structure (50), the TM helices form a cone-shaped chamber, with a wide opening from the intracellular side. In contrast, the structure of MsbA in the presence of ADP, vanadate, and a transported ligand (287) indicated a large rotation and translation of the TMDs, resulting in the opening and closure of the chamber to the periplasmic direction and the intracellular face, respectively. However, the question still remains open, are these major molecular movements indeed parts of the molecular mechanism, or do they just represent crystallization artifacts? A space-filling model of the hu-
III. METHODS FOR FUNCTIONAL INVESTIGATION OF HUMAN MDR-ABC TRANSPORTERS

A. Why Bother: Why So Special?

In addition to “curiosity-driven” basic research, the two major practical causes for measuring MDR-ABC transporter expression and function are related to cancer drug resistance and predicting/following the general fate and effect of pharmaceutical agents in our body. In both cases, the goal is to determine the actual drug transport capacity, that is, the functional expression level of the relevant transporter(s).

As described in the sections dealing with the individual transporters, this task is not easily achieved by the routinely available classical methods. MDR-ABC protein expression is often not correlated with mRNA levels, as translation, protein processing, localization, and degradation are all regulated processes. Moreover, the physiological or cancer cell expression level of an MDR-ABC transporter is often below the detection threshold, as relatively few active transporter molecules may cause major alterations in drug transport. Even if the overall determination of the transporter protein levels is successful, in many cases the variable, regulated localization of the protein may result in misleading conclusions. In addition, these proteins cannot be studied by the classical membrane transport methods, developed for, e.g., ion exchangers, P-type ATPases, or regulated channels. The major differences may lie in the hydrophobic or amphipathic nature of the transported substrates and the relatively loose coupling of transport and ATP hydrolysis in these proteins (see sect. II and below). Based on these problems, the quantitative, functional determination of MDR-ABC transporters gained a special emphasis in the research efforts.

Another major issue is the relevance of in vitro experiments to the in vivo role of MDR-ABC transporters. Although initial indications for the role of membrane transporters in cancer multidrug resistance originated from clinically oriented studies, it is important to emphasize that the role of these transporters in clinical anticancer drug resistance is still unsettled. According to our view, this is mostly due to the still underdeveloped methodological arsenal of the clinical laboratory studies, especially the lack of proper, quantitative assay methods directly applicable for studying the function of all relevant MDR-ABC proteins in human solid cancer tissues.

In the initial in vitro experiments, cellular multidrug resistance was modeled by the in vitro drug selection of the tumor cells. However, since the transported drugs/compounds are mostly hydrophobic, their cellular accumulation strongly depends on the availability of intracellular binding sites, sequestration, as well as the “passive” permeability of the cell membrane. Vesicular transport studies encountered similar problems; if there is no binding or sequestration, the “leakage” of the accumulated drug rapidly counteracts the accumulating transport process. This is the reason why many indirect methods (drug-stimulated ATPase activity, fluorescent dye transport, “nucleotide trapping,” drug binding, etc.) were developed to appreciate the drug transport and related drug resistance functions, but a consensus in their application is still lacking.

While this wide array of methods to investigate the function and substrate interaction of MDR-ABC transporters has been developed by various research laboratories, the pharmacological industry became a major consumer...
and contributor to these studies as well. This is partly due to the development of new anticancer agents, but even more to the interest in defining the role of ABC transporters in drug absorption, distribution, metabolism, excretion, and toxicology (ADME-Tox). As detailed in the relevant sections, MDR-ABC transporters are key determinants of drug permeation into different tissues, as these proteins are located in the absorption, secretion, and sanctuary barriers.

Based on the above-described questions, in section III we discuss the relevant in vitro systems, the various cellular and enzymatic/vesicular models applied for the studies of MDR-ABC transporters. We focus on in vitro functional assays, the analysis of cytotoxicity, translocation of substrates, and ATP hydrolysis and emphasize methods that can be used to quantitatively estimate transporter function. We refer to all in vivo investigations in the sections discussing the individual multidrug transporters.

B. Cellular Assay Systems

1. Drug resistance studies

Typically, in vitro studies use cell lines overexpressing a desired ABC transporter. Such cells may be readily engineered using routine molecular biology techniques. Alternatively, cell lines with pleiotropic drug resistance may be generated through exposure to increasing concentrations of antitumor drugs. Fulfilling their role in detoxification, several ABC transporters (such as ABCA2, ABCB1, ABCC1, ABCC2, ABCC4, and ABCG2) have been found to be overexpressed in cell lines cultured under selective pressure (367). For example, elevated levels of ABCB1 were found in cells selected with Vinca alkaloids, anthracyclines, and colchicine, among others. Similarly, ABCG2 was overexpressed in cells selected for resistance to topotecan and mitoxantrone. To some extent, in vitro selection of cells resembles the in vivo acquisition of the MDR phenotype. However, resistant cells have to be cultured under constant selective pressure to ensure a stable phenotype. Under these conditions, cells usually develop multiple mechanisms of resistance (15), involving the overexpression of further transporters. Discerning signal from noise in selected cells can be achieved by control experiments using parental cells, or cells treated with specific inhibitors. In general, availability of appropriate control cells is a limitation, since cells undergoing selection may have inherent differences that are not readily identified or controlled. Furthermore, inhibitors are rarely specific. Despite these constraints, selected cell lines overexpressing an ABC transporter are extensively used both in research and industrial settings.

Some of the ABC transporters implicated in MDR have never been found overexpressed in drug-selected cells. Still, these transporters (such as ABCB11, ABCC3, ABCC5, ABCC6, ABCC10, and ABCC11) could confer drug resistance when they were transfected into cells. Again, cell lines stably overexpressing these ABC transporters showed characteristic resistance to compounds that are substrates for transport. Thus cytotoxicity assay is a convenient tool that is often used to search for substrates and reversing agents. Cytotoxicity assays can be well quantitated, and “killing curves” provide proper IC50 values for the estimation of changes in cellular drug resistance. However, drug sensitivity may be entirely different in different immortalized or tumor-derived cell lines, and the complex cellular sensitivity and resistance mechanisms greatly modify the effects of such studies.

2. Transient expression systems

Studies using mammalian cells subjected to drug selection with or without the introduction of MDRI cDNA are subject to contention because of the pleiotropic effects of the drugs (12). To overcome this concern, ABC transporters may be expressed in transient expression systems. Gottesman and colleagues (283) adapted a vaccinia virus-T7 RNA polymerase hybrid transient expression system that does not involve selection for the functional expression of MDRI/Pgp. In this system, high levels of expression can be achieved within 48 h posttransfection, allowing the study of transport and drug-stimulated ATPase.

In the case of several ABC transporters, including MDRI/Pgp and its close relatives, expression of the gene products in insect (e.g., Spodoptera frugiperda, Sf9) cells, using recombinant baculoviruses, proved to be an efficient tool for analyzing various aspects of transporter function. This system allowed studying the protein interactions with substrates and also the substrate-stimulated ATPase activity (307). Although the baculovirus expression system ensures relatively high expression levels (4–5% of the total membrane protein), expression is transient, and the functional analysis of the expressed proteins in most cases has to be performed using microsomes prepared from the infected cells. Despite the lack of full glycosylation in insect cells (proteins are only core-glycosylated), we have successfully used this system for the expression and characterization of several ABC transporters (22, 260, 305, 307, 346). In case a mammalian protein has to be complemented by (an)other protein(s) for function, a heterologous system may not be suitable for functional analysis. Still, functional expression in insect cells can be used to characterize mammalian proteins outside the context of interacting networks. We have used this argument to show that ABCG2, an ABC half-transporter, functions as a homodimer (see sect. V). As mentioned below, transient expression systems are especially useful
for direct enzymatic or transport studies of MDR-ABC transporters.

3. Whole cell transport studies: fluorescent dyes and the calcein assay

The multidrug resistance phenotype suggests the overexpression of an MDR-ABC transporter and the decreased cellular accumulation of the toxic compounds. To verify this relation, experiments can be designed to follow the steady-state cellular accumulation of radioactively labeled or fluorescent compounds. In case a reduced accumulation or an increased extrusion is detected, experiments can be performed to define the kinetic parameters, energy dependence, and the specificity of the efflux. However, as mentioned above, intracellular binding, sequestration, and “membrane leakage” of the compounds are major difficulties in quantitating these studies. Therefore, a large number of indirect transport assays, where substrates and inhibitors are identified by following the transport of a reporter substrate, have been developed. Reporter substrates should be generally not toxic, their cellular fate should be well characterized, and an easily measurable fluorescence is a major advantage for such test compounds (Fig. 6).

The functions of MDR-ABC transporters have been characterized by measuring the cellular uptake, efflux, or steady-state distribution of a number of fluorescent substrates, including Hoechst dyes, fluorescent verapamil and prazosin derivatives, or various rhodamine derivatives. Several drawbacks have been noted relating to the sensitivity of most fluorophores because of protein binding, sequestration, or changes in the intracellular milieu (e.g., pH or free calcium) (124). Fluorescent indicators such as fura 2 are linked to an acetoxyethyl ester (AM) group to grant the access of the conjugates through the plasma membrane into the intracellular milieu. In an experiment in which our aim was to characterize the role of MDR1/Pgp in calcium homeostasis, we noticed that several fluorescent indicators used to measure intracellular calcium levels showed a decreased accumulation in MDR1/Pgp overexpressing cells (126). This observation prompted a search for a hydrophobic fluorescent indicator measuring the activity of MDR1/Pgp.

We found calcein-AM to be the dye of choice, because 1) the hydrophobic, nonfluorescent AM form is an excellent substrate for MDR1/Pgp; 2) the fluorescent, hydrophilic free acid is trapped inside the cells, does not bind to intracellular proteins, and is no longer a MDR1/Pgp substrate; and 3) the fluorescence of free calcein is not sensitive to changes in pH and ion concentrations. Due to the enzymatic enhancement of the dye-trapping process, the sensitivity of an assay measuring calcein accumulation highly surpasses that of other functional assays (Fig. 7, Ref. 124). Based on these considerations, we developed a quantitative calcein transport assay, which correlates with functional MDR1/Pgp expression. The assay kit is suitable for flow cytometry-based clinical laboratory applications and was found applicable in predicting multidrug resistance in acute leukemia (170). The calcein assay can be used for the estimation of the transport properties of certain MRPs as well, while ABCG2 does not transport either calcein-AM or free calcein (see sect. v).

4. Monolayer transport assays

In vivo, drugs have to cross pharmacological barriers to get absorbed (intestinal epithelial cells), distributed (blood-brain barrier endothelial cells), or excreted (hepatocytes, proximal tubule epithelial cells). This transcellular movement is modeled by cellular monolayer efflux (“vectorial transport”) assays. The cell lines used in these assays are polarized epithelial or endothelial cells, such as the human intestinal epithelial line Caco-2. Caco-2 cells have characteristics that resemble intestinal epithelial cells such as the formation of a polarized monolayer, with a well-defined brush border on the apical surface, and intercellular junctions. Measuring the rate of transport across the Caco-2 monolayer provides insight into absorption across the gut wall. Test drugs can be applied to either side of the cell layer, and the rate of transport across the monolayer is measured from the apical to basolateral (A-B) or from the basolateral to apical (B-A).
direction. The bidirectional apparent "permeability" of the test compound reflects the complex transport processes across the cell membranes. Measuring transport in both directions across the cell monolayer provides an indicator of active transport, chiefly mediated in Caco-2 cells by MDR1/Pgp (see sect. IV).

To establish specific roles of different MDR-ABC transporters, transfected versions of the canine kidney cell lines MDCKI, MDCKII, or the porcine kidney epithelial cells LLC-PK1 can be used. In these assays, polarized cells are grown on semipermeable filters, and the MDR-ABC transporters are localized to the proper apical or basolateral surfaces of the cells (for reviews, see Ref. 197). Fluorescent compounds can also be used in indirect transport assays in such polarized cells, e.g., the “vectorial calcein assay” can be applied for the estimation of drug interactions with various MDR-ABC proteins (33, 288). Especially in ADME-Tox drug research assays the application of cell lines with well-defined “efflux” and “influx” transporters can be an advantage. For such complex cellular or monolayer transport assays, the stable coexpression of various MDR-ABC transporters and a number of “influx” transporters are already available (70, 189, 206, 308, 309, 421).

5. Pharmacogenomic approach to identify ABC substrates

To determine substrate specificities of ABC transporters and their role in drug resistance of cancer cells, Szakacs et al. (363) have measured the expression profile of the 48 ABC transporters in the National Cancer Institute 60 (NCI-60) cancer cell panel. The NCI-60 cell panel was set up by the Developmental Therapeutics Program (DTP) of the National Cancer Institute (NCI), which has screened the cytotoxicity profiles of more than 100,000 chemical compounds in the 60 cell lines (97). Through the measurement of ABC transporter expression levels, it was possible to link ABC transporter function to a variety of already determined molecular, physiological, and pharmacological features of the cells. Analysis of the correlations between ABC transporter expression and known patterns of drug activity for 1,429 compounds across the 60 cancer cell lines yielded strongly inverse-correlated pairs, where the expression of an ABC transporter was strongly correlated with decreased sensitivity to a drug. As expected, good agreement was found between the mRNA expression of MDR1/Pgp and the reduced cellular sensitivity to anticancer drugs that are known to be substrates of this transporter. Furthermore, the method also allowed the identification of previously unknown MDR1/Pgp substrates (363). Interestingly, the same approach indicated that ABC transporters other than the well-characterized MDR proteins can provide resistance in naive (unselected) cancer cell lines. Follow-up experiments, using cells transfected with ABCC2, ABCC11, or ABCC4 (401), validated the predictions. These results suggested that this pharmacogenomic approach provides an unbiased method for discovering the substrate specificities of known, as well as yet uncharacterized members of the ABC superfamily.

C. MDR-ABC Enzymatic or Transport Assays

1. ATPase assay, detection of the catalytic cycle steps

As discussed in section II, MDR-ABC transporters exhibit a catalytic activity that is coupled to drug transport. Indeed, crude and purified preparations of various MDR-ABC transporters exhibit substrate-induced, vanadate-sensitive ATPase activities. The rate of ATP hydrolysis is easily determined by measuring the liberation of inorganic phosphate, using membrane vesicles prepared, e.g., from MDR-ABC expressing insect or mammalian cells. Isolated and reconstituted MDR-ABC transporters have also been successfully applied in this regard (10, 14, 81, 114, 219, 307, 329, 336).
The profile of the drug-stimulated ATPase reflects the nature of interaction: compounds may be substrates or inhibitors or may have no effect on the transporter. In the presence of transported substrates, the ATPase activity of the transporter increases (activation protocol). Noncompetitive inhibitors, or compounds transported at a lower rate, inhibit the ATPase activity of the stimulated transporter (inhibition protocol). In general, most of the efficiently transported compounds stimulate the ATPase. In the case of MDR1/Pgp, exceptions were noted, and some substrates were shown to stimulate activity at lower, and inhibit the ATPase at higher, concentrations. Further complicating the issues, the ATPase activity in most cases has a basal rate, probably related to an endogenous stimulation and/or a partial uncoupling (see below) and may also be affected by the lipid environment and the experimental conditions (9, 248, 330, 332). However, because of its simplicity and reproducibility, the ATPase assay is one of the most widely used assays to search for compounds that interact with various ABC transporters.

The application of the vanadate-sensitive membrane ATPase assay for drug interaction studies circumvents the problems of measuring the transport of hydrophobic substrate compounds, if indeed MDR-ABC ATPase is closely coupled to transport activity. While most studies agree that in general this is the case, a certain “slippage” between ATP hydrolysis and drug transport by the MDR-ABC transporters may be a basic feature of the catalytic mechanism (see sect. II). Studies on the wild-type and mutant variants of the major multidrug transporters may answer this basic question.

As described in more detail in the relevant sections, several methods have been developed for the analysis of the catalytic steps of the ATPase/transport reaction of MDR-ABC transporters. These include the determination of the modulation of the vanadate-dependent adenine nucleotide “trapping” (26, 362), and the vanadate-induced cleavage of the transporter protein (134), induced by transported substrates or modulators. Often applied methods are following the interaction of membrane-bound or isolated MDR-ABC proteins with labeled photoaffinity analogs of ATP or the transported substrates. The conformation-sensitive binding of specific monoclonal antibodies both in the case of MDR1/Pgp (267a) and ABCG2 (265) has been successfully applied to study substrate interactions and models of the catalytic cycle. While these methods may yield valuable information about the mechanism and transport properties of the given ABC protein, they are usually expensive with a low throughput; thus they may not be efficiently applied in cancer drug detection or drug research.

2. Vesicular transport

A more direct measurement of substrate translocation and its modulation can be achieved by the quantitation of the intravesicularly trapped substrates in vesicular transport assays. However, as noted above, this assay has major limitations when using hydrophobic substrates, due to significant nonspecific binding and rapid leakage of the compounds from the vesicles.

Successful vesicular transport studies using membranes from various sources (insect cells, transformed and selected cell lines, artificial membrane vesicles) have been reported by several laboratories (337, 422). Given the orientation of ABC transporters in cells (where the NBDs are in the intracellular compartment), in inside-out vesicles, the NBDs face the incubation media (accessible to ATP and other chemicals), and substrates are actively transported into the vesicles. Rapid filtration, using glass fiber filters or nitrocellulose membranes, is used to separate the vesicles from the incubation solution, and the test compound, trapped inside the vesicles, is retained on the filter. The quantity of the transported unlabeled molecules can be determined by high-resolution, high-sensitivity analytical methods. Alternatively, the compounds are radio-labeled or a fluorescent tag is attached, and the radioactivity or fluorescence retained on the filter is quantified.

In the case of MDR1/Pgp, which recognizes mostly hydrophobic compounds, a vesicular transport assay for most of the relevant substrates could not be established, due to the high nonspecific binding and passive diffusion of compounds. For some less hydrophobic, relatively low-affinity substrates, such an assay is available. Transport of hydrophilic quaternary drugs (such as N-methylated derivatives of quinidine) was demonstrated into vesicles isolated from MDR1/Pgp overexpressing insect cells (129). In another experiment, using rat hepatocyte canalicular membrane preparations, ATP-dependent uptake of radioactively labeled doxorubicin and N-pentylquinidium could be measured by centrifugation of the vesicles through a gel matrix (397). It has to be noted that vesicles prepared from hepatocytes contain additional ABC transporters, such as ABCB11 (BSEP, or “sister-of-Pgp”) and ABCG2/ABCG2. For these other ABC transporters, including members of the ABCC and the ABCG subfamilies, a number of transported substrates are less hydrophobic and therefore are trapped inside the vesicle compartment. Vesicular transport can also be performed in an “indirect” setup, where interacting test drugs modulate the transport rate of a labeled reporter compound.

As a general conclusion, a great variety of assays have been developed for functional studies on MDR-ABC transporters, but none of these methods can be singularly applied to answer all questions of functional expression, substrate handling, and regulation. The adaptation and/or the proper combination of these assays for detection of...
IV. ABCB1 (P-GLYCOPROTEIN, MDR1): THE CLASSICAL HUMAN MDR-ABC TRANSPORTER

A. Biochemistry and Cell Biology of MDR1/Pgp

Human ABCB1 (MDR1/Pgp) is the archetypal ABC transporter and has earned its reputation by being the first discovered, the most important medically, the most studied, and the one with the broadest substrate specificity (156). Models describing the function of MDR1/Pgp rely on biochemical experiments, mutagenesis studies, low-resolution structures, and the atomic level structures of various other ABC proteins.

ABCB1 is a member of the ABCB subfamily, which in humans has 11 transporters. The ABCB1 (MDR1) gene is located on chromosome 7q21. It consists of 28 exons, which encode a 1,280 amino acid glycoprotein (MDR1/Pgp). Analysis of the primary sequence delineates a tandem repeat of transmembrane domains and ATP-binding cassettes and a linker region connecting the two homologous halves of the protein. The two halves form a single transporter with a pseudo-twofold symmetry, in which the transmembrane helices define a "pore" for substrate translocation, and the nucleotide binding cassettes harvest the energy of ATP binding and hydrolysis.

The membrane topology of MDR1/Pgp has been elucidated by epitope insertion experiments (172, 173), fully supporting the original topology model of six TM helices in both TMDs of the protein (52). The linker region connecting the two halves of the protein plays a critical role in ensuring proper interaction of two subunits. The overall shape of the molecule has been revealed by low-resolution techniques. Cryoelectron microscopy images (at a resolution of ~8 Å) suggest that the transmembrane domains form a funnel-shaped aqueous chamber in the plane of the membrane (290). The chamber opens towards the extracellular compartment and seems to be closed at the intracellular end. The two NBDs are located intracellularly, in close proximity that allows extensive interactions between the two catalytic sites (Fig. 5).

ATP hydrolysis and drug transport are promoted by different segments of the protein, and the collaboration of these modules ensures that 1) ATP is hydrolyzed when a substrate is presented for transport, 2) the substrate is translocated as the energy of ATP is released, and 3) the two ABC units act in a concerted fashion (see sect. II). While a large body of biochemical experiments, coupled with mutagenesis studies summarized below, have elucidated some key features, in the absence of structural evidence the exact mechanism of how conformational signals are transmitted within the protein, resulting in coupling of ATPase and transport cycles, awaits more relevant and/or higher resolution structural data. In particular, there is much controversy regarding the mechanism by which transported substrates promote ATP hydrolysis (substrates may affect ATP binding, ATP hydrolysis, or both), and the details of how the energy of ATP hydrolysis is harnessed for transport.

