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Review

Structural, mechanistic and clinical aspects of MRP1

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Abstract

The cDNA encoding ATP-binding cassette (ABC) multidrug resistance protein MRP1 was originally cloned from a drug-selected lung cancer cell line resistant to multiple natural product chemotherapeutic agents. MRP1 is the founder of a branch of the ABC superfamily whose members (from species as diverse as plants and yeast to mammals) share several distinguishing structural features that may contribute to functional and mechanistic similarities among this subgroup of transport proteins. In addition to its role in resistance to natural product drugs, MRP1 (and related proteins) functions as a primary active transporter of structurally diverse organic anions, many of which are formed by the biotransformation of various endo- and xenobiotics by Phase II conjugating enzymes, such as the glutathione *S*-transferases. MRP1 is involved in a number of glutathione-related cellular processes. Glutathione also appears to play a key role in MRP1-mediated drug resistance. This article reviews the discovery of MRP1 and its relationships with other ABC superfamily members, and summarizes current knowledge of the structure, transport functions and relevance of this protein to in vitro and clinical multidrug resistance.

Keywords: Multidrug resistance; Organic anion transport; ATP-binding cassette transporter; Glutathione; Topology; Epitope mapping

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1. Introduction

The development of resistance to multiple drugs used in cancer chemotherapy is a serious limitation of this form of treatment. In vitro, broad resistance to multiple structurally and functionally unrelated drugs is often observed after intermittent or prolonged exposure of tumor cells to only a single agent. This 'classical' multidrug resistance is typically characterized by cross-resistance to four classes of commonly used natural product drugs, the anthracyclines, *Vinca* alkaloids, taxanes and epipodophyllotoxins. It was in such selected multidrug-resistant cell lines that in 1976, Juliano and Ling first noted a correlation between decreased drug accumulation and expression of a 170 kDa cell surface glycoprotein which they designated P-glycoprotein [1]. Since this time, the involvement of the type I P-glycoprotein isoforms in multidrug resistance has been characterized extensively, and gene transfection studies have demonstrated unequivocally that these proteins can confer a classical multidrug resistance phenotype on otherwise drug-sensitive cells [2,3].

For some time after its discovery, it was widely believed that P-glycoprotein (encoded by the *MDR1* gene) was the exclusive cause of multidrug resistance. However, increasing evidence in the mid to late 1980s suggested that this was not the case. Several cell lines were isolated that displayed a multidrug resistance phenotype in the absence of detectable P-glycoprotein expression, despite having undergone drug selection by protocols similar to those that often result in the elevated expression of this protein [4–8]. One such non-P-glycoprotein multidrug-resistant cell line was H69AR, which was derived from the parental NCI-H69 (H69) small cell lung cancer cell line by repeated, transient exposure to doxorubicin [4,5]. H69AR cells displayed moderately high levels of resistance (10–100-fold) to the *Vinca* alkaloids, epipodophyllotoxins, doxorubicin, and mitoxantrone. In addition, these cells were highly resistant to daunorubicin, epirubicin, and colchicine [5,8]. Prolonged growth of H69AR cells in the absence of drug resulted in a revertant cell line, H69PR, that had only 1.5–2.5-fold resistance to most of these drugs [9]. The multidrug resistance protein, MRP1, was discovered in 1992 during a differential hybridization screen aimed at identifying mRNA species whose levels changed in association with the gain and loss of the multidrug resistance phenotype in this panel of lung cancer cell lines [10]. MRP1 mRNA was overexpressed 100–200-fold in H69AR cells relative to H69 cells, and was downregulated 20-fold in the revertant H69PR cells relative to H69AR cells. It was also overexpressed in a doxorubicin-selected multidrug-resistant HeLa cell line that did not overexpress P-glycoprotein. Furthermore, the *MRP1* gene, localized to chromosome 16p13.1 by in situ hybridization, was amplified in both resistant cell lines [163]. Analysis of the 1531 amino acid MRP1 sequence identified the protein as a member of the ATP-binding cassette (ABC) superfamily of transporter proteins, to which P-glycoprotein also belongs. However, comparison of the MRP1 sequence with that of P-glycoprotein indicated that, although there were limited regions of similarity, the two proteins were only 15% identical [10,11].

Subsequent to its cloning, it was determined that MRP1 was probably identical to a 190 kDa protein (p190) overexpressed in a multidrug-resistant leukemia cell line that was identified on the basis of its cross-reactivity with a polyclonal antiserum raised against a peptide from a highly conserved region of human P-glycoprotein [12]. Immunoblot and immunoprecipitation analyses with MRP1-specific antibodies confirmed that MRP1 was a 190 kDa membrane protein [13,14]. Cell fractionation studies and immunocytochemistry indicated that the protein was predominantly localized to the plasma membrane in

drug-resistant cells, with detectable levels present in intracellular membrane compartments of some cell types [13–16].

Definitive proof that MRP1 is a cause of multidrug resistance came from transfection studies. HeLa cells transfected with MRP1 cDNA that overexpressed the MRP1 protein displayed a typical multidrug resistance phenotype, accompanied by reduced drug accumulation, relative to cells transfected with an 'empty' vector [17,18]. Similar findings were reported by several independent laboratories using different cell types [19–21]. It has also been shown that MRP1 antisense oligonucleotides can decrease MRP1 levels and reverse drug resistance in transfected and drug-selected cells [159].