MDR1/Pgp recognizes substrates that belong to very diverse chemical classes. Several investigators have attempted to catalog the chemical fingerprint of a "model substrate." The consensus that has emerged from these studies is that MDR1/Pgp substrates are amphipatic, with a molecular mass of 300–2,000 Da. Despite the wealth of mutagenesis and photolabeling studies (reviewed in Ref. 13), the structural basis of the transporter’s promiscuity remains unknown. Mutations affecting substrate specificity are clustered predominantly in transmembrane domains 5, 6 and 11, 12, but they are also found throughout the rest of the molecule, including the intracellular loops and the ATP binding domains (12). In a series of experiments, Loo and Clarke (217) have used cysteine scanning mutagenesis to assess the relative position of moieties involved in drug binding within the transmembrane regions. Models based on disulfide cross-linking experiments place transmembrane helices 6 and 12 in close proximity. Similarly, TM helices 5 and 8, as well as TM2/TM11, are close to each other, in agreement with the proposed funnel shape of the channel (214). During transport, drugs are translocated from a high-affinity "loading" site located in the intracellular or inner leaflet compartments to a low-affinity, outward-facing "unloading" site. This energy-dependent translocation event involves the repacking of the membrane-spanning α-helices (291, 293).

Crude membranes purified from insect cells expressing MDR1/Pgp show ATPase activity that is stimulated by transported substrates (307). Similarly, purified MDR1/Pgp reconstituted in proteoliposomes exhibit substantial drug-stimulated ATPase activity (14). The MDR1/Pgp ATPase has a low affinity and low specificity for nucleotides with a single apparent $K_m$ for MgATP of 0.5–1 mM that is not affected by the transported substrates. The turnover of the maximal, drug-stimulated ATPase is 10–20/s, the activity ranges between 5 and 22 μmol · min$^{-1}$ · mg MDR1/Pgp$^{-1}$, and the degree of drug stimulus is 2–11-fold. It is generally agreed that the stoichiometry of ATP hydrolysis to drug transport is in the range of 1–3 (14).
ATP binding and cleavage occur at the ABC units, and the close interaction of two ABC units results in the formation of a fully competent ATP-hydrolytic site (see sect. ii). MDR1/Pgp (as all ABC transporters) differs from P-type ATPases in that it does not show a high-affinity ATP binding and does not utilize a covalently phosphorylated protein intermediate. Theoretically, the ATPase cycle can be described as containing the following basic steps: ATP binding, cleavage of the terminal phosphate bond, and release of the catalytic products (Pi and ADP). Practically, these steps are empirically defined and characterized in experiments described below (for a summary, see Fig. 8).

To study ATP binding, researchers have used various ATP analogs that are either nonhydrolyzable (230), emit fluorescence (212, 334, 335), or covalently label nucleotide binding sites (252, 303). Under conditions preventing ATP hydrolysis (such as low temperature), two ATP molecules are seen bound to MDR1/Pgp. Compared with most ATPases, the affinity of MDR1/Pgp for ATP is low, with a $K_m$ of 0.3–1 mM. Binding curves can be described by a single $K_d$, indicating lack of cooperativity of the two

![ATP hydrolytic cycle of MDR1/Pgp. The ying and yang represent the two composite nucleotide binding folds. Step 1: ATP binding. Two ATP molecules are loosely bound to MDR1/Pgp (red circles). This is consistent with experimental data showing 2 ATP molecules bound to MDR1/Pgp with a low affinity. Step 2: ATP occlusion (“high-affinity binding”). ATP binding brings about a tighter interaction of the two NBDs, which results in the formation of the composite catalytic sites (dimerization). Although in the wild-type MDR1/Pgp ATP binding (step 1) is rapidly followed by ATP hydrolysis (step 3), mutational studies (312, 380, 381) strongly indicated the presence of such an ATP occlusion step. This prehydrolytic conformation is probably evoked by the binding of nonhydrolyzable ATP analogs. Experimental evidence suggests that in the “occluded state” only 1 ATP is bound tightly to MDR1/Pgp, consistent with the observation that only one catalytic site is committed to hydrolysis in the subsequent steps (red squares) (375). At present, it is not clear if the noncommitted site is unoccupied (as shown in this figure) or loosely binds a nucleotide. Step 3: ATP hydrolysis. This is probably a multistep process with minor conformational changes within the composite active center(s). Step 4: the cleaved terminal phosphate is released, and ADP still remains occluded. At this step, vanadate (and other phosphate mimicking transition state analogs) can replace the phosphate, thus stabilizing a complex containing ADP:Vi:Pgp (“trap,” step 4a). Ample experimental evidence suggests that only 1 ADP is trapped at a time (red hexagon). Moreover, in the trapped stage, the affinity of MDR1/Pgp for the nucleotide is drastically reduced (310), suggesting that no nucleotide is bound to the noncommitted (idle) site. Step 5: release of ADP. In the absence of vanadate, ADP dissociates. Step 6: ATP binding. With the renewed accessibility of the NBDs, 2 ATP molecules loosely bind to MDR1/Pgp. At this point, there are two possibilities. Step 7a: since steps 6 and 1 are identical, the cycle continues with the random recruitment of one of the composite catalytic sites. Step 7a would allow repeated hydrolysis at the same composite site. Step 7b: according to the widely accepted model proposed by Senior et al. (328), the two sites alternate in catalysis. Thus, in a “full cycle,” 2 ATP molecules are hydrolyzed in identical steps, alternating between the two sites (steps 7b–1). The original model suggested that 2 nucleotides may be bound to MDR1/Pgp at a time, and ATP binding at one site was proposed to promote hydrolysis at the other. Although this may indeed be the case, at present, there exists no experimental evidence to ultimately prove this interaction. Except for the initial loose binding of 2 ATP molecules (red circles, steps 1 and 6), consistently with experimental evidence, the model shown here involves steps where only one nucleotide is bound to MDR1/Pgp. This model is compatible with alternating catalysis (step 7b), if it is assumed that steps 6 and 1 are different, and in step 6, MDR1/Pgp “remembers” at which site hydrolysis has occurred in step 3 (this may be ensured, e.g., by the preferential binding of ATP to the previously idle site in step 5). At present, several contrasting views exist regarding the coupling of the ATPase to drug transport (see text). ATPase activity is clearly required for transport, and transported substrates typically increase the rate of ATP hydrolysis (steps that may be accelerated by transported substrate are shown by double arrows). It is not clear, however, which steps are associated with the binding, transport, and release of the transported drug (not shown, but see Ref. 310).
NBDs (205). The prevailing view in the literature is that transported substrates do not have any effect on ATP binding (205, 252), although this is still an unsettled dispute (see below).

The transport and ATPase cycle of MDR1/Pgp is blocked by phosphate-mimicking anions, such as orthovanadate. Being similar in size and charge, vanadate replaces the cleaved gamma phosphate and locks the complex consisting of MDR1/Pgp and ADP (Pgp-ADP-Vi). This complex is formed under conditions allowing hydrolysis of at least one ATP molecule, and it is generally accepted to closely mimic the conformation of a transition state. The trapped nucleotide can be visualized by covalent photoaffinity-labeling of the NBDs by α-32P-8-azido-ATP, which is an efficient energy donor substrate of MDR1/Pgp. In contrast to ATP binding, “ADP trapping” is accelerated by the transported drug substrates (176, 362). In the presence of drug substrates, a strong correlation exists between the increase in the rate of trapping and the steady-state fold stimulation of ATPase activity, indicating that they provide a measure of the same catalytic step (362).

Vanadate trapping has proven to be a very useful tool to dissect the catalytic cycle. The first model of the ATPase cycle, proposed by Senior et al. (328) in a seminal paper, suggested that both ABC domains bind and hydrolyze ATP in an alternating fashion. According to this model, ATP binding at one NBD promotes hydrolysis at the other, and parallel hydrolysis at the two NBDs is inhibited. This hydrolytic step leads to a high chemical potential conformation, the relaxation of which is coupled to drug transport. The basic paradigm of the Senior model has survived the test of time and has served as the framework for later refinements (157; Fig. 8).

The formation of the transition state clearly manifests in extensive conformational changes, which were detected by proteolysis experiments (165) and structural studies (293). Several authors agree that the decrease in drug binding affinity (“unloading”) is associated with this step of the catalytic cycle. Indeed, monitoring the binding of the substrate analog 125I-iodoarylazidoprazosine (IIAP), Sauna and Ambudkar (311) found that the vanadate-trapped transition state complex exhibited a reduced affinity to drugs and nucleotides. The reduced drug binding affinity persisted as the transition state collapsed (release of Vj and ADP), and only through the hydrolysis of a second ATP molecule did MDR1/Pgp regain its high-affinity binding site (282, 311). Thus a model was proposed in which a second ATP has to be hydrolyzed to reset the protein to the initial conformation with a high-affinity drug binding site. According to the Sauna model, release of the substrate occurs following hydrolysis of the first ATP molecule, and the resetting of the transporter requires the hydrolysis of a second ATP (13).

This model has been challenged by Sharom et al. (333), who used various fluorescent spectroscopic approaches to follow the catalytic cycle. They found that the stably trapped Pgp-ADP-Vi complex does not exhibit reduced drug binding affinity and that the affinity of NBDs for nucleotides does not change during conversion from the resting state (where 2 ATP molecules are bound) to the transition state (279). In addition, the same authors found a modest stimulation of ATP binding in the presence of transported substrates. Taken together, they interpret their data as signifying that the transported substrates are released before the formation of the transition state (ATP cleavage), suggesting a mechanism involving concerted conformational changes rather than a multistep process (279, 333).

Higgins and Linton (120) also observed an increase in ATP binding affinity of MDR1/Pgp in the presence of vinblastine. Furthermore, in experiments using nonhydrolyzable ATP analogs, the Higgins group found that ATP binding results in extensive conformational changes affecting the TMDs that eventually decrease the affinity of MDR1/Pgp to vinblastine (229, 230, 292, 349). These observations led to a model in which it is the binding, rather than the hydrolysis, of ATP that provides the “power stroke” for transport (120). According to the ATP switch model, in a “resting state,” the two NBDs are far from each other (“open conformation”). Substrate binding facilitates ATP binding, which in turn results in the dimerization of the two NBDs (closed formation). The extensive conformational changes prompted by ATP binding mediate signals to the drug binding domains and ultimately translate into the translocation of the drug substrate. Importantly, this model assumes that the conformational changes facilitating the translocation of the transported substrates are brought about by ATP binding, resulting in the switch between the closed and open states of the NBD interface. In this interpretation, hydrolysis of 2 ATP molecules (and the release of Pi and ADP) is triggered by the dissociation of the transported substrate, the result of which is the opening of the NBD dimer interface that resets the transporter to the starting conformation.

Concerning the mechanistic details of the communication between the NBDs and the drug-translocating domains and the role of ATP binding/hydrolysis in promoting drug translocation, the above-described models are clearly at odds with each other. The ultimate transport scheme will be based on detailed thermodynamic studies and crystal structures representing snapshots of MDR1/Pgp as it cycles through its physiological conformations. In the meantime, apparent discrepancies may be discussed in the context of the different experimental strategies used to define the catalytic steps. For example, advocates of the fluorescent nucleotide analogs argue that photolabeling techniques are subject to artifacts, and
reflect the variable efficiency of photoprobe cross-linking rather than the true binding affinities (279). On the other hand, it can be argued that fluorescent techniques (using fluorescent ATP analogs along with a quenching agent associated to the NBDs) provide but an indirect measure of ATP binding, especially as most of these analogs have clearly different affinities than ATP. In addition to specific artifacts related to these experimental protocols, another reason behind these contrasting views may be that these experimental techniques capture different steps of the catalytic cycle. At low temperatures, $\alpha^{32}$P-8-azido-ATP labeling of MDR1/Pgp is thought to represent ATP binding. As mentioned earlier, nonhydrolyzable ATP analogs are also used to study the same catalytic step. However, while neither condition is expected to allow ATP cleavage, nonhydrolyzable ATP analogs may nevertheless permit the formation of a prehydrolytic state (following binding but preceding hydrolysis) in which ATP is occluded (Fig. 8). Due to the high turnover of ATP hydrolysis, this state is difficult to capture in the wild-type protein. Interestingly, MDR1/Pgp variants containing combined mutations at the “catalytic carboxylate” and the contralateral signature region (E552A/S1173A) show vanadate-independent retention of ATP, suggesting that a closed formation of the dimer interface (with an occluded ATP) can indeed occur without ATP hydrolysis (374).

Most models agree that in MDR1/Pgp the two nucleotide binding domains are functionally equivalent and the integrity of both is needed for transport. Several lines of evidence prove the close interaction of the NBDs. Whereas (in saturating conditions) MDR1/Pgp may bind two ATP molecules, only one ADP is trapped at a time, suggesting that ATP hydrolysis at one site prevents ATP binding or hydrolysis at the other NBD (134). Also, the inactivation of one NBD (by mutations, chemical modifications, or ADP trapping) blocks catalysis at the other site. The structural basis for this interaction of the two NBDs is best explained by a model where the two NBDs form common ATP binding sites. This formation, with the ATP sandwiched between residues provided by both nucleotide binding domains, was predicted by Jones and George (160) and confirmed in several ABC crystals (see sect. ii; Figs. 3 and 4). The “open conformation” seen in crystals (in which the two NBDs are far apart) is in agreement with experimental evidence showing that $\alpha^{32}$P-8-azido-ATP binds to MDR1/Pgp with a low affinity and is readily exchangeable with unlabeled ATP (252). In the closed conformation, the bound ATP forms contacts with amino acids from both NBDs, which suggests that closing of the dimer interface precedes hydrolysis. Cross-linking studies (215, 218) have shown that at 37°C, cysteines located in the two NBDs can be within 1 Å from each other and that the “LSGGQ” motif in each nucleotide-binding domain is adjacent to the opposing Walker A sequence.

The very strong conservation of the ABC signature sequence implies an important role in catalysis.Mutational studies have confirmed that the conserved serine residues (“LSGGQ”) cooperatively accelerate ATP hydrolysis (374). Our own results showed that mutation of the conserved glycine residue (“LSGGQ”) in either NBD results in the loss of MDR1 ATPase. At the same time, ATP binding remained intact, and the formation of an AlF$_4^-$-dependent transition state suggested that the mutants can promote the hydrolysis of at least one ATP molecule (365). Similarly, mutating the key Walker A lysine to methionine at either NBD inactivates the transporter but nevertheless remains compatible with ATP binding (252) and the hydrolysis of at least one ATP (366). Thus the inactivation of a single NBD does not prevent ATP binding or the dimerization of the NBDs. However, when the conserved amino acids were changed in both NBDs, despite intact ATP binding, the mutants fell into a complete catalytic silence, suggesting that the inactivation of both ATP binding sites prevents the formation of the integrated catalytic site.

The ATPase activity of MDR1/Pgp is stimulated by the transported substrates. As demonstrated above, it has been difficult to assess whether it is the ATP binding, hydrolysis, or the release of the hydrolytic products that is facilitated by the transported substrates. It can be argued that, to increase the overall rate of the ATPase cycle, substrate stimulation has to increase the rate of the limiting step. Our own results (in agreement with other reports using $\alpha^{32}$P-8-azido-ATP-labeling under nonhydrolytic conditions as a measure of ATP binding) indicate that ATP binding is not affected by the presence of substrates even when the substrates are present at saturating concentrations (252, 366). In contrast, the formation of the Pgp-ADP-V$_i$ complex is accelerated by the transported drug substrates. Thus, with the assumption that the vanadate-trapped state reflects a physiological state in the catalytic cycle, the rate-limiting step has to precede the formation of the transition state. Therefore, it is unlikely that the release of ADP or P$_i$ could be the rate-limiting steps of the cycle (176), especially considering that these steps are not dependent on transported substrates (9). If we assume that in the presence of vanadate the catalytic cycle is arrested after the first turnover, this result implies that drugs exert their stimulatory effect at steps that follow ATP binding but precede the formation of the transition state. Such a state may involve the “occlusion” of the ATP, where the nucleotide is not yet hydrolyzed, but has already evoked a closer association of the dimer interface (Fig. 8).

One of the outstanding issues remaining to be clarified is the elucidation of the intramolecular network that propagates signals from the transmembrane domains involved in drug binding to the ATP-binding domains, fueling the transport. Insight into the TMD-NBD communica-
tion may be gained from the analysis of mutant MDR1/Pgp variants. A spontaneous glycine to valine mutation in the intracellular end of the third transmembrane helix of MDR1/Pgp (G185V) was shown to confer increased colchicine resistance to cells. Interestingly, improved resistance to colchicine is not accompanied by changes in apparent drug binding affinity or the velocity of the ATPase (9). Al-Shawi et al. (9) suggest that, rather, the activation energy of the coupled transition state is reduced, indicating that G185 has a pivotal role in transmitting signals from the drug binding to the catalytic domains. In structural models of MDR1/Pgp, the intracellular loop containing G185 interacts with the Q-loop of the NH$_2$-terminal catalytic site. The conserved glutamines of the Q-loop have been shown to play a role in interdomain communication (381). Interestingly, in MDR1/Pgp variants harboring mutations in the ABC-signature region in either one of the catalytic sites, the presence of the transported substrate strongly inhibited (rather than stimulated) the formation of the transition state, indicating that the conserved glycine residue in the signature region may be involved in the intramolecular signal transduction that carries information from the TMD to the NBDs. Based on these observations, we suggested a model where ATP hydrolysis is initiated by drug-induced conformational changes, in the course of which the ABC-signature region is juxtaposed to the ATP bound in the contralateral NBD, facilitating the formation of the ultimate dimer interface, and the hydrolysis of the ATP (see Fig. 8) (365, 368). Only one ATP is hydrolyzed at a time. Based on molecular modeling, Jones and George (157) speculate that, while an ATP is hydrolyzed at one NBD, the helical region of this NBD is “retracted” from the contralateral ATP to prevent parallel ATP hydrolysis at the other site (157).

B. Transported Substrates of MDR1/Pgp

MDR1/Pgp is an omnivore; it confers resistance to a vast array of clinically and toxicologically relevant compounds, including (but certainly not limited to) anticancer drugs, human immunodeficiency virus (HIV)-protease inhibitors, antibiotics, antidepressants, antiepileptics, and analgesics (Table 1).

Bacterial multidrug-binding proteins (such as the transcription regulator BmrR from Bacillus subtilis) may provide model systems for the analysis of these wide-range drug interactions. Crystal structures of these proteins indicate that they interact with drugs through van der Waals interactions and hydrophobic stacking (426). However, models based on chemical cross-linking studies, photolabeling experiments, homology modeling, and pharmacophore patterns (266) suggest that MDR1/Pgp makes different interactions with different drugs, implying the involvement of several, partially overlapping residues. Thus each substrate appears to define a unique niche in the complex binding pocket through an “induced-fit’” mechanism (216, 272).

MDR1/Pgp substrates are expected to freely diffuse into the cells, and MDR1/Pgp may recognize them in the context of the plasma membrane (268). Studies using fluorescent substrates and FRET have confirmed that the drug binding sites are located within the membrane plane (225). There are strong indications that this type of recognition makes the MDR1 protein a highly effective multidrug resistance pump, preventing the cellular entry of toxic compounds. Theoretical models (353) and in vitro experiments (211) indicated that MDR1/Pgp may recognize its substrates before they reach the cytoplasm (“preemptive pumping”). This is in accordance with our observation that calcein-AM and other fluorescent methyl esters are extruded from the cell membrane, before the cleavage of the AM group occurs in the cytoplasm (126). The finding that the binding site of MDR1/Pgp for the transported substrate Hoechst 33342 is localized in the cytoplasmic leaflet of the bilayer (333) is in keeping with this proposal.

If MDR1/Pgp reacts with its substrates within the membrane lipid phase, it is still a question if drug removal occurs to the external water phase (“hydrophobic vacuum cleaner” model, Refs. 32, 268) or the drugs are only flipped from the internal membrane leaflet to the external leaflet, which promotes their overall removal from the cell (drug flippase model, Ref. 119). Most probably both mechanisms may occur, differently for different substrates of

<table>
<thead>
<tr>
<th>TABLE 1. Substrates of MDR1/Pgp</th>
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<tr>
<td><strong>Anticancer drugs</strong></td>
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<tr>
<td>Anthracyclines</td>
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<td>Daunorubicin</td>
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<tr>
<td>Doxorubicin</td>
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<td>Epirubicin</td>
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<td>Anthracenes</td>
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<td>Bismantrene</td>
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<td>Mitoxantrone</td>
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<td>Vinca alkaloids</td>
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<td>Vinblastine</td>
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<td>Vinristine</td>
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<td>Vinorelbine</td>
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<td>Vindesine</td>
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<td>Tubulin polymerizing drugs</td>
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<td>Colchicine</td>
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<td>Paclitaxel</td>
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<td>Docetaxel</td>
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<td><strong>HIV protease inhibitors</strong></td>
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<td>Ritonavir</td>
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<tr>
<td>Indinavir</td>
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<tr>
<td>Saquinavir</td>
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<tr>
<td>Hoechst 33342</td>
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<tr>
<td>Rhodamine 123</td>
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<tr>
<td>Calcein-AM</td>
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<tr>
<td><strong>Other compounds</strong></td>
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<tr>
<td>AM, acetoxymethyl ester. [Data are from Ambudkar et al. (12) and Litman et al. (210).]</td>
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</tbody>
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variable size and hydrophobicity. In fact, it has been proposed that MDR1/Pgp evolved through mutations that expanded its substrate specificity from lipids to lipidsoluble toxic chemicals (194). Flippase activity has been attributed to other related transporters (see sect. vi), indicating a possible conservation of function between ABC proteins.

Besides the broad substrate specificity, MDR1/Pgp’s unique feature is its basal ATPase activity. Basal ATPase activity is usually attributed to the transport of an “endogenous” substrate, present in the membrane or the lipid environment. However, purified MDR1/Pgp shows ATPase activity even in the absence of added lipids (205). A detailed analysis of purified MDR1/Pgp indicated that the drug-induced and basal ATPase activities are influenced by a single overall rate-limiting step that shows significantly different thermodynamic properties depending on the presence of a transported substrate (9). To avoid futile cycles, ABC transport engines should harvest the energy of ATP only in the presence of transported substrates. However, the distinct thermodynamic profile of the basal ATPase activity indicated that it is not linked to the transport (or flipping) of an endogenous substrate. Why is MDR1/Pgp “wasting” ATP molecules in uncoupled cycles? To maximize the chance of successful transport, MDR1/Pgp has to remain in a “transport-competent” conformation even in the absence of transported substrates. Al-Shawi et al. (9) speculate that this conformation has only one bound ATP (9). Therefore, in the event another ATP binds to MDR1/Pgp in the absence of the transported drug, MDR1/Pgp “resets” the transport-competent conformation by “wasting” an ATP. In the presence of saturating drug concentrations, the transporter follows the coupled pathway, where ATP is utilized exclusively for transport.

A partially uncoupled power source may be ideally suited for a transporter whose task is to handle compounds newly presented to the organism (194). As a prominent multidrug transporter, MDR1/Pgp has to retain a very low selectivity to substrates. Low selectivity comes at a price; in the absence of specific interactions, the transport process cannot benefit from the energy of drug binding. Therefore, the energy-dependent reorientation of the binding sites (the conformational changes necessary for transport) should also occur in the absence of transported substrates in an autonomous, although in a slow manner (uncoupled activity), during which a high-affinity loading site for drug binding is exposed. Thus, for the sake of broad substrate specificity, coupling efficiency is sacrificed and the need for specific substrate interactions is reduced.

Several groups have attempted to define the common pharmacophore of MDR1/Pgp substrates and inhibitors. One approach is based on the systematic modification of known inhibitors and substrates, such as chloroquine (420), vinblastine (3), reserpine (273), colchicines (372), verapamil (373), as well as of several peptides (337) (for review, see Ref. 12). No clear consensus has emerged, except for the finding that substrates are hydrophobic and tend to have planar aromatic domains and tertiary amino groups (326). The analysis of three-dimensional structures revealed that substrates typically contain hydrogen bond acceptor (or electron donor) groups with defined spatial separations. Modeling indicates that the rate-limiting step for the interaction of a substrate with MDR1/Pgp is the partitioning of the compound into the lipid membrane. Conversely, dissociation of the Pgp-substrate complex is determined by the number and strength of the hydrogen bonds formed between the substrate and the transporter. Thus a compound with a higher potential to form hydrogen bonds with MDR1/Pgp generally acts as an inhibitor (326).

A variety of in vitro assays have been used to classify compounds as substrates, nonsubstrates, or inhibitors (see also sect. iii). Each approach has strengths and limitations, depending on the research setting and the questions asked. Monolayer efflux (“vectorial transport”) systems are regarded as standard, because they measure efflux in the most direct manner, and they closely model pharmacological barriers. Indirect tests, such as the ATPase and calcein assays, are readily automated and offer higher throughput, but if used alone, they are less reliable in classifying compounds. Classification of compounds depends on the assay types. Admittedly, there is confusion. For example, verapamil is not predicted to be a substrate in the monolayer system (270), whereas, ironically, it was one of the first compounds to be identified as a stimulator of the MDR1/Pgp ATPase (307). Further complicating issues, above a certain concentration, verapamil inhibits the basal ATPase activity (see preceding section). From a cancer drug resistance viewpoint, verapamil is one of the most potent in vitro MDR1/Pgp inhibitors, tested even in clinical trials (27).

Using a systematic and uniform approach, Polli et al. (278) compared these methods using a set of structurally diverse compounds. Of the 66 compounds studied, the 3 assays (i.e., the monolayer efflux, ATPase, and calcein-AM assays) showed concordant results with only 35 drugs. Differences in predictions in the remaining set related to the apparent permeability \( P(\text{app}) \) of the compounds. Detection of Pgp-mediated efflux was associated with compounds having low/moderate \( P(\text{app}) \) values, whereas inability to detect Pgp-mediated efflux was associated with compounds having high \( P(\text{app}) \) values. Interestingly, the calcein-AM and ATPase assays revealed Pgp interactions for highly permeable compounds but were less responsive than monolayer efflux for low/moderate \( P(\text{app}) \) compounds. All assays detected substrates across a broad range of \( P(\text{app}) \), but the efflux assay was more prone to fail at high \( P(\text{app}) \), whereas the calcein-AM and ATPase assays were more prone to fail at low \( P(\text{app}) \). These
results indicate that when the apparent permeability of a compound is relatively low, efflux is a greater factor in the disposition of Pgp substrates (278).

It should be noted that transcellular flux assays are carried out under nonsink conditions, that is, in the absence of physiological binding proteins or hydrophobic components, usually examining the transport of only one compound at a time. As a consequence, efflux assays are more reliable at low/moderate $P_{\text{app}}$ and can be the method of choice for evaluating the absorption and distribution of such drug candidates. However, they may provide false-negative results in the case of compounds with high $P_{\text{app}}$, when MDR1/Pgp interaction is masked by a rapid back-leakage of the compound. Still, this interaction may significantly modulate in vivo drug absorption where acceptor molecules are present, or strongly modulate the transport of other transporter-interacting compounds. We certainly suggest that a proper combination of direct transporter interaction and flux assays should be applied to model any pharmacological relevance of the MDR-ABC proteins.

C. MDR1/Pgp in Cancer Multidrug Resistance

Despite recent developments, effectiveness of chemotherapy is still rather limited for most types of cancer, including tumors of the colon, lung, kidney, pancreas, and liver. Why some cancers respond better than others may be explained by factors relating to the anatomy and physiology of the cancer-ridden organ or the pharmacokinetics of the drugs used to combat the disease. In addition, tumors may resort to cellular mechanisms, which may prevent the accumulation of cytotoxic drugs in the cancer cells. Whereas the plasma membrane efficiently prevents the entry of hydrophilic drugs, the free diffusion of hydrophobic compounds is limited by active transport mechanisms.

With the discovery of MDR1/Pgp, it became evident that even a single protein can be responsible for the increased efflux of structurally unrelated compounds. As we have seen, MDR1/Pgp can export most neutral and cationic hydrophobic compounds, and cancer cells readily co-opt this mechanism as a primary shield against chemotherapy. This “first-line” defense is reasonably successful, as most of the routinely used agents of the current chemotherapy regimens are MDR1/Pgp substrates (see Table 1).

Certain tumors (including hepatomas and lung or colon carcinomas) often show intrinsic resistance to cancer chemotherapy. However, tumors may also develop resistance over the course of the disease progression and the treatment. Cancer cells, often heterogeneous with respect to initial MDR1/Pgp expression, are constantly selected for increased survival and proliferation. In line of the general protective function of ABC transporters, cells with higher levels of MDR1/Pgp have a selective advantage during adaptation to the hostile environment (such as hypoxia or inflammation). Thus, at the time of diagnosis, tumors may be prepared to cope with the additional stress of chemotherapy. Cytotoxic agents may eliminate most of the tumor mass, but the surviving cells usually give rise to clones that are resistant to repeated treatment efforts (Fig. 9).

Although in vitro models clearly show that MDR1/Pgp can protect tumor cells, the relevance of MDR1/Pgp function in clinical oncology remains controversial. To date, clinical researchers have failed to embrace universally accepted guidelines to standardize methods for the measurement and evaluation of the impact of MDR1/Pgp in therapy. There are several challenges pertinent to the estimation of expression levels, acquisition of tumor cells, and the evaluation of the results (an important pitfall is that MDR1/Pgp may be present in stroma). There are at least three different approaches to detect clinical MDR at the mRNA, protein, or functional level. Assays measuring mRNA levels benefit from the specificity and sensitivity of the available technologies that are, however, too expensive and time consuming for the routine clinical practice. Measuring protein levels seems to be a more relevant

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**Fig. 9.** Evolution of MDR1/Pgp positive cancer. Tumor cells arise by a complex mutation and induction pathway. Cells that do not express multidrug transporters are sensitive to chemotherapy and are eliminated. In the course of chemotherapy, further mutations and selection may greatly increase the expression of multidrug transporters, which protect the tumor cells against chemotherapy.
option, although it has proven difficult if not impossible to standardize procedures and evaluation criteria. Finally, tests measuring the capacity of cells to extrude drugs (daunorubicin, doxorubicin) or fluorescent dyes (rhodamine-123, calcein, DiOC2) estimate the functional impact of transporters, which is arguably most relevant to clinical MDR. Our laboratory has pioneered the application of calcein-AM as a functional marker of the drug resistance phenotype. We have established a simple assay and demonstrated its applicability and predictive power in adult acute myelocytic leukemia (170). The development of this assay for solid tumors is in progress.

It is generally agreed that proper evaluation of clinical MDR should lead to a more efficient treatment of malignant diseases. Furthermore, chemotherapy-containing drug resistance-modulating agents should be planned and performed on the basis of a proper diagnosis of the actual presence and relevance of MDR (367). Because ABC transporters mediate resistance to a whole array of drugs, they should be an attractive target for the improvement of anticancer therapy. In theory, coadministration of MDR1/Pgp inhibitors with cytotoxic agents could reverse MDR and improve treatment outcome. There is no lack of compounds that have the capacity to inhibit MDR1/Pgp function. Nevertheless, despite promising in vitro results, successful modulation of clinical MDR through the chemical blockade of drug efflux from cancer cells remains elusive. Over the years, several generations of MDR1 modulators have raised hopes only to fail in clinical trials. The negative results may be explained by several factors, such as the intrinsic toxicity of the modulators and the unwelcome inhibition of MDR1/Pgp residing in pharmacological barriers, resulting in the altered distribution of the simultaneously administered chemotherapy. In our view, the lack of a serious effort to connect proper MDR-ABC transporters to pharmacological modulation of human MDR1/Pgp is a feasible strategy to treat multidrug-resistant cancer.

It is important to note that laboratory mice are raised in a well-controlled environment, where the function of the ABC transporter-based “chemoimmunity network” may be less relevant. This was proven in a legendary (and unplanned) experiment, in which the caretakers of the Netherlands Cancer Institute’s animal department took center stage. Without prior notice, they sprayed the animal cages with ivermectin, a potent GABA receptor agonist of natural origin, routinely used to treat mite infestation. Ivermectin was supposed to kill only nematodes and arthropods, since mammalian neurons were expected to be protected by the blood-brain barrier. However, the treatment left all the knockout mice dead. When the scientists reproduced the experiment, the detailed toxicity assays showed that the Mdr1a (−/−) mice were 50- to 100-fold more sensitive to orally administered ivermectin, due to an increased accumulation in the brain (319, 321). These results were consistent with the high MDR1/Pgp expression in brain capillary cells and suggested that MDR1/Pgp plays a predominant role in the blood-brain barrier of mice. Interestingly, the well-known sensitivity of inbred dogs, such as collies and Australian shepherds, to ivermectin was also linked to a 4-bp deletion mutation resulting in a frame-shift and a lack of canine MDR1/Pgp expression (296). On the other hand, ivermectin is widely used to treat river blindness (onchocerciasis) in Africa. Interestingly, more than 20 million patients have been treated with ivermectin without any documented central side effects (208).

MDR1/Pgp is expressed in the apical (luminal) membranes of epithelial cells, lining organs regulating drug distribution. The expression pattern and the hypersensitivity of the knockout mice to xenobiotic toxins suggest a major physiological role of this protein in the protection of the organism against orally ingested natural toxins. MDR1/Pgp influences drug distribution in three ways: it limits drug absorption in the gastrointestinal tract; it promotes drug elimination in the liver, kidney, and intestine; and it regulates drug uptake into cells, tissues, or pharmacological compartments. The gastrointestinal tract, which represents the first line of defense against orally ingested toxins and drugs, is well equipped with a range of ABC transporters (see sects. vi and viii). The AUC (area under the plasma concentration vs. time curve) of orally administered taxol is significantly higher in the knockout mice, suggesting that intestinal MDR1/Pgp is a major determinant of the reduced uptake of orally administered drugs (319).

D. Physiological and Pharmacological Functions of MDR1/Pgp

MDR1/Pgp shows high expression in the apical (luminal) membranes of epithelial cells lining the lower gastrointestinal tract, in the apical surface of proximal tubule cells of the kidney, in the canicular membranes of hepatocytes, and in the capillary endothelial cells in the brain and testes. Lower levels are expressed in the placenta, the adrenal cortex, and CD34+ hematopoietic stem cells. Crucial data regarding the physiological relevance of MDR1/Pgp in these locations came from knockout mice studies. Mice have two Mdr1 genes (Mdr1a and Mdr1b), both of which were inactivated by insertional mutagenesis (321). Surprisingly, loss of either or both genes did not result in an obvious phenotype: the knockout mice were viable and fertile, almost indistinguishable from their wild-type littermates. These results were interpreted to suggest that pharmacological modulation of human MDR1/Pgp is a feasible strategy to treat multidrug-resistant cancer.

It is generally agreed that proper evaluation of clinical MDR should lead to a more efficient treatment of malignant diseases. Furthermore, chemotherapy-containing drug resistance-modulating agents should be planned and performed on the basis of a proper diagnosis of the actual presence and relevance of MDR (367). Because ABC transporters mediate resistance to a whole array of drugs, they should be an attractive target for the improvement of anticancer therapy. In theory, coadministration of MDR1/Pgp inhibitors with cytotoxic agents could reverse MDR and improve treatment outcome. There is no lack of compounds that have the capacity to inhibit MDR1/Pgp function. Nevertheless, despite promising in vitro results, successful modulation of clinical MDR through the chemical blockade of drug efflux from cancer cells remains elusive. Over the years, several generations of MDR1 modulators have raised hopes only to fail in clinical trials. The negative results may be explained by several factors, such as the intrinsic toxicity of the modulators and the unwelcome inhibition of MDR1/Pgp residing in pharmacological barriers, resulting in the altered distribution of the simultaneously administered chemotherapy. In our view, the lack of a serious effort to connect proper MDR-ABC transporters to pharmacological modulation of human MDR1/Pgp is a feasible strategy to treat multidrug-resistant cancer.

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Clinical studies indicate that the inhibition of intestinal MDR1/Pgp increases the oral bioavailability of substrate drugs, such as cyclosporin A (CsA). CsA is a critical immunosuppressive drug with a narrow therapeutic range and wide interindividual variation in its pharmacokinetics. Amlodipine, which is a substrate of MDR1/Pgp, significantly increases CsA absorption (198). As oral bioavailability is an important parameter in the development of drug candidates, the role of MDR1/Pgp-mediated transport in the gastrointestinal tract bears significant consequences in the design and formulation of pharmacological agents. To account for MDR1/Pgp-mediated restriction of oral absorption, in vitro models are used to follow the transcellular transport of drugs through the MDR1/Pgp-expressing intestinal cell line Caco-2 (see sect. iii). Interestingly, intestinal MDR1/Pgp is also responsible for the excretory function of the gut wall mucosa (155, 234).

Exquisitely sensitive compartments, such as the testes and the brain, are protected by a second line of defense presented by the blood-tissue barriers. The central nervous system is shielded from the circulating blood by a complex physiological interface. In addition to the barrier formed by the tightly adhered capillary endothelial cells (blood-brain barrier), the brain is further protected by the selective permeability of the epithelial cells in the choroid plexus (blood-cerebrospinal fluid barrier). At both locations, ABC transporters play a prominent role in restricting the passage of hydrophobic compounds that would otherwise cross these barriers by passive diffusion. The role of MDR1/Pgp was discerned in studies examining the brain accumulation of compounds in the knockout mice. Compared with wild-type mice, MDR1/Pgp substrates (such as ivermectin and vinblastine) show one to two orders of magnitude higher accumulation in the brain of the knockout animals (319).

This is relevant to human pharmacology, as illustrated by an experiment where the effect of loperamide on the central nervous system was followed. Although the antidiarrheal loperamide is a potent opiate, it does not produce opioid central nervous system effects at usual doses in patients. However, when loperamide was coadministered with the MDR1/Pgp-inhibitor quinidine, central opioid effects, such as respiratory depression, became prominent (299). The pharmacological relevance of MDR1/Pgp expands beyond the protection of the brain from ingested toxins and drugs of serious abuse potential. MDR1/Pgp represents an obstacle to the brain penetration of agents targeted to the central nervous system (CNS) to treat epilepsy, central infections (such as HIV), or brain tumors. Thus there is a pharmacological need to overcome barriers maintained by MDR1/Pgp in tissue-blood and tumor-blood interfaces. Because selective modulation of MDR1/Pgp in cancer cells is difficult to achieve, attempts to circumvent MDR will have to face the profound effects of MDR1/Pgp inhibition on the distribution of concomitantly administered drugs.

In addition to pharmacological barriers, expression of MDR1/Pgp can be detected in the adrenal gland, the pregnant uterus, the placental trophoblasts, and hematopoetic stem cells. Perhaps MDR1/Pgp has a specific role in these locations (such as the excretion of steroid hormones). Alternatively, it may just stand guard to protect valuable physiological sanctuaries (see sect. vii).

As we have seen, the majority of experimental data support that MDR1/Pgp is an active transporter and that the energy of ATP hydrolysis is used for the removal of cytotoxic compounds from the cells. However, it is particularly tempting to speculate that MDR1/Pgp (or other ABC transporters) regulate important physiological mechanisms, such as the self-renewal of stem cells. Recent observations support the possibility that MDR1/Pgp can regulate cell fate. In these experiments, MDR1/Pgp was found to confer drug resistance to cancer cells through the inhibition of caspase-dependent apoptosis (155). In fact, this antiapoptotic effect persisted even when MDR1/Pgp was rendered inactive by mutation of key lysine residues of the Walker A region. Intriguingly, these data suggest a dual activity model for MDR1/Pgp-induced MDR, involving both ATPase-dependent drug efflux and ATPase-independent inhibition of apoptosis (369). Although direct interaction between MDR1/Pgp and apoptotic enzymes has not been documented, it has also been hypothesized that MDR1/Pgp acts as a primary antiapoptotic regulator by reducing ceramide levels either through the reduction of inner leaflet sphingomyelin pools or through the modulation of the glyco-sphingomyelin pathway (222, 377). The validity of these interesting conjectures awaits further confirmation and back up by experiments using, e.g., knockout models.

It has been advocated that in mammalian cell membranes most of MDR1/Pgp is localized in “raft” domains, that is, in cholesterol-rich, relatively detergent-insensitive, light membrane fractions, and this localization and/or the level of lipids, especially cholesterol, drastically modulate MDR1/Pgp transport activity (see Refs. 19, 101, 226). However, functional reconstitution studies of purified MDR1/Pgp into different lipid mixtures argued against a profound effect of specific lipids or cholesterol on MDR1/Pgp function (248). A number of studies still suggest that a rather complex effect of the membrane environment, including the possible “flipping” of some membrane lipids, may modify the transport capacity or the substrate affinity of MDR1/Pgp (see Refs. 88, 335).
E. Regulation of MDR1/Pgp Expression and Function

1. Transcriptional regulation

Undoubtedly, MDR1/Pgp-mediated resistance of in vitro engineered cells is due to the wide substrate recognition and the high efficiency of the pump. In addition, the predominant clinical (in vivo) role of MDR1/Pgp in multidrug resistance is ensured by mechanisms regulating its expression. In that setting, regulatory mechanisms enable adaptation of cancer cells, and also orchestrate the detoxification machinery that involves drug metabolism and the efflux systems (see sect. VII).

Data from the literature suggest a coordinated regulation of MDR1 promoter activity, with several overlapping binding sites for many different transcription factors. It is likely that through the competition for binding sites, transcription factors regulate transcription in a highly complex and interactive manner, where the combination, rather than the individual elements of the myriads of factors shape the ultimate response and provide specificity. In this review, we only provide a brief description of the structure of the promoter, in addition to the list of cellular factors and mechanisms that are involved in the transcriptional regulation of human ABCB1. For more details, we refer the reader to an excellent recent review (325).

The MDR1 gene, as is true for all of the human multidrug transporters examined to date, has a “TATA-less” promoter, where, instead of a TATA box, the transcription complex is controlled by an initiator (Inr) element. The transcriptional factors acting on the MDR1 promoter can be classified into several major groups, which include the inverted CCAAT-box (Y-box) binding proteins (NF-Y, YB-1), the CAAT-box interacting proteins (c-fos, NFκB), and the GC-box interacting proteins (Sp1–3, EGR1, WT1). The requirement of each element in the constitutive expression of ABCB1 has been demonstrated in several cell lines.

The high-level expression of MDR1/Pgp in tumors raised the possibility that oncogenes or tumor suppressor genes may regulate constitutive ABCB1 expression. p53 plays a complex role in the regulation of ABCB1, which may depend on the cellular environment, the cytotoxic drug used during selection or treatment, and mutations in p53 (43). The negative regulatory role (i.e., suppression) of wild-type p53 was published more than a decade ago (59), but the interacting site (a novel head-to-tail site, HT-site) in the ABCB1 proximal promoter region was identified much later (153). Surprisingly, several common p53 mutants activate, rather than suppress, the ABCB1 promoter (302). The effect of two members of the p53 family (p63 and p73) on ABCB1 transcription has been discovered recently. Overexpression of p63 and/or p73 in certain types of tumors may facilitate ABCB1 expression, thus influencing multidrug resistance in cancer. Both p63 and p73 activate, rather than repress, the ABCB1 promoter (154). The activation is independent of the p53-interacting (“repressor”) site or the “activator” site involved in the effect of the mutant p53.

Overexpression of MDR1/Pgp (or other multidrug transporters) in drug-resistant cells is either due to gene amplification or to elevated level of transcription. As mentioned above, a multiple start site element is present in the ABCB1 promoter, which was found to be functional in the activation of the gene in drug-resistant cell lines (228). In a recent report, this site (invMED1) was precisely localized between −105 and −100 bp, and the LRP130 protein was identified as a component of the nuclear factor that binds to the invMED1 site (199). It was also demonstrated that the invMED1/LRP130 complex formation increased with the chemoresistance level. The presence of another regulatory site between −118 and −111 bp was identified, binding a 130-kDa nuclear protein, named MDR1 promoter-enhancing factor (MEF1) (258). This interaction results in the upregulation of the ABCB1 gene. Recently, it was reported that RNA helicase A (RHA) is a component of the multiprotein complex that interacts with the −118 to −111 site in the ABCB1 promoter of a drug-resistant cell line (427). It was also shown that RHA is involved in upregulation of the ABCB1 expression in drug-resistant but not in drug-sensitive cells.

Permanent genomic alterations, like translocations or mutations in regulatory sequences, provide a possible mechanism for transcriptional alterations. Fojo and colleagues have used fluorescence in situ hybridization (FISH) to reveal breakpoints between MDR1 and sequences 500–1,000 kb telomeric to it. The rearrangements resulted in the capture of MDR1 by constitutively expressed genes, suggesting that random chromosomal rearrangement is a mechanism for activation of MDR1 in drug-selected cell lines and patient samples acquiring anticancer drug resistance (182, 238, 352).

The dynamic changes in the structure of chromatin can fundamentally influence gene expression. Chromatin-modifying enzymes, like histone acetylases (HATs) and histone deacetylases (HDACs), were found to be involved in the regulation of the ABCB1 gene. It was observed that upon treating the cells with specific HDAC inhibitors, the endogenous ABCB1 promoter as well as ABCB1 promoter/reporter constructs were activated (152). It was also shown that this activation is dependent on both an intact inverted CCAAT box and the transcription factor NF-Y.

Several studies have shown that the transcription of ABCB1 is regulated by DNA methylation. One consequence of CpG methylation is the silencing of ABCB1, which correlates with binding of methyl-CpG binding protein 2 (MeCP2) to the promoter, as was demonstrated by...
and upon demethylation, activation of ABCB1 control is managed by two epigenetic events; when MeCP2 is released. This means that the transcriptional transcription unless chromatin is demethylated and hyperacetylated core histones, but this does not activate chromatin immunoprecipitation (94). According to the ABCB1 expression of various human ABC transporters. The constitutive androstane receptor, CAR; and the farnesoid receptor, RAR; the steroid-activate receptor, SXR; the shock element (HSE) is localized in the toxic metabolites, or ultraviolet (UV) irradiation. A heat shock, inflammation, hypoxia, exposure to xenobiotics, 1 (HSF-1) with this element results in activation of ABCB1 promoter, which overlaps with the GC site playing a role in constitutive and in inducible expression, and activates the promoter (325).

Under acute phase conditions (i.e., in response to inflammation), when inflammatory cytokines act on the gene expression profile of the liver, ABCB1 is also induced in this organ (256). The interleukin-6-induced CAAT enhancer-binding protein (C/EBPβ) activates human ABCB1 via interaction with the −147 to −139 bp region of the promoter (63). In rodents, a homologous site is present that is involved in the glucocorticoid receptor-mediated activation of the hamster Mdr1 gene (325). This may suggest that in hamster, inflammatory and anti-inflammatory signals act in a concerted manner on the Abcb1 promoter. It was also shown that both mouse Mdr1 and Mdr3 and human ABCB1 can be activated in hepatoma cells exposed to dexamethasone (a synthetic glucocorticoid), demonstrating that the expression of these genes can be modulated by glucocorticoids. Glucocorticoids are used in chemotherapy of cancer, and better understanding of their regulatory role in ABCB1 transcription would be important (425).

2. Posttranscriptional regulation of MDR1/Pgp

Increased mRNA stability was identified as the basis of MDR1/Pgp overexpression in certain drug-selected cell lines (405). On the other hand, several studies indicate that the correlation between the level of mRNA and functional MDR1/Pgp expression is relatively poor, and highly variable under different in vitro and in vivo conditions. This discrepancy may be one of the causes why the role of MDR1/Pgp in cancer resistance is still debated.

As mentioned above, MDR1/Pgp is extensively glycosylated, but this glycosylation is clearly not a major factor either in the localization or the transport activity of the protein. According to recent information, MDR1/Pgp localization and stability may be more efficiently regulated by ubiquitination (424). MDR1/Pgp has also been shown to be phosphorylated at numerous sites, especially in the cytoplasmic linker region, connecting the two homologous halves (2, 103, 105). In early experiments the potential role of protein kinase C (PKC)-dependent phosphorylation (2, 410) in the function of MDR1/Pgp was advocated, especially because several PKC inhibitors also inhibited MDR1/Pgp function (see Refs. 23, 109). However, these results may have reflected a direct interaction of PKC inhibitors with MDR1/Pgp, and not a regulation through protein phosphorylation (135, 347). In detailed experiments, involving mutagenesis of the potential phosphorylation sites and direct measurement of MDR1/Pgp function, it became clear that phosphorylation has no essential role in the transport capacity of this protein,
while a slight modulation of substrate affinity or selectivity may involve such a covalent modification (46, 103, 106, 347, 361).

V. THE ABCG2 (MXR/BCRP) PROTEIN

A. Biochemistry and Cell Biology of ABCG2

The ABCG2 protein is a member of the ABCG sub-family, which in humans contains five homologous half-transporters, with the ABC domains located toward the NH₂ terminus of the polypeptide chain (see sect. II). Within this group we have no detailed knowledge about the physiological function of ABCG1 and G4, while ABCG5 and G8 are known to work as heterodimers, transporting various cholesterol-related molecules and providing resistance to plant-derived sterols (see sect. VI). As far as we currently appreciate, the only bona fide plasma membrane multidrug transporter in this group is ABCG2.

1. Structure and cellular localization

ABCG2, a 655-amino acid glycoprotein, was cloned independently from two drug-selected cell lines and a human cDNA library and was given three different names. ABCG2 cloned from a heavily drug-selected breast cancer cell line was named breast cancer resistance protein (BCRP; Ref. 83). The cDNA obtained from cells selected by mitoxantrone in the presence of verapamil (to block MDR1 function) codes for a protein denoted as mitoxantrone resistance protein (MXR) (245). The putative protein product of the cDNA obtained from a human cDNA library and showing enrichment in the placenta was named ABCP (8). Interestingly, the only cDNA coding for the wild-type protein was this latter one, while both cDNAs obtained from drug-selected cells contained a single nucleotide mutation, coding for altered amino acids at position 482 (R in the wild-type protein, T in BCRP, and G in MXR). These cloning differences produced a lot of controversy in the literature related to the substrate recognition and transport properties of ABCG2 (see below). The suggested membrane insertion pattern and topology of ABCG2 have been already shown in Figure 2, with six TM helices and a conserved ABC domain, although in a reverse arrangement compared with the ABCB or ABCC groups of transporters. It should be mentioned that membrane topology predictions for the ABCG group indicate a major difference in the interhelical loop arrangements compared with those in other subfamilies. Namely, the members of the ABCG family contain a relatively large extracellular loop between TM5 and TM6, and this arrangement prevents proper homology modeling for ABCG2, based on related ABC structures.

ABCG2 is physiologically expressed in a variety of tissues, most abundantly in the liver and intestinal epithelia, the placenta, the blood-brain barrier, and various stem cells. ABCG2 is a plasma membrane glycoprotein, in polarized cell types localizing to the apical regions (161, 227). ABCG2 is extensively glycosylated (from among the three predicted N-glycosylation sites) on asparagine-506, which is located within the third extracellular loop of the polypeptide (78, 250, 370). The extent of glycosylation of ABCG2 is variable in different tissues, but it has been clearly established that ABCG2 glycosylation, similarly to that found for MDR1 or MRPs, is not required either for the proper expression, function, or routing of this protein. Despite the lack of glycosylation, ABCG2 could be functionally expressed both in insect cells (260, 261) and in Lactococcus bacteria (151), and the removal of the predicted glycosylation sites did not modify either the membrane localization or the transport function of the protein in mammalian cells (78, 370). Moreover, in glycosylation-deficient mutants, both the steady-state expression and the half-life of the protein were unchanged (78).

An interesting feature of the ABCG2 protein that its structure and/or dimer form is stabilized by S-S bridges within the third extracellular loop of its transmembrane domain (see below). Recent biochemical studies established that alterations or removal of the NH₂-terminal 5–10 amino acid regions are not harmful for ABCG2 localization or function, while the COOH terminus (which, according to the membrane topology model has only a few amino acids outside the transmembrane region) is highly sensitive. Decoration of this area by conventional tags (e.g., 10-His or GFP) drastically reduces transport function and in most cases alters the membrane localization of ABCG2 (370 and C. Özvegy-Laczka and B. Sarkadi, unpublished experiments).

2. Transport properties

ABCG2 is an active transporter for many different drugs and metabolites, by extruding these compounds from the cells through a process energized by ATP hydrolysis. As discussed in detail below, the transported substrates of ABCG2 include many cytotoxic drugs, their partially detoxified metabolites, toxins, and carcinogens found in food products, as well as endogenous compounds. Similarly to all ABC multidrug transporters, drug extrusion by ABCG2 is closely coupled to a drug-stimulated, vanadate-sensitive ATPase activity, which requires the presence of Mg²⁺ (260). The direct, ATP-dependent transport of several less-hydrophobic substrates of ABCG2, e.g., methotrexate (264, 394), glucuronidated methotrexate, or sulfated estrogens and xenobiotics (56, 143, 359, 394) has been directly demonstrated in inside-out membrane vesicles. In polarized cell lines, obtained from MDR/MRP knock-out mice and transfected with human ABCG2, the transepithelial transport of a dietary
carcinogen and an H2 receptor antagonist, cimetidine, was demonstrated (271).

The substrate transport and ATP cleavage cycle of ABCG2 has not been investigated as yet in such detail as for MDR1 or MRP1, but we suspect no major differences in the basic steps. Mutation of the key lysine residue within the Walker A domain (K86) to methionine (similarly to that found for MDR1 or MRP1) abolishes both transport and ATPase activity of ABCG2. ATP binding and the formation of a reaction cycle intermediate, occluded ADP, stabilized (trapped) by vanadate in this protein have also been documented (261). Recently, we found that the binding of the anti-ABCG2 monoclonal antibody 5D3, recognizing an extracellular epitope of this protein, can be applied to investigate the catalytic cycle-dependent conformational changes of ABCG2. The ATP-bound nucleotide-trapped forms of ABCG2 show low-affinity antibody binding, while the ADP-bound and substrate-inhibited forms show maximum antibody-binding (265). These binding differences suggest major conformational movements within ABCG2, which have also been suggested by the molecular models for various ABC transporters detailed in section II.

In the case of ABCG2 we have the advantage of having a specific, high-affinity inhibitor molecule. Rabin dran and co-workers (280, 281) first observed that a micotoxin, Fumitremorgin C, was a strong inhibitor of drug resistance in ABCG2-expressing tumor cells, and Koomen and co-workers (386) developed derivatives of this molecule with potent and selective inhibitory action on ABCG2. One of these molecules, Ko143 (7), inhibits ABCG2 in nanomolar concentrations, whereas it has practically no effect on other multidrug transporters or on cellular functions up to micromolar levels. As shown in several studies, Ko143 blocks both the transport and the ATPase activity of ABCG2 at the same low concentrations. According to our unpublished studies, Ko143 is a high-affinity competitive-type inhibitor, probably interacting with the “off-site” of drug binding in the ABCG2 protein (Özvegy-Laczka, Glavinas, and Sarkadi, unpublished data).

ATP cleavage and the transport cycle, as in all ABC multidrug transporters, seem to be coupled to events in the ABCG2 protein. However, when the ATPase activity of the human ABCG2 protein was examined in isolated Sf9 cell membranes, we found that the ATPase activity of the wild-type protein was high and could be hardly stimulated by substrates of the transporter (260, 261). In contrast, in membranes isolated from ABCG2-overexpressing mammalian cells, the ABCG2-ATPase activity was significantly stimulated by many transported substrates (67, 247). The lack of glycosylation of ABCG2 in Sf9 membranes cannot be responsible for this effect (370), and we suspect that the lipid composition of the insect cell membrane results in an endogenous modulation of the ABCG2 ATPase activity (see below). As judged from expression levels and transport/ATPase measurements in isolated cell membranes, human ABCG2 has a similar, or even higher turnover than the MDR1 protein (260).

3. Dimer formation

As detailed in section II, according to our current knowledge the minimum requirement for a functional ABC transporter is the cooperation of two ABC and two transmembrane domains; thus the functional form of ABCG2 must be at least a dimer. Most experimental data indicate that in the case of ABCG2, the prevalent form is a homodimer; at least a homodimer can carry out all the relevant transport functions. In drug-selected cell lines, the upregulation of the ABCG2 gene expression alone was sufficient to cause drug resistance (183, 245), and transfection by the ABCG2 cDNA alone produced massive drug resistance in various mammalian cells (82). When human ABCG2 was expressed in heterologous Sf9 cells (260), or even in Lactococcus (151), the protein was fully functional, although unlikely to find any endogenous dimerization partners in the insect cells or bacteria, respectively. A dominant negative effect of a nonfunctional ABCG2 mutant on drug resistance and transport function (93, 166) is also indicative of protein homodimerization.

Homodimerization of the ABCG2 protein was directly demonstrated by using nonreducing SDS gels, where a disulfide-bridged dimer of ABCG2 was found to occur (166, 277, 370). Due to the reducing intracellular environment, such a physiological disulfide link can only be formed between SH groups present on the extracellular loops of the protein. Indeed, three cysteines, conserved in most mammalian ortholog ABCG2 sequences in the third extracellular loop, were found to play a key role in the dimerization, expression, and localization of this protein (117, 370). According to our unpublished studies (Özvegy-Laczka and Sarkadi, unpublished data), chemical cross-linking of ABCG2 dimers may still preserve transport function, while various modifications of the cell surface S-S bridges may result in the loss of ABCG2 transport activity. Several sequence motifs within ABCG2 have been suggested to influence ABCG2 function and dimerization (277), but a clear-cut picture has not yet been established in this regard. As suggested by one experimental study, higher order oligomerization of ABCG2 may be involved in the function of this protein (404).

B. Transported Substrates of ABCG2 and Its Mutants/Variants

ABCG2 is a drug transporter with a wide substrate specificity, that includes large molecules, both positively and negatively charged, with amphiphilic character. The reported cytotoxic drugs extruded by the wild-type hu-
man ABCG2 are mitoxantrone, topotecan, the active metabolite (SN-38) of irinotecan, camptothecin, flavopiridol, and methotrexate. The transported substrates of this protein also include sulfated hormone metabolites, antibiotics such as nitrofurantoins, antihelminthic benzimidazoles, various flavonoids, the food carcinogen PhIP, the chlorophyll metabolite pheophorbide a, fluorescent dyes such as Hoechst 33342 and BODIPY-prazosin, or the H2 receptor antagonist cimetidine (24, 146, 161, 162, 210, 235, 254, 271, 287, 384, 393). The pharmacological and possible physiological substrates are discussed in detail in the relevant following sections.

There is little information on the structural elements responsible for the substrate recognition and transport by ABCG2. In the case of MDR1 and MRP1 (see above). The transmembrane domains were found to be responsible for the recognition of transported substrates. In the case of ABCG2, the artificial R482 mutants and the naturally found polymorphic variants of this protein might give a lead to study these recognition sites and patterns.

1. Mutant variants of ABCG2

The cloning variants first characterized in drug-selected mammalian cells (R482G and R482T) caused a lot of uncertainty regarding the substrate profile of ABCG2, but became also educative regarding the substrate handling of this protein. ABCG2 variants containing either R482G or R482T conferred increased mitoxantrone resistance to transfected cells; moreover, they introduced anthracyclin (doxorubicin) resistance and rhodamine-123 extrusion capacity, which were not found in the case of the wild-type protein (see Fig. 10, Refs. 127, 261). In contrast, the R482G and R482T mutants were not able to extrude methotrexate, which is a transported substrate of the wild-type ABCG2 (56, 242, 393). Interestingly, the R482G mutant variant of ABCG2. The major differences in this selectivity were the removal of the positive charge or alteration of the recognition site for the ABCG2 protein. ABCG2-specific ATPase activity (inhibited by Ko143). Mitoxantrone was transported by all ABCG2 variants, except by R482K. Rhodamine-123 was extruded by most of the mutants, except by R482K, R482Y, and the wild-type ABCG2. Interestingly, the R482K variant had relatively low activity in all assays, although this mutation (Arg to Lys) represents only a minor change without charge alteration. In contrast, the removal of the positive charge or even the introduction of a negative charge at this site, in several cases greatly increased or in other cases did not alter the transport activity. While no detailed structural data for ABCG2 are currently available, according to the topology models amino acid 482 is localized near to, the cytoplasmic end of the third transmembrane helix of the protein. The above functional data may become even more valuable when the substrate recognition site of ABCG2 can be properly modeled.

Still, the role of this mutation in ABCG2 may give important information on the structure-function aspects. Recently two detailed studies (244, 264), examining the effects of artificial amino acid 482 mutations in ABCG2 by using various cellular expression and transport systems, have clearly demonstrated a key role of this site in the ABCG2 transport function. In intact, transfected cells, these mutants showed significantly different drug resistance patterns, some with a gain-of-function while several others with a loss-of-function character (244). When expressing nine Arg-482 mutants of ABCG2 in Sf9 cells (264), we found that none of these changes was detrimental for the correct folding, cell surface expression, or ABCG2-specific ATPase activity (inhibited by Ko143). Mitoxantrone was transported by all ABCG2 variants, except by R482K. Rhodamine-123 was extruded by most of the mutants, except by R482K, R482Y, and the wild-type ABCG2. Interestingly, the R482K variant had relatively low activity in all assays, although this mutation (Arg to Lys) represents only a minor change without charge alteration. In contrast, the removal of the positive charge or even the introduction of a negative charge at this site, in several cases greatly increased or in other cases did not alter the transport activity. While no detailed structural data for ABCG2 are currently available, according to the topology models amino acid 482 is localized near to, the cytoplasmic end of the third transmembrane helix of the protein. The above functional data may become even more valuable when the substrate recognition site of ABCG2 can be properly modeled.

A key observation in these studies was that methotrexate transport was singularly supported by the wild-type ABCG2. This finding suggests that, although the natural R482-ABCG2 protein has a lower turnover and narrow substrate recognition, a group of transported molecules, including folates, methotrexate, and its conjugated variants, may be preferential substrates of ABCG2 under in vivo conditions. This special recognition might give a strong selective force in evolution to keep this variant uniformly present in the population. No apparent mutation hot spots such as CpG islands can be identified in this particular region, and these mutations were probably generated as a combined result of the mutagenic
effect of the cytotoxic drugs and a long-term in vitro drug selection. As mentioned later, these variants with a selected transport profile may be helpful for various cell protection applications.

It is worth noting that in addition to the amino acid 482 ABCG2 mutations, several other variants, A149P, T153M, R163K, and P269S, were identified in different cell lines that were also not detected in healthy individuals (188, 247) (Fig. 11). In the case of in vitro expression, the above ABCG2 variants did not show any difference from the wild-type ABCG2 protein, neither in their subcellular localization in LLC-PK1 polarized cells, nor in their estrone sulfate, dehydroepiandrosterone sulfate (DHEAS), methotrexate, or p-aminobenzylamine (PAH) transport. Therefore, it is not clear why these variants appeared in drug-selected cell lines (188), although it is interesting that three out of four mutations identified to date affect nonconserved amino acids of the ATP binding domain. In the first cloning of the ABCG2 cDNA from human placenta (8), several sequence alterations causing amino acid changes, including A24V, Q166E, and F208S, were recorded, compared with the database reference sequence. In subsequent studies, these nucleotide substitutions were not detected in healthy individuals or patients (see Ref. 418), and we are unaware of any functional consequences of these alterations. In contrast, the polymorphic variants of the ABCG2 protein, appearing in >1% in the human populations, have attracted a lot of attention in the past few years. We discuss these variants in section V, C and D.

C. ABCG2 in Cancer Multidrug Resistance

1. Anticancer agents and ABCG2

As mentioned above, ABCG2 transports a wide variety of anticancer agents. A key group in this regard includes topoisomerase I inhibitors, e.g., topotecan, irinotecan, and its active metabolite SN-38 (255, 317). From the discovery of the protein we know that ABCG2 extrudes the important anticancer agents mitoxantrone, camptothecin and its analogs (e.g., ifosfamide, a synthetic derivative of camptothecin), as well as flavopiridol, a promising drug under clinical development (96). As described in section IV, the MDR1 protein has an overlapping transport profile with ABCG2, and the relative role of the different MDR-ABC transporters in the extrusion of a given compound is difficult to assess.

A potentially clinically important role of ABCG2 is to transport methotrexate (MTX) and its polyglutamated forms, thus greatly modifying both short-term and long-term methotrexate resistance in various cancer cells. Among the MDR-ABC transporters, several MRPs can also transport methotrexate, but these pumps are usually unable to extrude the intracellularly glutamated forms of folates or folate antagonists, while ABCG2 is a highly active pump for all glutamated folates. Moreover, ABCG2 has a relatively high capacity and affinity in transporting newly developed antifolate agents and folate derivatives (399) as well. The in vivo relevance of MTX transport is suggested by the finding that in cancer patients the coad-
ministration of benzimidazoles and MTX can result in severe toxicity, with an increase in the serum concentrations of MTX. Competition of MTX and the ABCG2 substrate benzimidazol on this transporter may explain this pharmacological interaction (37).

The chemically heterogeneous group of anticancer agents interacting with ABCG2 includes a variety of tyrosine kinase inhibitors (TKIs). In current antitumor drug research, TKIs are highly promising agents for specific inhibition of malignant cell growth and metastasis formation. However, their therapeutic potential also depends on access to intracellular targets, which may be significantly modulated by ABC membrane transporters. Several research groups have recently shown that the human ABCG2 multidrug transporter interacts with a number of anticancer TKIs, including Imatinib (STI-571) and Iressa (ZD 1839).

According to our in vitro experiments (263), both Iressa and Imatinib inhibit the transport function and cell protecting activity of ABCG2 in submicromolar concentrations. Because low Iressa concentrations stimulated the ABCG2-ATPase activity, we suggested that Iressa may be a transported substrate. Stewart et al. (355) examined the effect of Iressa in tumor xenografts and Saos2 cells and found that this TKI reversed ABCG2-mediated resistance to irinotecan and SN-38. Still, their experiments indicated that Iressa is not a transported substrate for ABCG2.

To answer the question if Iressa is extruded by ABCG2, two recent studies examined a pharmacologically relevant model system, that is the growth of A431 tumor cells, depending on epidermal growth factor (EGF) receptor signaling. Yanase et al. (406) reported that in transfected A431 cells, ABCG2 provided a significant resistance against Iressa. In our experiments (93), the retroviral expression of ABCG2 protected A431 cells from Iressa, while this protection was absent in the presence of the selective ABCG2 inhibitor Ko143 or in cells expressing an inactive ABCG2 mutant. ABCG2 function also prevented EGF receptor dephosphorylation and the extracellular appearance of phosphatidylserine, a marker for early apoptosis (93).

When studying the action of Imatinib in Saos2 osteosarcoma cells expressing ABCG2, Houghton et al. (133) found that this TKI reversed SN-38 and topotecan resistance in submicromolar concentrations. However, the sensitivity to Imatinib, or the accumulation and efflux of labeled Imatinib, was not modulated by ABCG2 expression; therefore, these authors concluded that Imatinib is a high-affinity inhibitor, and not a transported substrate of ABCG2. In contrast, by using labeled Imatinib and ABCG2-expressing MCF-7 or HEK cell lines, Burger et al. (40) showed that the accumulation of Imatinib was significantly lower in ABCG2-expressing cell lines than in the parental cells. Moreover, when the specific ABCG2 inhibitor Ko143 was added, Imatinib accumulation in ABCG2-expressing cell lines reached the same high level as in parental cells. Breedveld et al. (36) found an active, vectorial transport of Imatinib in an epithelial cell monolayer, transfected with the mouse Abcg2. Moreover, according to their results, ABCG2 function limits the penetration of Imatinib through the blood-brain barrier (see below).

These experimental data suggest that both Iressa and Imatinib interact with ABCG2 at low, pharmacologically relevant concentrations and while they inhibit the transport of other substrates, these TKIs are also transported by ABCG2. These data also indicate that the expression and function of ABCG2 may significantly alter the absorption, metabolism, and toxicity of TKIs (262).

Some of the above discussed results suggest that there may be a narrow concentration range in which multidrug resistance proteins can transport the TKIs, while above these concentrations TKIs inhibit the function of these proteins (93, 262). Therefore, it is possible that ABCG2 may not influence the intestinal absorption of, e.g., Iressa, present at this site in high concentrations, but may play a role in cellular extrusion of this compound where its local concentration is lower.

2. ABCG2 expression in tumors

Although experimental data for cytotoxic drug transport by ABCG2 are convincing, a key question for the in vivo relevance is the functional expression of ABCG2 in various human tumors. Overexpression of ABCG2 was first documented in drug-selected cell lines from ovary, lung, breast, colon, and gastric cancer, mediating in vitro resistance against various cytotoxic compounds (6). According to the analysis of ABCG2 mRNA and protein levels in blast cells from acute leukemia patients, ~30% of the patient samples were identified with relatively high expression of ABCG2 (295, 304). In acute lymphoblastic leukemia (ALL), ABCG2 expression was more abundant in B-cell lineage ALL, with a function to produce significant drug resistance (275). More and more data indicate that individuals with higher ABCG2 expression in their leukemic blast cells have a higher probability of poor response to chemotherapy (28, 354, 358, 378).

Immunohistochemical studies in human tumors revealed frequent expression of ABCG2, especially in adenocarcinomas of the digestive tract, lung, and endometrium (76). However, both intracellular and plasma membrane staining was observed; thus the drug extrusion relevance of this protein expression may be questionable. In non-small cell lung cancer (NSCLC) tissue extracts, the expression and function of ABCG2 were analyzed in parallel experiments (174), and the authors concluded that these tumors expressed sufficient functional ABCG2 to confer drug resistance. In a retrospective study in NSCLC, the chemotherapy response rate in patients was found to
be correlated with ABCG2 expression (while not with MDR1, MRP1, or MRP3 expression), suggesting that the inhibition of ABCG2 function may help overcome drug resistance in such patients (408).

ABCG2 expression was greatly increased in irinotecan- and SN 38-resistant colon cancer cell lines, and the in vivo overexpression of ABCG2 in the metastases of irinotecan treated patients was also detected (44). Still, the controversy about the actual role of ABCG2 in cancer multidrug resistance is unresolved, and the major hope in this regard is the introduction of proper, quantitative laboratory diagnostic methods for the detection of ABCG2 in tumor samples.

3. Diagnostics of ABCG2 expression and function

On the basis of the above-described role of ABCG2 in tumor drug resistance, the selective and sensitive detection of the ABCG2 protein may have a major importance in cancer diagnostics and treatment. Determination of RNA or total protein levels in the case of MDR-ABC transporters may lead to seriously misleading results, as the active form of these transporters is only the protein properly localized on the cell surface and in polarized cells in the right membrane compartment. One possible way is thus to determine the cell surface expression level by using monoclonal antibodies, while the other approach is to directly measure the transport function of the protein in intact tumor cells.

As to the determination of ABCG2 cell surface expression, a group of monoclonal antibodies have been developed that specifically recognize this protein on extracellular epitopes in intact cells (430). Interestingly, the antibody, named 5D3, inhibits the transport and ATPase function of ABCG2 in intact cells (265, 430), and both antibody binding and the inhibition of ABCG2 function depend on the actual conformation of the transporter (265). Thus the proper detection of quantitative ABCG2 expression by this antibody requires fixation of the protein in a high-affinity conformation, either by chemical cross-linking or by stabilization of a specific step within the transport cycle. The increasing knowledge on the antibody-reactive conformations of ABCG2 should improve the use of this important diagnostic tool.

The other major way to characterize the relevant multidrug transporter activity of ABCG2 is to measure the active extrusion of a labeled or fluorescent compound. In practical laboratory diagnostics, the use of a nontoxic fluorescent dye, with a unique interaction with ABCG2, and providing high level fluorescence in a convenient wavelength area, would be most advantageous both in flow cytometry and fluorescence microscopy studies. The most often applied method in this regard is the measurement of the fluorescent dye Hoechst 33342 by flow cytometry (430). Several other fluorescent substrates of ABCG2 (e.g., topotecan, flavopiridol, BODIPY-prazosin, or mitoxantrone) have also been applied in this regard. However, these dye transport assays are not specific for ABCG2, and the combined use of ABCG2-specific inhibitors is advocated in these methods. A recent report suggests that the chlorophyll derivative phaeophorbid a is a selective ABCG2 substrate that can be used for flow cytometry analysis of this protein (287).

As a summary, the laboratory detection of functionally relevant ABCG2 expression in cancer cells has several encouraging options, but a highly sensitive and selective morphological and/or functional recognition of this transporter is still to be introduced.

D. Physiological and Pharmacological Functions of ABCG2: Drugs, Sex, and Survival

Both the transport properties and the tissue distribution of ABCG2 indicate a key role of this protein in the protection of our body against xenobiotics (227, 316). Since ABCG2 transports a variety of amphipathic molecules, we can expect that both natural and artificial toxins can be eliminated by its function. Moreover, ABCG2 is physiologically expressed in relatively high levels in the canalicular membrane of the liver, in the epithelia of small intestine, colon, lung, kidney, adrenal, and sweat glands, as well as in the endothelia of veins and capillaries, including the capillary endothelial cells of the blood-brain barrier, and in stem cells (66, 227, 430).

The apical membrane expression of ABCG2 in epithelial cells along the gastrointestinal track suggests a major role of this protein in the first line of defense against xenobiotics. This protection is further extended by the function of ABCG2 in the biliary and renal luminal epithelia, responsible for toxin clearance. Interestingly, issues related to drugs, sex differences, hormonal regulation, and protection of the fetus and stem cells all seem to be connected to ABCG2 function.

Again, it is important to note that ABCG2 has an overlapping transported substrate profile and localization with other major multidrug transporters, especially that of MDR1 and MRP2. Therefore, the appreciation of the physiological and pharmacological role of ABCG2 requires special experimental approaches, that is, the use of inhibitors or various transporter knock-out conditions.

1. First line defense and secretion: general xenobiotic resistance

As to the role of Abcg2/ABCG2 in xenobiotic (toxin and drug) absorption and bioavailability, the first important information was provided by investigations carried out in Mdr1a knock-out mice. When topotecan (an ABCG2 substrate) and GF120918, an inhibitor of ABCG2, were coadministered orally to these animals, the bioavail-
ability of topotecan in the blood plasma was drastically increased. The hepatobiliary excretion of intravenously administered topotecan was decreased by oral GF120918, consistent with an excretory role of canalicular Abcg2 into the bile (163). Based on these experiments, similar studies were carried out in human patients treated with topotecan. The oral administration of the ABCG2/MDR1 inhibitor GF120918 significantly increased the oral absorption and systemic bioavailability of topotecan (192). These data indicate that ABCG2 function limits the uptake of topotecan from the intestinal lumen, while it increases the hepatobiliary excretion of this compound.

To appreciate this physiological role, the development of the Abcg2 knock-out mice was initiated (161, 429). The nonessential nature of Abcg2 in these knock-out animals, that is, the lack of any phenotype in a controlled environment, was the first key observation. However, under various environmental or pharmacological challenges, several alterations have been observed connected to the lack of functional Abcg2.

The Dutch group first reported that in the Abcg2 knock-out mice the combination of exposure to UV light and an alfalfa diet, rich in chlorophyll, led to serious phototoxic skin lesions (161). These lesions were caused by the accumulation of a chlorophyll degradation product, pheophorbide a, found in various plant-derived nutrients and food supplements. Normally, Abcg2 actively transports pheophorbide a, efficiently limiting the uptake of this compound from ingested food, while in the knock-outs there was an increased intestinal absorption and decreased biliary secretion of this toxin. Based on these studies, it has been suggested that the malfunctioning of ABCG2 may cause an increased risk for developing protoporphyria and diet-dependent phototoxicity. This finding is a striking illustration of the importance of drug transporters in protection from toxic compounds (161). Indeed, van Herwaarden et al. (384) demonstrated that Abcg2 knock-out mice have elevated plasma levels and decreased intestinal, fecal, and hepatobiliary excretion of the food carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). A recent study of Adachi et al. (1), using either MRP2-deficient or Abcg2 knock-out animals, demonstrated that ABCG2 has a key role in extruding glucuronide and sulfate conjugates of drugs and hormones, formed in the enterocytes, into the intestinal lumen.

There are numerous ABCG2 substrate compounds that could compete with each other during absorption or secretion, and this competition may involve various food constituents. Flavonoids are important modifiers of several physiological processes (see the “French paradox,” probably caused by flavonoids in red wine), and they also interact with ABCG2. As shown by Imai et al. (146), several flavonoids, including genistein and naringenin, increased the cellular accumulation of topotecan in ABCG2-expressing cells. A direct transport of labeled, unmetabolized genistein has also been documented. Thus flavonoid consumption may significantly alter the pharmacokinetics, increasing the toxicity or the antitumor action of ABCG2 substrate compounds. In summary, all these data suggest that ABCG2 has a major role in the modulation of the absorption of toxic materials from food, as well as of their excretion mostly through the biliary pathway (77).

2. Prevention of endogenous toxin accumulation

The Abcg2 knock-out mouse study, carried out in the United States, revealed a hypersensitivity of the tissues in these animals to hypoxic challenges (191). Progenitor cells obtained from Abcg2 knock-out mice showed a reduced ability to form colonies under hypoxia. Moreover, blocking of Abcg2 function in normal progenitor cells reduced survival under hypoxic conditions. As it turned out, the primary cause of this hypoxic susceptibility was the accumulation of cellular heme and/or porphyrins. The blockade of heme/porphyrin synthesis reversed this condition, while cells overexpressing ABCG2 accumulated lower amounts of porphyrin. Heme was shown to be specifically bound by ABCG2, and drug transport by ABCG2 was significantly modulated by heme.

These studies suggest that ABCG2 permits enhanced cell survival in oxygen-poor environments by reducing the accumulation of toxic heme metabolites; thus ABCG2 expression provides an important cell survival advantage under hypoxic conditions. Of course, the chemical similarity of pheophorbide and porphyrin is evident and should direct our interest to possible ABCG2-dependent transport of various related compounds.

3. ABCG2 in the placenta

The wild-type human ABCG2 was first cloned from a placenta library (8), and the protein is abundantly expressed in chorion epithelial cells. The maternal-fetal barrier in the placenta, protecting the fetus from drugs and environmental toxins, has been indicated to involve the function of MDR1/Pgp. Further studies in Mdr1 knock-out mice indicated an important role of ABCG2 in this tissue. In pregnant, Mdr1-deficient mice, when topotecan was coadministered with the inhibitor GF120918, the fetal penetration of topotecan was significantly higher than without this inhibitor (163). In the Abcg2 knock-out mice, the fetal exposure to topotecan and dietary toxins greatly increased, compared with normal mice (320). The selective inhibition of Abcg2 by fumitremorgin C also produced a large increase of the topotecan and mitoxantrone levels in the fetus (187). All these data indicate that ABCG2 plays a major role in protecting the fetus against toxic compounds ingested by the mother (Fig. 12).
4. ABCG2 in the blood-brain barrier

The presence of ABCG2 in the brain endothelia was indicated by RNA expression measurements (90, 423), and the expression of the ABCG2 protein was directly demonstrated in the luminal surface of brain microvessels, forming the blood-brain barrier (BBB), by immunofluorescence confocal microscopy (66). The vectorial drug transport by ABCG2 overexpressed in immortalized endothelial cells indicated a functional role of this protein, limiting the brain penetration of hydrophobic compounds (115). By using in situ brain perfusion techniques, in Mdr1 knockout mice an efficient extrusion of prazosin and mitoxantrone by Abcg2 in the BBB was clearly demonstrated (62). Interestingly, flavonoid transport through the BBB was also modified by the function of ABCG2 (409). In a rat BBB model, the expression and function of rABCG2 was upregulated by astrocyte-derived soluble factors (132), and the application of an siRNA reduced both the expression and the transport function of endogenous rABCG2 in capillary endothelial cells (131). Recently, Breedveld et al. (36) have shown that the brain penetration of intravenously administered Imatinib was significantly increased in Abcg2 knockout mice. All these data strongly indicate an important function of ABCG2 in the BBB, protecting the brain from potentially toxic agents (Fig. 12).

5. ABCG2 in stem cells

A fascinating development in the field of stem cell research was the finding that a high-level expression of the ABCG2 protein and its fluorescent dye extrusion function could identify hemopoietic stem cells (177, 314, 430). The so-called "side population (SP)" of progenitor cells, actively extruding the fluorescent Hoechst 33342 dye, seems to represent pluripotent stem cells in a variety of tissues (177, 201, 314, 357, 430) (Fig. 12). Although we do not know as yet why ABCG2 expression is high in these cells, it seems likely that it is required for the metabolic protection of stem cells. ABCG2 expression decreases during stem cell differentiation. Still, both the knock-out mouse studies and recent transplantation experiments suggest that the lack of ABCG2 expression or ABCG2 overexpression do not directly influence stem cell function and differentiation (249, 429) (O. Ujhelly, unpublished data). As mentioned above, ABCG2 function may protect against local hypoxia; thus its presence may be a survival factor for stem cells under unfavorable conditions. This role may have important implications for increasing medical activities connected to stem cell transplantation.

6. Sex differences in ABCG2 expression and function

Recent studies demonstrated a sex-dependent expression and function of ABCG2 in various organs. In mice a significant difference was observed in the pharmacokinetic behavior of ABCG2 substrate drugs between male and female animals, leading to much higher plasma levels in female mice after a similar drug exposure (237). The main cause of this difference was the higher liver expression and biliary clearance function of Abcg2 in male mice, while this pharmacokinetic sex difference was absent in Abcg2 knockout animals. The authors also found a much higher hepatic expression of ABCG2 in men than in women (237). Imai et al. (144) reported that estrogens, like 17β-estradiol, downregulate ABCG2 expression through an estrogen receptor α-interaction pathway in various cell lines (see below). Another recent study (371) has found a much higher ABCG2 expression in the intestine and kidney of male rats compared with females, and a male-dominant expression was observed in the mouse liver. Moreover, when following ABCG2 expression, a suppressive effect of estradiol and an induction by testosterone (371) were observed. These data may have a major importance in characterizing the often ob-

![Diagram](image-url)
erved sex differences in drug effectiveness and toxicity. Importantly, the sex hormone-dependent regulation of ABCG2 expression may be connected to its role in the transport of various hormone metabolites, e.g., estrone sulfate.

In connection with sex-related differences, ABCG2 was shown to have an important role in drug and toxin secretion into milk. As reported recently by Jonker et al. (162), ABCG2 expression (as examined in mice, cows, and humans) is greatly increased in the mammary gland during lactation, and the ABCG2 protein actively secretes various drug and toxin substrates into the milk. The secretion of the antibiotic nitrofurantoin into milk has been reported to be 80 times higher in wild-type mice compared with the Abcg2 knock-out animals (236). Thus the regulation and function of ABCG2 may significantly modulate contamination of milk with drugs and xenobiotics, leading to toxin exposures in infants or consumers of dairy products.

E. Polymorphisms and Regulation of ABCG2

Both the pharmacological and physiological functions of the ABCG2 protein have been indicated to be significantly influenced by interindividual variations and regulatory responses. In this section we discuss the two main apparent sources of these variations: a widespread genetic polymorphism, probably affecting both protein expression and function, and the response to toxin exposure, stress, or hormonal changes by a variable expression and processing of ABCG2.

1. SNPs in ABCG2

Analysis of the ABCG2 sequences in the human population identified several single nucleotide polymorphisms (SNPs), and their effects on protein expression and function have been the subject of numerous recent studies. Earlier reports indicated considerable interindividual variations in the oral bioavailability and clearance of drugs that are ABCG2 substrates, such as topotecan (163, 419), which may reflect such a polymorphism.

The ABCG2 gene is located on chromosome 4q22, spans over 66 kb, and consists of 16 exons, ranging from 60 to 532 bp in length (20). In healthy individuals or patients, altogether eight nonsynonymous (V12M, Q141K, I206L, F431L, S441N, F489L, N590Y, D620N), five synonymous (silent) (c. 114T>C, c. 369C>T, c. 474C>T, c. 1098G>A, c. 1425A>G) mutations, one nonsense (Q126X, c. 376C>T), and one frameshift (c. 1515delC) mutation were identified in the coding region of ABCG2. The sequence variant Q126X, leading to premature termination of protein synthesis, was consistently observed in certain Japanese cohorts, while absent in different Caucasian and African American groups (145, 148, 185). From these numerous reported alterations, two protein variants, V12M, and Q141K, were found in relatively high frequencies, with significant differences in allele frequencies in different areas of the world (Fig. 11).

The V12M polymorphism affects the N-terminus and the signature region (amino acids 83–89) and the Walker A motif (amino acids 38–47) of the uncharged glutamine residue with a positively charged lysine within the ATP-binding domain, between the Walker A motif (amino acids 83–89) and the signature region (amino acids 186–189). The Q141K variant was also detected in all ethnic groups tested: the allele frequency ranged between 1 and 35% (the African and African-American subjects with low, while the Japanese and Chinese populations with high allele frequencies; see Refs. 45, 74, 185).

To clarify the possible physiological or pathological relevance of the ABCG2 polymorphisms, several studies attempting the functional characterization of the variants were performed. Interestingly, the results of the different research groups, regarding expression levels, localization, and functionality, are quite contradictory. Imai et al. (145) and Morisaki et al. (251), by using stable mammalian expression systems, found that in PA317 or HEK-293 cells the expressed Q141K ABCG2 protein had a lower expression level than the wild-type ABCG2, or the V12M variant. Morisaki et al. (251) demonstrated that both the V12M and Q141K ABCG2 could reach the plasma membrane in the HEK-293 cells, while a significant portion of Q141K remained intracellular. Other studies found a 30–40% reduction in cell surface expression of the Q141K variant, despite a similar mRNA level than the wild-type ABCG2 (145, 188).

Recent investigations of 99 Japanese placenta samples revealed that individuals homozygous for the Q141K variant showed significantly lower expression levels of this transporter protein, while the heterozygous samples displayed an intermediate expression level (185). In contrast, another study, investigating the expression of natural allelic variants of ABCG2 in the human intestine, demonstrated no significant differences in mRNA and protein levels between subjects expressing the K141 allele in heterozygous form, or the Q141 (wild-type) allelic variant (418).

Mizuarai et al. (247) expressed ABCG2 in polarized LLC-PK1 cells, and by using confocal microscopy, the authors observed that the wild-type ABCG2 and Q141K showed mainly apical staining, while the V12M variant...
showed mostly intracellular localization. In a recent study, Kondo et al. (188) also used LLC-PK1 cells to express the V12M and Q141K variants and found that all polymorphisms, including V12M and Q141K, had an apical localization. These contradictory expression and localization data for the ABCG2 variants, even when similar cell lines were applied, indicate that differences in culture conditions or other cellular determinants may variably affect the cellular processing of these proteins.

When the functions of the ABCG2 variants were examined in cytotoxicity assays, a 10-fold decrease in drug resistance, compared with the wild-type ABCG2, was reported by Mizuarai et al. (247), when the V12M or Q141K-transfected LLC-PK1 cells were challenged by mitoxantrone, topotecan, or an indolocarbazole topoisomerase I inhibitor. In contrast, Morisaki et al. (251) found that only the Q141K variant had a moderately lower level resistance against mitoxantrone, topotecan, or SN-38, compared with the wild-type ABCG2-transfected cells. Moreover, in experiments comparing the transport activities of the wild-type protein and its variants for estrone 3-sulfate, dehydroepiandrosterone sulfate, methotrexate, or PAH, no significant differences were reported, when the transport activities were normalized for the expression levels of ABCG2 proteins (188).

Q141K is mapped to a functionally important ABC region of ABCG2; therefore, it is possible that the ATPase activity of this variant is altered. Two studies compared the vanadate-sensitive ATPase activity of ABCG2 V12M and Q141K variants, using Sf9 (Spodoptera frugiperda) cell membranes (247, 251). A reduced basal ATPase activity was observed by both groups for the Q141K variant. On the other hand, the V12M ABCG2 showed a similar ATPase activity as the wild-type protein.

All these transport, ATPase, and cell survival data suggested that the investigated SNPs may not substantially alter the substrate specificity of ABCG2. On the other hand, if their expression level is indeed lower, or their membrane localization is impaired, these alterations may modify ABCG2-dependent drug transport. Clearly, more detailed studies are required to clarify the mechanism of a reduced protein expression for Q141K, and the altered cellular localization found for the V12M and Q141K variants under certain conditions. It is important to note that in heterozygous individuals, different ratios of the ABCG2 dimers may be present. The above in vitro experiments investigated homodimers of the variants, which is a model applicable only for the rare cases of homozygotes.

In addition to the above-described in vitro investigations, several pharmacokinetic studies are in progress to evaluate the potential functional differences of various ABCG2 genotypes. De Jong et al. (74) investigated patients with solid malignant tumors receiving irinotecan treatment. In 84 European Caucasian patients, the frequency of the K141 allele was 10.7%, with 14 patients carrying heterozygous alleles and 2 patients homozygous for the variant. According to this study, the pharmacokinetic parameters of irinotecan and SN-38 were not significantly different between patients carrying the wild-type ABCG2 or at least one polymorphic allele. However, one of the two homozygous individuals showed increased accumulation of SN-38 and SN-38 glucuronide, indicating that the K141 homodimer may have an impaired function.

A recent investigation performed an exploratory, retrospective evaluation of the functional consequence of the ABCG2 Q141K variant in 20 adult patients, treated with diflomotecan, a synthetic derivative of camptothecin (350). In five patients carrying one K141 variant allele, after oral administration of diflomotecan, no difference was found in serum levels compared with individuals with the wild-type genotype. However, after intravenous administration of the drug, the plasma concentration in the heterozygous patients was markedly increased compared with the individuals with the wild-type genotype. This observation is in harmony with studies indicating a reduced protein expression and function for the Q141K variant, while it seems to contradict the results of De Jong et al. (74).

As mentioned above, the allele frequency of Q141K varies in diverse populations, and in Japan and China, this polymorphism appears to be common, with an overall frequency of \( \approx 30\% \). The resulting frequent occurrence of the wild-type/K141 heterodimer and even the K141 homodimer ABCG2 transporters in this population, as well as the appearance of other mutations, leading to a reduced ABCG2 protein level, may strongly modify the ADME-Tox properties of many different drugs. Since the recently introduced anticancer tyrosine kinase inhibitor Iressa (Gefitinib) is a transported substrate of ABCG2 (93, 406), polymorphisms in this protein may be involved in the different effectiveness and toxicity of this drug in distinct populations. Moreover, because ABCG2 was demonstrated to have a protective role against food toxins, especially plant metabolites, a lower expression or impaired function of ABCG2 variants may affect the diet-related health conditions in a huge human population (161).

2. Regulation of ABCG2

A) TRANSCRIPTIONAL REGULATION. Relatively little is known as yet about the transcriptional regulation and promoter structure of ABCG2. In their pioneering work, Bailey-Dell et al. (20) have analyzed the 5’-upstream \(-2\)-kb region of the human ABCG2 by bioinformatic tools and localized positive and negative regulatory elements by a luciferase reporter assay. They showed that, similar to other ABC protein coding genes, the ABCG2 gene lacks the canonical TATA box within the region 100 bp up-
stream to the transcriptional start site. Several Sp1 sites were found within the corresponding −222 to −49 bp region (relative to the transcriptional start site). The presence of Sp1 sites is a common feature of genes without TATA box, and ABCB1 and ABCC1, both lacking TATA boxes, possess several Sp1 binding sites. A CCAAT-box is present in the −274 bp position, and CpG islands are found in its downstream proximity. These elements predict a putative promoter/enhancer in the −266 to −36 bp region. The reporter assays identified potential positive regulatory elements between −1285 and −62 bp, and potential negative regulatory elements between −628 and −31 bp. Removal of the CCAAT box and part of the predicted region reduced and further truncation of this region further decreased transcription activity.

A novel estrogen response element has been identified in the −188 to −172 bp segment of the gene (89), and it has been demonstrated that the 17β-estradiol-ligated estrogen receptor (E2/ER) binds directly to this element and, by interacting with components of the RNA polymerase II transcriptional machinery, triggers an enhanced transcription of human ABCG2 in ER-positive T47D: A18 cells (89). The same element has been found by a systematic genome-wide bioinformatic study by analyzing promoter elements for transcription factor binding sites with the aim of identifying estrogen-responsive genes (169). However, the regulation of ABCG2 seems to be cell line specific, because it is actually downregulated following estrogen treatment in ER-positive MCF7 cells (169). Moreover, as mentioned below, estrogen regulation of ABCG2 may be mostly of posttranslational nature.

ABCG2 is probably also regulated by drug treatment and cellular stress, but this phenomenon has not yet been explored as critically as in the case of MDR1 or MRPs. Benzopyrene conjugates were reported to induce ABCG2 expression in the Caco-2 intestinal cells (87). Burger et al. (41) demonstrated that in the same cell type, the addition of Imatinib, an ABCG2-substrate TKI, upregulates the transporter expression through an SXR-dependent mechanism.

As mentioned above, ABCG2 may have a key role in protecting the cells against porphyrin metabolites during hypoxia, and this coincides with an upregulation of this transport protein (191). ABCG2 expression was found to be upregulated through an interaction of the hypoxia-inducible transcription factor complex HIF-1 with the ABCG2 promoter region. Because MDR1/Pgp is also upregulated during hypoxia by HIF-1 through the activation of a stress-activated kinase pathway (64, 65), this could be a general type of stress response also involving the ABCG2 protein.

Interestingly, the lack of Mdr1 expression in knockout mice greatly upregulates Abg2 expression in a functionally important area, the BBB (62). This excessive overproduction of a related transporter in a given tissue and its role in a defense network is discussed in detail in section VII.

B) POSTTRANSLATIONAL REGULATION. One possibility for a posttranslational regulation stems from the dimerization properties of this ABC half-transporter. In heterozygotes, dimerization of polymorphic variants in fact results in functional heterodimer formation, and thus may alter the localization and/or function of the protein (see above). Clearly, a nonfunctional mutant (K86M) ABCG2 variant induces a dominant negative effect, that is, a nonfunctional dimer formation suppresses the activity of the wild-type protein (93, 166). Such a dominant negative effect may be a rare case in vivo, but the polymorphic heterodimerization may be pharmacologically relevant.

Similarly to that found for MDR1/Pgp (see sect. iv), the transport function or localization of ABCG2 is not significantly modulated by protein glycosylation (78, 250, 370). There are no published experimental data as yet on the possible phosphorylation-dependent regulation of ABCG2. Based on earlier observations when ABCG2 was expressed in Sf9 cells (260, 261) and in Lactococcus (151), as well as on our recent experiments (Sarkadi, unpublished data), membrane lipid composition, especially the local concentration of cholesterol, has a key regulatory effect on ABCG2 transport activity. This may be most relevant in transport function, when ABCG2 is retained in the cholesterol-poor endoplasmic reticulum membrane, or transferred to the cholesterol-rich plasma membrane.

As mentioned above, sex hormones have a regulatory effect on ABCG2, that is, estrogen in general downregulates ABCG2 protein expression and function (144). This regulation involves estrogen receptor alpha and is reversed by the antiestrogen tamoxifen. Interestingly, according to this study, estrogen does not affect the level of ABCG2 mRNA, but the regulation is posttranscriptional, by decreasing ABCG2 protein biosynthesis and maturation. According to Tanaka et al. (371), based on gonadectomy and hypophysectomy experiments, the lower level ABCG2 expression in female rat kidney is probably caused by estradiol suppression, while higher ABCG2 expression in male mouse liver is induced by testosterone.

These data raise the possibility that ABCG2 is under a complex posttranslational regulation, which involves the interaction of this protein with cytoskeletal and other intracellular elements that may significantly influence the cell surface exposure, that is, the drug expulsion function of this protein.

F. ABCG2 in Medical Applications: Drug Development and Gene Therapy

1. ABCG2 in drug screening and development

Based on the above-described drug interactions, ABCG2 plays a major role in tumor drug resistance, as
well as in the absorption, metabolism, excretion, and toxicity (ADME-Tox) of various pharmacological compounds. Therefore, an in vitro prescreening of new therapeutic compounds and their metabolites may provide a great advantage; all these parameters could be predicted without tiresome and expensive in vivo screening, costing a lot of animal lives.

As described in section iii, the major MDR-ABC transporter screening methods include direct enzymatic (ATPase, nucleotide trapping, fluorescence-based enzyme activity measurements, etc.) and vesicular transport assays, by using isolated membrane vesicles exclusively or predominantly expressing the desired transport protein in a functional form. The overexpression of the ABCG2 protein in the heterologous insect cell/baculovirus system is a well-established possibility for such screenings, and isolated membranes of mammalian cells could also be used for this purpose (see Ref. 104). Another type of basic drug screening can be performed by using cultured mammalian cell lines, specifically engineered to express the given transporter. In this case both the direct transport measurements for labeled compounds and indirect assays by using fluorescent or otherwise labeled reporter compounds are available. Without detailing these assays here (see sect. iii), we feel that these methods should be rapidly optimized and standardized by the research and development community, to provide the drug development industry with a well-established toolkit for prescreening new drugs in their pipelines. Since, as demonstrated above, ABCG2 is a “transporter for all seasons” (306), performing numerous tasks in a number of organs, screens for ABCG2 should be in the first line of such development strategies. The excessive polymorphisms and the sex-dependent distribution of this transporter add an extra task to design such a meaningful in vitro screening panel, but may also lead to a major increase in the cost-to-benefit ratio of drug development.

2. ABCG2 in gene therapy

The physiological presence of ABCG2 in stem cells provides the basis for a gene therapy application of this transport protein. The introduction of a selectable marker in ex vivo stem cell gene modification provides a selective advantage of the modified cells after transplantation. The coexpression of a drug-resistance protein with a therapeutic gene product should allow both an enrichment of the corrected cells and an in vivo drug selection during clinical gene therapy. The use of the MDR1-Pgp as such a selectable marker has been widely investigated and advocated, while other studies reported major problems with this approach (39, 113, 318). Recent studies in our laboratory suggest that a mutant ABCG2 protein is an ideal candidate for human stem cell protection and for use as a selectable marker in gene therapy. The cDNA encoding this protein is relatively small (~2 kb), and the active dimer is spontaneously formed in the overexpressing cells (379). Because the substrate specificity of the R482G variant of ABCG2 differs from that of the wild-type protein, this mutant has a special advantage in gene therapy applications.

We have documented that when the mutant ABCG2 was coexpressed with a therapeutic gene, the expression of the therapeutic gene in hematopoietic progenitor cells corrected the loss-of-function mutation responsible for human chronic granulomatous disease (379). At the same time, the mutant ABCG2 protein selectively protected the transduced cells against clinically applicable cytotoxic agents. Overexpression of ABCG2 did not affect in vitro hematopoietic cell maturation or the restoration of granulocyte function by the therapeutic gene. The first in vivo mouse studies (Ujhelly et al., unpublished data) have demonstrated that the ABCG2 expressing bone marrow stem cells can be used for efficient transplantation, the production of all blood cells remains normal, and there is no change in cell repopulation or differentiation patterns.

VI. ADDITIONAL TRANSPORTERS IN CANCER DRUG/XENOBIOTIC RESISTANCE

Up to now only three human ABC transporters, MDR1/Pgp (ABCB1), MRP1 (ABCC1), and ABCG2, have unambiguously been shown to contribute to cancer multidrug resistance. However, as mentioned earlier, there are several other ABC proteins that were implicated in cancer drug resistance and xenobiotic transport. In this section we give an overview of selected ABC transporters that are major candidates for participating in drug and xenobiotic resistance.

A. The MDR3 (ABCB4) Protein

P-glycoproteins (Pgps) form a small subfamily within the superfamily of ABC transporter proteins. In mice, there are three genes that encode for Pgps, Mdr1a, Mdr1b, and Mdr2. In humans, in addition to MDR1, only one other Pgp gene has been identified: MDR3 (also known as MDR2, PGY3, ABC21, or PFIC-3). According to the classification of ABC transporters, MDR3 belongs to the ABCB subfamily and is called ABCB4. Shortly after the identification of MDR1, the human MDR3 gene product was cloned from a liver cDNA library (382). The protein encoded by the MDR3 gene consists of 1,279 amino acids and has a deduced $M_r$ of 140,000, which is increased by glycosylation. The MDR3 protein is a prototypical ABC transporter, consisting of two cytosolic nucleotide-binding domains (ABC units) and two large transmembrane domains, each composed of six transmembrane helices. It shows a high, 77% amino acid sim-
ilarity with the product of the MDR1 gene (52). The level of sequence conservation is the highest in the ABC units and in the transmembrane domains and the lowest at the NH₂ terminus and in the 60-amino acid linker region connecting the two halves of the protein.

The MDR3 gene covers 78.74 kb and maps to chromosome 7, at 7q21.1, in the proximity of the MDR1 gene (209). The two genes are separated by a 34-kb intergenic region. It has been postulated that the MDR3 gene evolved from the MDR1 sequence by gene duplication. This hypothesis is supported by the observation that the exon/intron structure of these genes is almost identical, each containing 28 exons, of which 27 encode the functional protein (209).

1. Tissue distribution and cellular localization of MDR3

The MDR1 and MDR3 gene products have different tissue distributions. In contrast to the wide-range expression of MDR1 in a number of organs (see sect. iv), the expression of MDR3 protein is constrained predominantly to the canalicular membrane of hepatocytes. Low levels of MDR3 RNA have been found in some tissues other than liver, namely, in the heart, skeletal muscle, and spleen, although the MDR3 protein was not detected by Western analysis (341). A high-affinity antibody, however, was able to demonstrate MDR3 protein expression in the glomeruli of the kidney (315). The regulation of expression of MDR3 is not clarified. It has recently been reported that the MDR3 protein, along with other major canalicular transporters, is expressed at midgestational stage during human fetal development. However, the level of MDR3 expression was found to be much lower in fetal liver than in the adult tissue (53).

The sequences and protein-protein interactions that are responsible for proper maturation and accurate targeting of MDR3 to the canalicular membrane are also yet to be discovered. Epitope insertion studies have revealed that intracellular loops between transmembrane helices 3 and 4 as well as between 4 and 5 are crucial for proper targeting (172). Mutation in the ABC unit of MDR3 also prevents correct trafficking and results in the loss of function of the protein (79). The MDR3 protein colocalizes with MDR1 and with the bile salt transporter (BSEP/ABCB11) in the apical compartment of hepatocytes. Binding of these ABC transporters to regulatory proteins such as HAX-1 and myosin II regulatory light chain (Mlc2) has also been demonstrated (47, 259). These observations suggest that HAX-1, Mlc2, and possibly cortactin are involved in the trafficking of these ABC transporters. It has been proposed that canalicular ABC transporters, like several canalicular ectoenzymes and cell adhesion molecules, transcytose from the basolateral plasma membrane to the apical surface. However, evidence has been provided for the fact that newly synthesized MDR1 and MDR2 are directly targeted from the Golgi apparatus to the canalicular membrane without entering the basolateral compartment (179).

2. Transport properties and the physiological function of MDR3

Since the MDR3 protein was found to be sequentially similar to MDR1, MDR3 was also predicted to be an efflux pump, possibly a drug transporter with broad substrate specificity. However, this Pgp, in contrast to MDR1, was proven to be a highly specialized transporter which translocates solely phospholipids with a choline head group (297, 298, 345). The restricted tissue distribution, particular cellular localization, and specialized transport properties suggest a key physiological function for MDR3 in the bile formation. The generation and phenotyping of the Mdr2 −/− mouse unambiguously demonstrated that this transporter is essential for biliary phosphatidylcholine extrusion (342), since the bile of Mdr2-deficient mice is devoid of phosphatidylcholine. MDR3 is a selective transporter regarding the fatty acid moieties of the transported lipid. Lipid analogs with C₆ and C₁₆ fatty acids as well as with a ceramide backbone are transported by MDR3, whereas a phosphatidylcholine analog with two C₆ fatty acids is not recognized (383). This substrate selectivity of MDR3 is assumed to be responsible for the particular fatty acid composition of the phosphatidylcholine pool in the bile, which differs from that in the liver tissue.

A suggested model for MDR1 transport mechanisms is that this protein acts as a flippase, i.e., it translocates amphipathic molecules from the inner leaflet of the plasma membrane to the outer leaflet (119). Based on the homology between MDR1 and MDR3 proteins, the latter one was also predicted to function as a flippase, specialized for phosphatidylcholine (PC) (see Fig. 13). This hypothesis was supported by detailed transport studies demonstrating an ATP-dependent translocation of fluorescent PC analogs from the outer to the inner leaflet of yeast secretory vesicles containing Mdr2 (298). This model system was used in a subsequent study to reveal that the flippase activity of Mdr2 was stimulated by the bile salt taurocholate but not by the nonmicelle-forming counterpart taurodehydrocholate (297). Asymmetric translocation of PC analogs was also demonstrated by Nies et al. (257) using rat canalicular membrane vesicles. This transport was also increased by taurocholate but not by taurodehydrocholate. The flippase model for MDR3 transport mechanism was also supported by studies by Smith et al. (345) using fibroblasts from transgenic mice. In these experiments mouse fibroblasts were labeled with [¹⁴C]PC, and the efflux of the labeled substrate from the cells was monitored by adding purified PC transfer protein and acceptor liposomes into the bath. Compared with
normal mouse fibroblasts, which do not express Mdr2, cells expressing the human MDR3 exhibited a threefold increase in the rate of translocation of PC. All these data support the concept that MDR3 is an ATP-dependent PC flippase, which translocates its substrate from the inner to the outer leaflet of the canalicular membrane of hepatocytes.

Smith et al. (344) generated a transgenic mouse deficient in murine Mdr2, but expressing the human MDR3 protein under the control of an albumin promoter. The expression of the transgene restored PC excretion in the transgenic animals. The lack of other defects in these mice raises doubt about the significance of low level Mdr2/MDR3 expression in extrahepatic tissues. Moreover, these studies have clearly demonstrated that PC secretion depends both on Mdr2/MDR3 expression and the availability of bile salts. The current understanding of biliary PC excretion can be summarized as follows (35, 92). The Mdr2/MDR3 protein, residing in the canalicular membrane of hepatocytes, flips PC into the outer hemileaflet. Bile salts destabilize the PC-containing micromdomains, which grow into a vesicular structure by the ongoing translocation of PC, driven by Mdr2/MDR3. Finally, the biliary vesicle pinches off the canalicular membrane. This hypothesis was supported by the observations that vesiculation of the canalicular membrane is dependent both on bile salt secretion (69) and Mdr2 expression (68). It should be noted that, in addition to PC deficiency, Mdr2 −/− mice also exhibited reduced biliary cholesterol excretion. However, the role of Mdr2 was proven to be secondary in this effect.

3. Clinical relevance of MDR3

Lack of PC in the bile of the Mdr2-deficient mice results in a mild liver disease. Since bile salt secretion is normal in these animals, the high bile salt concentration causes damage in the hepatocytes and the small bile ducts, resulting in extensive proliferation of bile duct epithelium, portal inflammation, and fibrosis (92). Because human bile salts are more hydrophobic than their murine counterparts, lack of MDR3 expression results in a more severe liver disease in humans. It was first shown by de Vree et al. (75) that mutations in the MDR3 gene are the primary cause of type 3 progressive familial intrahepatic cholestasis (PFIC). This autosomal recessive liver disease is characterized by an early onset of cholestasis that progresses to cirrhosis and liver failure before adulthood. Patients with type 3 PFIC are distinguished from the other two types of PFIC by high γ-glutamyltransferase activity and a bile that lacks phospholipids but has a normal bile acid concentration. These patients exhibit portal duct inflammation and ductular proliferation, reminiscent of the phenotype exhibited by the Mdr2 −/− mice. Several MDR3 mutations have been identified in type 3 PFIC patients (150). Most of these mutations result in truncated proteins, which do not reach their destination in the canalicular membrane. However, a recent
study reported that MDR3 protein expression was detectable in the canalicular membrane of a few patients with type 3 PFIC (175).

Two other diseases are associated with mutations in the MDR3 gene, namely, intrahepatic cholestasis of pregnancy (ICP) and low phospholipid-associated cholelithiasis. ICP is a liver disorder characterized by cholestasis during pregnancy in women with otherwise normal medical history and by the occurrence of generalized pruritus mostly in the third trimester. ICP is frequently associated with fetal distress, spontaneous immature delivery, and idiopathic intrauterine fetal death in the third trimester. Heterozygotes for either nonsense or missense mutations in the MDR3 gene are susceptible to develop intrahepatic cholestasis of pregnancy (79, 149, 223). Recently, a detailed study has demonstrated the close correlation between the genetic variations in MDR3 gene and the pathophysiology of pregnancy-associated cholestasis (269).

Mutations in the MDR3 gene lead to the absence or low level of phospholipids in the bile. The elevated cholesterol-to-phospholipids ratio promotes lithogenicity of the bile with crystallization of cholesterol. Therefore, mutations in the MDR3 gene represent a risk factor for symptomatic cholelithiasis (294). Recently, a novel phenotype has been described in the Mdr2−/− mice. By the end of the 15th week of age, half of the animals kept on chow diet develop gallstones with cholesterol crystals (200). Thus these mice can serve as an animal model for low phospholipid-associated cholelithiasis.

4. MDR3 and drug resistance

Since the MDR3 gene most likely evolved from the gene encoding for the drug-transferring MDR1, it has been postulated that MDR3 also possesses drug-pumping activity. However, initial studies using cells transfected either with the murine Mdr2 or the human MDR3 failed to confirm this hypothesis. It has been shown that Mdr2 exhibits reduced drug binding capacity compared with that of Mdr1 (42). Surprisingly, Ruetz and Gros (298) found that the PC translocation in yeast secretory vesicles containing Mdr2 was inhibited by verapamil, a known competitive inhibitor of the drug-pumping activity of MDR1.

The first study, demonstrating MDR3-dependent drug resistance, was provided by Kino et al. (178). Yeast cells, transfected either with the human MDR1 or with the human MDR3, acquired resistance against Aureobasidin A, an antifungal antibiotic. By using polarized cultures of pig kidney epithelial cells transfected with MDR3, Smith et al. (346) have shown MDR3-dependent vectorial transport of digoxin, paclitaxel, and vinblastine. The transport of digoxin was inhibited by verapamil, cyclosporin A, and PSC833, compounds that are known MDR1 inhibitors. The interaction between MDR3 and these drugs was also supported by the observation that nucleotide trapping in MDR3 is reduced by paclitaxel, vinblastine, verapamil, cyclosporin A, and PSC833 (346). However, no ATPase activity of MDR3 was detected in the same experimental setup. The most plausible explanation for these observations is that MDR3 has a relatively high affinity for drugs but shows a low transport rate, which is not sufficient for conferring drug resistance to the cells. In contrast, MDR1 possesses high transport activity, which allows effective extrusion of hydrophobic drugs from the cells. This concept is supported by the recent finding that targeting of MDR3 RNA with siRNA results in a minor decrease of paclitaxel resistance in human ovarian cell lines, whereas MDR1 siRNA strongly reduced drug resistance in these cells (86).

These findings raise the question whether the observed affinity for drugs and the poor drug pumping activity of MDR3 is only an evolutionary relict or it has a relevant physiological role. It has been suggested that MDR3 serves as a “dual-function protein,” which transports PC and also some toxins that are especially threatening to the liver (35). However, these two types of transport activities of MDR3 can be combined in a single function, if we consider PC translocation as a protective mechanism against toxic compounds. MDR3 plays a key role in bile formation, which represents a central component of detoxification processes. The protective role of MDR3 becomes evident from the phenotype of the Mdr2-deficient mice and the human diseases associated with MDR3 dysfunction. In these cases, the toxic bile acids are not sequestered in mixed micelles by phospholipids, and this causes extensive damage to hepatocytes and bile duct epithelial cells. The composition and cytotoxicity of bile salts greatly varies from species to species. The lack of phospholipids in the Mdr2−/− mice results in a mild liver disease, whereas in humans it leads to complete loss of liver function. Interestingly, no biliary lipid excretion was found in the little skate Raja erinacea, implying that these primitive animals possess no functional ortholog of MDR3 (91). The bile salt excreted by the little skate was still found to be as cytotoxic as taurocholate, and in experiments it stimulated phospholipid and cholesterol secretion in isolated mouse liver. It has been proposed that cell membranes in little skate are protected against bile salt mainly by their high sphingomyelin content, and Mdr2/MDR3-like protective mechanism evolved later in evolution.

In addition, it has been postulated that phospholipid vesicles and mixed micelles contribute to the biliary excretion of organic anions and serve as a “micellar sink” (Fig. 13). It has been reported that the biliary excretion of protoporphyrin and indocyanine green is almost abolished in the Mdr2-deficient mouse, whereas estradiol-17β-d-glucuronide and glutathione excretion were reduced to a lesser extent (31, 136). The decrease in the number of...
phospholipid vesicles in the canalicular lumen of these animals was found to be similar to the reduction in biliary excretion of protoporphyrin and indocyanine green. These observations strongly support the hypothesis that phospholipid vesicles participate in biliary excretion of certain organic anions.

Collectively, MDR3 either directly or indirectly contributes to the elimination of a number of toxic compounds from the body; therefore, this transporter should be considered as an important component of xenobiotic resistance and the proposed chemoimmunity network.

B. The ABCG5 and ABCG8 Proteins

The ABCG subfamily of ABC transporters consists of five members. All of them are half-transporters, i.e., they are composed of only one NBD and a single TMD. In addition to ABCG2, discussed in section v, two other members of the ABCG subfamily, ABCG5 and ABCG8, may contribute to the chemoimmunity network by controlling the selective absorption and excretion of important dietary components.

The ABCG5 and ABCG8 transporters were identified by Berge et al. (29), based on a disease condition: mutations in either the ABCG5 or ABCG8 genes were found to cause sitosterolemia, an autosomal recessive disorder. This serious disease is characterized by the accumulation of plant-derived toxic sterols (phytosterols) and cholesterol in a large variety of tissues. As documented below, the active phytosterol and cholesterol extrusion by a heterodimer of ABCG5 and ABCG8 proteins is the protective mechanism in our body to prevent such a disease condition.

The deduced 651- and 673-amino acid sequences for ABCG5 and ABCG8 share 28% identity, and both show a “reverse order half-transporter” structure, like other members of the ABCG subfamily (see Fig. 2). Both genes map to chromosome 2, at 2p21, and are tandemly arranged in a head-to-head orientation, separated only by 374 bp. The genomic organization of ABCG5 and ABCG8 genes are similar; they both contain 13 exons and span ~28 kb each. The murine orthologs of ABCG5 and ABCG8 exhibiting ~80% identity with the corresponding human proteins were identified by Lu et al. (221).

It has been reported that cholesterol feeding induces a coordinate increase in ABCG5 and ABCG8 expression, suggesting the possible involvement of liver X receptor (LXR) in the regulation of these genes (29). Direct evidence for LXR regulation of ABCG5 and ABCG8 genes was provided by several studies (193, 284). It has been demonstrated that cholesterol, LXR agonists as well as retinoid X receptor (RXR) agonists upregulate ABCG5 and ABCG8 expression in mice, whereas these changes were not observed in LXRα-deficient mice (284). These studies also pointed toward the possible involvement of the bile acid receptor (FXR) in the ABCG5 and ABCG8 gene regulation. This hypothesis was justified by a recent study demonstrating FXR-dependent increase in ABCG5 and ABCG8 mRNA in response to cholate feeding (411).

In contrast, diosgenin, a plant sterol that activates pregnane X receptor (PXR), failed to increase ABCG5 and ABCG8 expression levels (190, 411).

Although no obvious LXR response element was identified in the sequence (29, 284), a binding site for orphan nuclear hormone receptor homolog 1 (LHR-1) has been identified in the intergenic region of ABCG5 and ABCG8 genes (98). It has been shown that mutation in this binding site greatly reduced the promoter activity, whereas LHR-1 overexpression increased the expression of ABCG5 and ABCG8, unambiguously demonstrating that LHR-1 is a positive transcription factor for these genes.

1. Tissue distribution and cellular localization of ABCG5 and ABCG8

Initial studies demonstrated that ABCG5 and ABCG8 exhibit a contiguous and restricted tissue distribution. These transporters are expressed congruently in the liver and the intestine, since the mRNA of both ABCG5 and ABCG8 was found to be abundant in these tissues (29, 204, 220). However, the tissue- and cell-specific distribution of these proteins exhibits a more complex pattern. A detailed analysis revealed that the mRNA expression profiles are not uniform along the intestinal tract (253). Although the results of microarray and RT-PCR techniques did not correlate closely, the trend indicated low expression levels in the colon and elevated levels in the jejunum, when compared with duodenal expression. Additionally, a physiological study demonstrated that the functional presence of ABCG5 and ABCG8 can be detected in the jejunum and the ileum rather than in the duodenum (85). A low-level expression for both ABCG5 and ABCG8 has also been detected in the choroid plexus (61).

For studying the expression pattern of ABCG5 and ABCG8, in situ localization studies were performed with liver and intestinal sections from mice (284). In the liver, both proteins were uniformly distributed along the hepatic lobule, indicating expression in the hepatocytes, whereas in the intestine, ABCG5 and ABCG8 were found in the enterocytes lining the villi. To circumvent the unavailability of good antibodies for these proteins, the subcellular localization of ABCG5 and ABCG8 has been studied with epitope-tagged proteins in cultured hepatocyte models (111). The coexpressed ABCG5 and ABCG8 proteins were localized to the apical (canalicular) membrane of hepatocytes. This finding was further supported by subsequent in vivo experiments, when antibodies for these proteins became available. In these studies, Abcg5/
Abcg8-deficient (G5G8 −/−) double-knockout mice were infected with adenovirus constructs for the human ABCG5 and ABCG8 (112). The human ABCG8 and an apical marker exhibited a similar distribution pattern in plasma membrane sheets isolated from the liver of the transgenic mice.

A more straightforward and detailed biochemical and immunolocalization analysis was performed by Klett et al. (180) by using human liver, gallbladder, and intestine samples. They raised peptide antibodies against ABCG5 and ABCG8 and demonstrated that these proteins are mostly cofractionated and colocalized to the apical surface in the liver and the intestine. Nevertheless, the distribution of the two proteins was not completely contiguous: ABCG5 was found primarily in the canalicular cells, whereas ABCG8 was more readily detectable in the bile duct cells. In addition, ABCG5 was seen in the canalicular membrane, whereas ABCG8 in hepatocytes was expressed in a more diffuse pattern. Conversely, in the intestine, ABCG8 was found in the apical membrane of enterocytes, whereas the expression of ABCG5 was found to be more diffuse. An interesting addition to the list of diverse expression pattern of ABCG5 and ABCG8 was also provided by Klett et al. (181), demonstrating that both hepatic and intestinal expression of Abcg5 remained apical in the Abcg8-deficient mice.

2. Dimer formation

As discussed earlier, it is commonly accepted that ABC half-transporters must homo- or heterodimerize to yield a functioning unit. The genomic arrangement of ABCG5 and ABCG8 genes, which is typical for coordinately regulated genes that encode for subunits of functional complexes, suggests that these proteins may form heterodimers. The similar tissue distribution and cell-specific expression of these proteins is also indicative for heterodimerization. In addition, mutations in either ABCG5 or ABCG8 genes cause a disease of a clinically identical phenotype (29, 204, 220). By using epitope-tagged versions of ABCG5 or ABCG8 proteins, Graf et al. (111) have demonstrated that proper targeting of these transporters to the apical plasma membrane requires co-expression of both proteins, as single expression of either of the proteins resulted in retention in the endoplasmic reticulum. Further support for heterodimerization came from colocalization and communoprecipitation of these tagged proteins and from the observations that they undergo posttranslational modification only when co-expressed (111).

Even more compelling confirmation of heterodimerization was provided by Graf et al. (112) in a subsequent study, showing the requirement of coexpression of ABCG5 and ABCG8 for function. They introduced either the human ABCG5 and ABCG8 alone or both proteins into the liver of G5G8 −/− mice. The phenotype of markedly reduced biliary cholesterol secretion in the G5G8 −/− mice was restored only when both ABCG5 and ABCG8 proteins were expressed. In contrast to coexpression of ABCG5 and ABCG8, the coexpression of ABCG2 either with ABCG5 or ABCG8 in the G5G8 −/− mice failed to promote sterol excretion into the bile, indicating that ABCG5 and ABCG8 are obligate heterodimers.

3. Physiological function and clinical relevance of ABCG5/ABCG8

The cholesterol homeostasis in our body is determined by three processes: de novo synthesis, intestinal absorption, and biliary excretion. Under normal circumstances, our diet contains about equal amounts of animal-derived sterol, mostly cholesterol, and plant-derived sterols, that is, sitosterol, campesterol, or stigmasterol. These phytosterols are selectively eliminated from our body, and only regulated amounts of cholesterol are retained.

Mutations in either ABCG5 or ABCG8 genes are associated with sitosterolemia (29), a rare autosomal recessive disorder characterized by accumulation of both plant-derived sterols and cholesterol in the blood and different tissues. Due to sterol deposition in various tissues, patients with sitosterolemia frequently develop tendon and tuberous xanthomas, as well as premature coronary atherosclerosis. The accumulation of sterols in the blood is a consequence of both intestinal hyperabsorption and reduced biliary excretion (239). Hitherto, six mutations in ABCG5 and nine mutations in ABCG8 genes have been identified in patients with sitosterolemia (29, 204, 220, 398). Graf et al. (110) have demonstrated that most disease-causing missense mutations prevent the formation of stable ABCG5/ABCG8 heterodimers and result in impaired trafficking from the endoplasmic reticulum to the cell surface.

Six missense polymorphisms, one in the ABCG5 gene and five in the ABCG8 gene, have been identified, four of which were proven to be associated with changes in plasma sterol levels, either in sitosterol, campesterol, or cholesterol concentration (30, 137, 243). Two of these polymorphisms have been recently demonstrated to be responsible for the gender-specific response of plasma cholesterol levels after changes in dietary composition (138). A third one, which was previously shown to be linked to altered plasma campesterol and sitosterol levels (137), has been found to be associated with a more profound response to statin therapy, i.e., atorvastatin treatment, in patients with hypercholesterolemia (167).

Direct evidence for the involvement of ABCG5/ABCG8 in fractional sterol absorption was provided by Yu et al. (412) by generating and phenotyping G5G8 −/− mice. These mice exhibited a 3-fold increase in intestinal absorption of plant sterols and a 30-fold increase in the
plasma sitosterol level. Interestingly, the accumulation of plant sterols in these animals was associated with a compensatory decrease in plasma cholesterol levels (by ~50%); thus the total sterol content of the blood was not significantly altered (415). However, the knockout mice were found to be extremely sensitive to changes in dietary sterol content. When they were challenged with a 2% cholesterol diet, the plasma cholesterol increased 2.4-fold in the G5G8 −/− animals but not in controls.

According to our current understanding, the intestinal uptake of sterols is primarily controlled by the Niemann-Pick C1 Like 1 protein (NPC1L1) (11, 72), a transporter residing in the apical membrane of the jejunal enterocytes, whereas the ABCG5/ABCG8 heterodimer is responsible for selective extrusion of plant sterols and resecretion of cholesterol into the gut lumen. Exclusion of plant-derived sterols from the body seems to be crucial, since accumulation of these sterols profoundly perturbs cholesterol homeostasis not only in the blood and the liver but also in the adrenal gland (407), a tissue/organ in which sterols have a very special role.

The net luminal absorption of cholesterol in enterocytes is primarily determined by the steady state of fluxes driven by the NPC1L1 protein and the ABCG5/ABCG8 heterodimer, both transporters residing in the brush-border membranes. This scenario is supported by several experimental observations. Overexpression of ABCG5 and ABCG8 results in about a 40% reduction in fractional cholesterol absorption (413). Conversely, impaired function of ABCG5/ABCG8 leads to hypercholesterolemia and hyperabsorption of plant sterols in patients with sitosterolemia (239), as well as increased plasma levels of plant sterols in the G5G8 −/− mice (412). Despite the fact that ezetimibe, a known inhibitor of intestinal absorption of both animal- and plant-derived sterols, selectively blocks NPC1L1 (100), this drug also corrects the impaired fractional absorption and plasma levels of sterols in patients with sitosterolemia (301) and in the G5G8 −/− mice (414), demonstrating the importance of balance between uptake and resecretion processes. It should be noted, however, that the total intestinal absorption of sterols in enterocytes is influenced by several other factors, i.e., the activity of carriers from and to the brush border membrane, the esterification of cholesterol in the endoplasmic reticulum, as well as the serosal transport processes, probably involving another ABC transporter, ABCA1.

In addition to their role in the intestinal absorption, the involvement of ABCG5 and ABCG8 in biliary excretion of sterols is also supported by a growing body of evidence. In the G5G8 −/− mice, secretion of sterols into the bile was markedly reduced (by 91%) (412). Transgenic mice overexpressing human ABCG5 and ABCG8 show increased biliary cholesterol levels (5-fold) and a reduced intestinal absorption of sterols (413). Selective hepatic overexpression of human ABCG5 and ABCG8 in mice results in elevated biliary excretion of cholesterol and plant-derived sterols without affecting their intestinal absorption (402). In addition, overexpression of ABCG5 and ABCG8 in the livers of G5G8 −/− mice restores biliary cholesterol levels (112). Moreover, hepatic mRNA levels and biliary cholesterol concentrations were shown to be directly proportional in genetically modified mice containing different copy numbers of the ABCG5 and ABCG8 genes (411). All these observations imply that the ABCG5/ABCG8 heterodimer plays a crucial role in the biliary excretion of sterols.

It should be noted, however, that although Abcg8-deficient, single-knockout mice also exhibited impaired biliary cholesterol excretion, sitosterol secretion into the bile was still maintained (181). Additionally, biliary cholesterol excretion in Abcg5-deficient mice remained inducible by LXR activation (276). These data can be explained either by independent function of these proteins or by the existence of an alternative sterol excretion pathway. The latter hypothesis is consistent with the finding that at least two loci distinct from the ABCG5/ABCG8 locus in mice are associated with plant-derived sterol levels (327).

4. Substrates and transport mechanism of ABCG5/ABCG8

The key substrates of the ABCG5/ABCG8 heterodimer are sterols and possibly sterol derivatives. These transporters are clearly responsible for the vitally important exclusion of plant-derived sterols from our body; thus they must selectively recognize and remove phytosterols even in the presence of high concentrations of cholesterol. At the same time, ABCG5/ABCG8 also seem to participate in cholesterol transport at various sites, including the gut and the bile canaliculi. How important this latter function is in humans has not yet been clearly established. Interestingly, our recent, unpublished data indicate that the ABCG5/ABCG8 heterodimer may also be involved in the transport of sterol derivative sex hormones.

Two distinct transport models for the operation of ABCG5/ABCG8 heterodimer have been suggested. In analogy with the transport mechanism of MDR1 and MDR3, Wittenburg and Carey (400) proposed a flippase model for ABCG5/ABCG8. Cholesterol is equally distributed in both hemileaflets of the membrane. According to the flippase model, the ABCG5/ABCG8 heterodimer generates an uneven distribution of cholesterol by facilitating its translocation from the inner to the outer hemileaflet, from which cholesterol is extracted by acceptors. Based on energetic considerations and experimental observations, Small (340) favored another, so-called “activation-collision” model for the operation of ABCG5/ABCG8. In this model, the energy of ATP hydrolysis is used by the transporters.
to partially push cholesterol from the inner leaflet to the lumen (activation). If this occurs without flipping the molecule, the isooctyl tail of cholesterol is presented for the acceptors. Thus cholesterol can be readily transferred to small mixed micelles and phospholipid vesicles in the lumen.

In summary, ABCG5 and ABCG8 proteins form functional heterodimers in the brush-border membrane of enterocytes, and in the canalicular membrane of hepatocytes. They extrude sterols from these epithelia to the lumen (gut or bile canaliculus). These transporters are responsible for the exclusion of plant-derived sterols from the body. This physiological role seems to be vital, since the accumulation of these sterols is toxic and profoundly perturbs cholesterol homeostasis.

VII. ROLE OF ABC TRANSPORTERS IN XENOBIOTIC METABOLISM: THE CONCEPT OF A "CHEMOIMMUNITY" DEFENSE SYSTEM

In this review we suggest that the ABC multidrug transporters are essential parts of an immune-like defense system, and their network is a major contributor to "chemoimmunity" in living organisms. It has been documented in detail in the preceding sections that substrate recognition and ATP-dependent toxin extrusion by the MDR-ABC transporters are directly relevant to xenobiotic resistance. How are these resistance proteins organized in our body to become part of a coordinated defense system against toxic compounds? In the following passages we describe this concept, currently with a lot of uncertainties and without a quantitative model. Still, we feel that this may stimulate further work in establishing a quantitative systems biology approach and detailed modeling.

Classical immunology is concerned with the response of the organism to an environmental challenge by mostly water-soluble toxic compounds, microorganisms, or other living agents, e.g., cancer cells. The ultimate function of the immune system is to seek and destroy foreign agents and substances in our body. The classical immune system, however, is practically ineffective in the case of hydrophobic toxic invaders molecules, which rapidly cross cell membrane-based tissue barriers. The lipid core structure of the biological membranes makes these barriers essentially freely permeable for hydrophobic compounds, and it is only the cellular toxin transport and metabolism that can protect our body against such chemicals.

The essential basis of the classical immune defense is the discrimination of the self from the nonself, followed by an immediate response to the acute challenge of any possible invaders by the apparatus of the innate immune system. If this response is weak or not fully successful, it is followed by an adaptive, amplified response, more specific for the intruders that could not be eliminated by the first line of defense. The adaptive form of the immune system shows memory for invader recognition and a complex regulation of the interacting elimination pathways. An overreactive response by the adaptive system causes hypersensitivity or allergy to certain immune challenges. We try to demonstrate below that the proposed chemoimmunity system has many similar features to these classical pathways.

A. Chemoimmunity and Toxin Metabolism

Chemoimmunity, as appreciated here, is based on the coordinated action of specific transport systems and cellular xenobiotic metabolism in a defense against hydrophobic or amphipathic compounds. In textbooks, toxin metabolism is usually divided into two major steps. Phase I metabolism is characterized by the oxidation of the toxic compounds, essentially based on the function of cytochrome P-450 (CYP) enzymes. This phase may also include the reduction or chemical cleavage of certain toxic compounds. Phase II metabolism involves conjugation, mostly of the already oxidized chemicals, with cellular glutathione, glucuronide, or other small hydrophilic molecules. The abundance and variety of CYP superfamily enzymes in our tissues makes xenobiotic oxidation an effective means of detoxification. The large conjugation capacity of phase II enzymes, especially in the liver cells, provides the following step for the elimination of these toxins.

In this review we do not detail these cellular toxin metabolizing systems but concentrate on the role of MDR-ABC membrane transporters. These proteins are involved in two additional, essential steps of the xenobiotic defense mechanisms that may be called phase zero and phase III.

1. Toxin extrusion at the gates

Phase zero has been described as the cellular uptake step of the toxic compounds (see Refs. 356, 387). In this phase, the above-described MDR-ABC proteins pump out several hydrophobic compounds before they would reach the intracellular compartments and consequently the "milieu intérieur" of our body ("preemptive pumping"). These transporters extrude xenobiotics directly from the plasma membrane bilayer or from the vicinity of this lipid bilayer, in cells predominantly located in tissue barriers.

As discussed above, multidrug transporters preferentially interact with substrate molecules concentrating in the hydrophobic membrane environment of the lipid bilayer. The pumps remove the accumulated hydrophobic compounds from the inner membrane leaflet and either pump these compounds into the external leaflet or di-
rectly into the extracellular water-phase. We have no means as yet to distinguish these particular pathways, but this is the mechanism after which the multidrug transporters have been named “hydrophobic vacuum cleaners” (see Refs. 107, 108, 119). It is clear though that their function in phase zero is a most effective way to keep chemoinvaders out of our cells or entirely out of our body, without allowing toxin interaction with intracellular enzymes or compartments.

2. Extrusion of modified toxins

The need for a phase III of toxin metabolism, an additional key step in the xenobiotic defense, was first emphasized by Ishikawa (147). He predicted that during toxin metabolism the efficient elimination of the intracellular, already detoxified (oxidized and/or conjugated) molecules requires an active, ATP-dependent transport mechanism. This active extrusion has to help these metabolites to enter the extracellular fluids and eventually the bile or urine, and thus reduce their accumulation in the key detoxifying cell types. Indeed, with the discovery of the ABCC-type and the ABCG2 multidrug transporters, the identity of these efflux pumps was revealed, and it has become clear that these proteins perform a crucial task in eliminating conjugated toxins.

In this review we do not detail the information related to the transport function of the key conjugate export transporters, the MRPs, as this is extensively provided by the review of Deeley et al. (73). We only emphasize here that the transport features of MRPs are most adapted to this type of phase III metabolic function. Although the relative transport capacity (turnover) of the MRP pumps is much lower than that of MDR1/Pgp, their combined action allows the rapid transport of conjugated metabolites in the required direction with a flexible metabolic control; that is, MRPs are variably located in the apical or basolateral membranes of the cells, their transport is allosterically regulated by cellular metabolites through multiple binding and transport sites, and their expression is also strongly modified depending on the actual metabolic conditions within the cell. As described in the relevant section, ABCG2 may be an important additional player in phase III reactions.

As a conclusion, phase zero and phase III, and the multidrug transporters involved in these reactions, seem to be just as important in xenobiotic elimination as the generally acknowledged phase I and phase II metabolic reactions. In a composite toxicology approach, all these pathways should be included and their interactions analyzed. In addition, both MRPs and ABCG2 have a major role in the elimination of the endogenous toxic metabolic products, also using the phase III pathway (see Ref. 73).

Of course, in xenobiotic transport processes, the “passive” permeability of the respective cell membranes for xenobiotics, based on physical solubility and diffusion criteria, as well as the possible involvement of other transporters should also be considered. Many pharmaceutical agents and toxins are transported by multispecific solute carrier (SLC) transporters for organic anions (e.g., OATP-SLCO/SLC21 and OAT-SLC22A1–3) and organic cations (OCT-SLC 22A4–5). These solute carriers, often called “uptake transporters,” include a large variety of related membrane proteins, and in many cases possess overlapping substrate profiles with the MDR-ABC proteins (for recent reviews, see Refs. 196, 231, 246, 360). Some of these transporters perform obligatory exchange of organic compounds (e.g., OAT3), while in others transport is modulated and/or driven by monovalent ions and the membrane potential (e.g., OCTN). Moreover, there is a coordinated expression pattern for various SLC and ABC transporters in the liver, kidney, and BBB, which may govern the overall vectorial transport of many compounds that are mutual substrates of MDR-ABC and SLC transporters (see Ref. 196).

At the time of writing this review there is an accelerating accumulation of permeability and membrane-mediated transport data that may elucidate the complex pattern of drug absorption and distribution. Moreover, expression systems with coordinated expression of MDR-ABC and SLC transporters are becoming available (70) for such detailed studies. We are aware of several consortial efforts in this regard; major international groups are set to perform this enormous task, by using a variety of screening methods, establishing standard, comparative protocols and joint computer bases. A systems biology approach, based on the above experimental work, should soon allow a better understanding of this drug metabolism and transport network.

B. Innate Chemoimmunity

In the complex functioning of this xenobiotic defense mechanism, the analogy to the classical immune system is rather compelling. Similarly to the immune system, the transport and metabolic machineries have to react with an enormous number of compounds, previously not met by the organism. During this step, the immediate recognition of the nonself invader and an innate type of acute defense reaction are both essential.

The first major question is how this selective, still wide-range recognition of the toxic, nonself molecules by the transporters and metabolic enzymes occurs. In the case of the CYP system, the solution is basically similar to that of any metabolic pathways. There are numerous forms of these enzymes; in seven superfamilies a large number of related proteins carry out somewhat different tasks and recognize different substrate molecules (for reviews, see Refs. 60, 207, 376, 403). Still, the CYP pro-
proteins are relatively nonspecific enzymes and recognize a variety of substrate molecules. This is further emphasized by the fact that, e.g., in humans, most of the oxidative drug metabolism is carried out by only a limited number of the members of the CYP1, CYP2, and CYP3 families, and a few key enzymes (like CYP1A2, CYP2A6, CYP2C9, and CYP3A4) perform most of the metabolic tasks. Thus selectivity and overlap in drug recognition are both important features in this case.

As far as the functioning of the intracellular conjugation systems is understood, these enzymes preferentially recognize molecules already “decorated” by phase I metabolism, while they also conjugate a large variety of molecules that have available valences with little specificity.

In the case of the multidrug transporters, especially when extruding foreign chemicals (in the phase zero reaction) at the initial tissue barriers, e.g., in the intestine and the fetomaternal or blood-brain barrier, the recognition of the foreign molecules is still a puzzling process. MDR1/Pgp, MRP1, and ABCG2 are the key transporters involved, and there is only one gene in the human genome for each of these transporters. There are no known isoforms produced, e.g., by alternative splicing or other mechanisms; thus one single protein, such as MDR1/Pgp, has to recognize and eliminate thousands of different hydrophobic agents. In addition, a major task is the recognition of the “self” molecules to prevent the removal of essential elements.

As described in the sections dealing with the individual transporters, the molecular mechanisms of the discrimination between the self and nonself compounds by the MDR-ABC transporters is still far from being resolved, and we have no definite clue as yet how the “receptor” and “effector” functions, that is, the recognition and elimination of the foreign materials, respectively, are engineered into one single machinery within these proteins. In a phenomenological approach, however, we can state that the function of MDR-ABC proteins in xenobiotic metabolism is based on an extremely wide-range recognition and extrusion of any possible foreign hydrophobic compounds.

From an evolutionary viewpoint, there are indications that MDR-ABC transporters have evolved from early bacterial transporters devoted to specific solute transport, initially using the energy of the protonmotive force (see Ref. 390). In the continuous fight for survival, both attacking and protecting armors have developed, and microorganisms possessing efficient toxins and antitoxin defense systems (at least protecting themselves from these toxins) had a selective advantage. A possible way of producing a promiscuous “defense” mechanism was to decrease the selectivity of a transporter by making the substrate recognition site larger, more complex, and able to accommodate a variety of toxic compounds. This might have led to a loss of efficiency in transport, but provided survival. The forthcoming acquisition of the ATP-binding and hydrolytic domains as energy providers for this transport may have led to less dependence on the actual membrane potential and allowed the eukaryotic cells to use this invention in their plasma membrane.

Regarding the physiological role of the mammalian/human ABC-MDR transporters, we have to deal with the following two important issues.

1) Knockout animals lacking MDR1/Pgp, ABCC1, or ABCG2 (including a variety of combined MDR1/Pgp and ABCC1 knockouts) are practically healthy and have no pathological alterations in the controlled environment of an animal house. These knockout animals are sensitive to various toxins that are normally harmless to the same species, but there are no major physiological alterations even after long-term observations (see above). Another example is the occurrence of homozygous MDR1/Pgp loss of function in the collie dogs, which are healthy, but very sensitive to certain drugs, e.g., ivermectin (298).

2) There are no data in the literature describing human diseases based on a nonfunctional MDR1/Pgp protein, or disease conditions caused by any MDR1/Pgp mutation. An extensive search for null alleles of MDR1 has also remained negative. Thus the lack of MDR1/Pgp in humans may be either early lethal (very unlikely) or harmless. In the case of ABCG2, individuals with genes coding for a nonfunctional ABCG2 (a truncated form of the protein) have been found in a low percentage (141, 428), with no apparent disease condition. However, it has been suggested that drug hypersensitivity (188, 242, 251) and even alfalfa hypersensitivity (see Refs. 271, 385) in some Japanese cohorts may be due to the absence of the polymorphisms of ABCG2.

These data may suggest that, in a general sense, MDR-ABC transporters are not essential for the basic physiological processes in mammalian life. Nevertheless, it is quite clear that they are key players in the “chemo-defense” mechanisms.

C. Adaptive Chemoimmunity Response, Memory, and Hypersensitivity

Similarly to that seen in the classical immune system, there is a possibility that xenobiotic resistance based on transporters may show an adaptive phase, with increasing specificity and efficiency. In this review we have already described the regulation of the individual MDR-ABC proteins, and in this context we only emphasize a few key points, concerning the adaptive nature of this network.

Several data indicate that the expression of the MDR-ABC transporters in tumors is characteristically induced by cytotoxic agents. This finding is not directly relevant to physiology, but the same may be true in normal tissues.
exposed to xenobiotics, and there are indications that several tissues react to a toxin exposure by a rapid over-expression of the relevant multidrug transporter. In bacteria, most multidrug transport systems are under the tight control of specific regulatory elements, composed of toxin-recognition and DNA-binding domains and (usually located within the same operon) that significantly modulate the expression of the relevant transporter protein(s) (see Refs. 323, 388, 426). Toxin recognition by these regulatory proteins is just as promiscuous and/or specific as that by the regulated transporter, providing a very efficient way to increase or decrease the defense capabilities of the microorganism. In eukaryotes, exemplified, e.g., by yeast, these regulatory and multidrug transport systems are combined into a complex network, containing a number of overlapping elements.

With regard to the human MDR-ABC transporters, transcriptional and posttranscriptional regulatory mechanisms have been already detailed in the respective sections. Here we just mention again that many stress factors, including steroid hormones, heat shock, or hypoxia (for MDR1/Pgp, see Refs. 64, 65; for ABCG2, see Ref. 191), upregulate these transporters, which may thus be considered as parts of a general cellular stress response. It is assumed that cellular toxin activated receptors, including the PXR-SXR (pregnane X receptor) and the CAR (constitutive androstane receptor), play a major role in this response (see Refs. 195, 351, 403), which is an integrated phenomenon including the regulation of metabolic enzymes as well (25, 55, 195). A general and coordinated upregulation of the multidrug transporters and CYP enzymes may significantly alter several drug effects. This seems to be the case, e.g., during the application of a widely used herbal extract from Saint John’s wort (SJW), a holistic medicine to accelerate wound healing, treat nerve pain, or depression. Chronic exposure by SJW reduces the bioavailability for many drugs because of the induction of both CYP3 and MDR1/Pgp expression and activity (274).

Interestingly, under conditions resulting in major cell damage and increased endotoxin exposure (55, 168), or in various forms of inflammatory bowel disease (IBD) (267), the expression of several MDR-ABC transporters is down-regulated, which makes the affected tissues even more defenseless. The stress responsiveness of MDR1 expression is interestingly exemplified by the observation that this protein is measurably upregulated in nerve cells (normally hardly expressing MDR1) after limbic seizures (395).

In connection to the general stress responsiveness, the classical immune system also has a strong influence on the chemoinnunity transporters, as several immune mediators directly influence MDR-ABC transporter expression. As examples, transforming growth factor-β up-regulates MDR1/Pgp in the BBB (80), the cytokines interleukin (IL)-1β, IL-6, and tumor necrosis factor-α reduce MDR1/Pgp expression in HepG2 cells (202) and in Caco2 cells (26), while MDR1/Pgp expression is upregulated again by interferon-γ (26). Inflammatory cytokines, including IL-1β, inhibit MR2 expression in the liver (122), while IL-6 transcriptionally upregulates various MRPs in normal epidermal fibroblasts (84), through the activation of the STAT1, STAT3, and MAPK pathways. Lipopolysaccharide exposure regulates the expression of several hepatic MDR-ABC proteins, including MDR1/Pgp and MRP2 (57).

It has been established that hormones and mediators also have important regulatory effects on MDR-ABC transporters. Thyroid hormones upregulate the expression of MDR1/Pgp (241) in several tissues, while in the BBB MDR1 expression and related transport is reduced by endothelin through endothelin B receptors (116). Interestingly, sex hormones have major regulatory effects on ABCG2 expression, that is, testosterone increases while estradiol decreases ABCG2 expression in various animal models (144, 371). As mentioned above, pathological activation of brain tissues increases MDR1/Pgp expression, and this regulation may occur through glutamate release. This mediator was shown to upregulate MDR1/Pgp expression through an N-methyl-D-aspartate receptor mechanism (431). Increasing glutamate release during ischemic, anoxic brain injury may thus increase the level of MDR-ABC transporters and provide a defense mechanism against accumulating toxins. Interestingly, serotonin may have an opposite effect: in the small intestine serotonin depletion induced an increased MDR1/Pgp expression (121).

As a summary, the expression and function of MDR-ABC transporters are subject to complex regulation, which differently modifies their presence and efficiency in different tissues. Moreover, this regulation is in close coupling with that of the metabolic enzymes and other membrane transporters. It seems to be a general rule that MDR-ABC transporters are regulated in a positive correlation with the key metabolic enzymes of antitoxin defense, while uptake (SLC) transporters, which in most cases accelerate cellular toxin exposure, are coordinately downregulated (4).

Interestingly, the MDR-ABC transporters seem to “feel” the presence of their transporter network partners, and the reduction of one transporter modifies the expression of a related other pump protein. This seems to be the case in the Dubin-Johnson disease, or other alterations of conjugate transport by MRP2, when conjugate export into the bile is insufficient. In this case, several studies observed an upregulation of other MRP proteins, especially MRP3, transporting conjugates into the bloodstream. Although this does not help the biliary clearance of conjugated toxins, it at least protects the hepatocytes from a harmful accumulation of these metabolites. Another interesting example of this adaptive cross-regulation may be the large increase of MDR1.
protein expression in ABCG2 knockout animals, especially observed in the BBB (62).

The above-described, sometimes long-term modulation by hormones, mediators, immune-components or even by the altered expression of corresponding transporters causes an adaptive memory in the chemoimmunity transporter network. We still have only very limited information about the molecular details of this adaptation. At the cellular level, gene amplification, epigenetic, or other transcriptional modulation of gene expression, posttranscriptional, and even posttranslational modulation of various MDR-ABC transporters have already been described (see above). On the basis of environmental toxicology studies and clinical experience, it is quite compelling that long-time exposure to toxic compounds results in a new steady-state, with higher metabolic enzyme and MDR-ABC transporter expression levels all over our body. Although with little evidence as yet for humans, this may be an important mechanism for survival in a polluted environment, or for tolerating medical or nonmedical drug abuse.

At the same time, a hypersensitivity of our tissues to certain chemicals may occur under conditions when elevated levels of the MDR-ABC transporters ensure the adaptation to a toxic environment. In the continuous presence of various MDR-ABC transporter substrate toxins, the upregulation of the transporters provides a new steady-state, in which an increased toxin and toxin metabolite extrusion allows proper adaptation. However, cells may become “addicted,” and the appearance of a new toxic compound, which inhibits the function of a given ABC transporter, may disturb the balance in this environment and cause an apparent hypersensitivity to a large number of chemicals well tolerated before. Such a phenomenon has already been explored in several animal species (see Refs. 224, 339). Although with little evidence as yet in humans, this chemosensitization effect may be an increasing danger in our over-polluted world. The complex, harmful effects of MDR-ABC transporter inhibitor toxins can only be investigated in the presence of other, unrelated toxic compounds (see Refs. 17, 224, 339, 343). The clinical consequences of this phenomenon, often denoted as “collateral sensitivity,” have just begun to be appreciated. With as yet unknown mechanisms, a similar hypersensitivity was observed in several MDR1/Pgp-expressing tumor cell lines against completely unrelated chemicals, which are not MDR1/Pgp substrates but cause cell destruction in close correlation with the MDR1/Pgp expression (363).

D. Chemoimmunity Transporters: Why the MDR-ABC Proteins?

As already mentioned above, defense against internal and external toxins is very old and efficient. Over 60% of the current anticancer drugs are derived from natural sources. These chemicals have evolved to provide an evolutionary preference for their hosts, which also had to develop self-protecting mechanisms. Natural products are “privileged structures” selected by evolutionary pressure to interact with a wide array of biological targets (186). Perhaps not surprisingly, evolution produced equally effective countermeasures against these noxious chemicals. MDR-ABC transporters are present from bacteria to human; thus their structure and function survived all pressures during evolution. This requires a very efficient and adaptable mechanism of action. In fact, according to a whole-genome analysis, in Caenorhabditis elegans, the 60 ABC transporters evolved with higher than expected rates of duplication (338), suggesting that ABC transporters are particularly adaptable evolutionary modules, used for variable functions. How do we think MDR-ABC transporters could cope with all these requirements?

The trick of this efficiency could have been to couple ATP hydrolysis to substrate binding and export within one protein complex, or later during the evolution within one protein. In bacteria and lower eukaryotes, many ABC transporters are formed from independent polypeptides representing the transmembrane domains or the ABC units. In higher eukaryotes, these are combined into major units, containing at least one transmembrane and one ABC domain, but in most cases the two TM and two ABC domains are within one polypeptide chain.

In section II we provided a description of our current knowledge about the molecular mechanism of MDR-ABC transporters. Although we are far from understanding all details, some principles of this mechanism of action can be appreciated. These suggest that ATP hydrolysis is coupled to drug substrate transport in a relatively loose fashion, that is, the ATPase machinery has a low basic activity even in the absence of transported drug substrates. This may be ensured by a relatively low rate of lipid flippase activity or by interactions with endogenous substrates. In accordance with this notion, the drug binding region in the MDR-ABC transporters is a relatively large cavity with multiple binding sites of variable polarity. According to recent models, this is the way substrate recognition may be very wide and still efficient.

For the construction of efficient target/receptor interactions, nature has many different solutions. In the classical immune system, foreign agent recognition is ensured by a huge repertoire of antibody proteins, formed by millions of variants from relatively few genes through gene rearrangement. Thus there is a good chance for every single intruder “epitope” to find a recognition partner, which then can be selected and expressed in large quantities.

In the case of the chemoimmunity transporter network, the relatively few MDR-ABC proteins involved share a wide substrate recognition; thus only a few genes
are handled to require the huge number of possible toxins. One trick for this “promiscuity” or multispecificity might be the composition of the substrate binding site (see above), and also, that this drug binding site is buried in the plasma membrane (see the “hydrophobic vacuum cleaner” model). In the case of the transport of amphipathic, and even more of hydrophobic toxic agents, this results in a relatively high local concentration of the substrates at the binding site. Thus even a relatively low-affinity binding allows the recognition of the transported molecules.

In the years to come we expect to learn a lot more about the structure and molecular mechanism of action of the human MDR-ABC transporters, which will solidify or discard some of the speculations presented here. Still, we believe that the concepts described above may stimulate the analysis of these systems as interacting members of a general physiological defense network.

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