2. The MRP branch of ABC transporter superfamily

The ABC superfamily of transporters, to which MRP1 and P-glycoprotein belong, is among the largest and most widespread protein superfamilies known [22] (reviewed elsewhere in this volume). Its members are responsible for the transport of a wide variety of substrates, including ions, phospholipids, steroids, polysaccharides, amino acids and peptides, across biological membranes. Interest in these proteins has grown enormously in recent years, as superfamily members including the cystic fibrosis transmembrane conductance regulator (CFTR) [23], the sulfonylurea receptors (SUR1 and SUR2) [24], the organic anion transporter cMOAT or MRP2 [25], ABCR [26], and ABC7 [27] have been implicated in the etiology of various inherited disorders.

ABC transporters contain two types of structural domains: hydrophobic, polytopic membrane spanning domains (MSD), and hydrophilic, cytosolic nucleotide binding domains (NBD). The typical functional ABC transporter has two MSDs and two NBDs [22], and the majority of identified eukaryotic ABC transporters are composed of tandemly arrayed pairs of subunits (MSD-NBD or NBD-MSD) expressed as one continuous or two separate polypeptides [28]. In most ABC transporters, the binding and subsequent hydrolysis of ATP by the NBDs is believed to be coupled to, and provide the energy for, substrate transport [22]. These domains are highly conserved, typically showing 30–40% identity among different superfamily members in a core region of about 200 amino acids. The NBDs of ABC superfamily members share two sequence motifs, designated 'Walker A' and 'Walker B', with many other nucleotide binding proteins [29]. Mutational analysis of a number of ABC proteins indicates that these two regions are critical for ATPase function [30]. In addition, ABC transporters possess a characteristic conserved fourteen amino acid 'active transport family' signature (or 'C') motif located between the Walker A and B motifs. In contrast to the NBDs, the MSDs of ABC transporters are highly divergent. Current evidence suggests that the majority of these MSDs possess either four or six membrane-spanning helices; however, the MSDs of different family members generally show little sequence identity to one another. This sequence divergence is consistent with the notion that the MSDs are important determinants of the different substrate specificities of various ABC transporters, and in cases where it has been examined, this appears to be true [31–33].

When the sequence of MRP1 was first analyzed in 1992, the two most closely related members of the ABC superfamily were the CFTR chloride channel (19% identity) and LtPgpA (30% identity) [10]. The latter is a *Leishmania tarentolae* protein that was thought to be a P-glycoprotein homolog [34]. Since this time, many proteins more closely related to MRP1 have been identified in a wide variety of eukaryotic organisms, ranging from plants and yeast to mammals (reviewed elsewhere in this issue). Included among these are five human MRP1-related proteins, designated MRP2, MRP3, MRP4, MRP5, and MRP6 [35–42].

Phylogenetic analyses indicate that the MRP1-like transporters belong to a novel branch of the ABC superfamily (Fig. 1). In general, the MRP1-like proteins possess two features that distinguish them from other ABC superfamily members [10,43–45]. The first feature pertains to the structure of the NH_2-proximal NBD (NBD1). Alignment of the primary sequences of MRP1, LtPgpA, and CFTR with the human P-glycoprotein encoded by the *MDR1* gene revealed that, in comparison to P-glycoprotein, these transporters all contain a 'deletion' of 13 amino acids located between the Walker A and B motifs of NBD1. The corresponding 13 amino acids are present in the COOH-proximal NBDs of these pro-

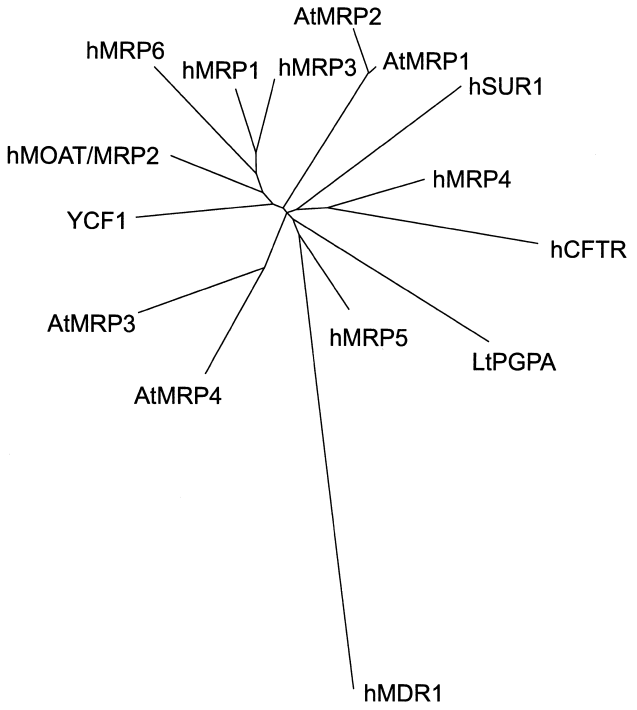

Fig. 1. Similarity dendrogram of selected MRP-related ABC transporters. The dendrogram illustrates the similarity between the amino acid sequences of selected members of the MRP branch of the ABC transporter superfamily. Human P-glycoprotein (hMDR1) was included for comparison. Multiple sequence alignment was carried out using CLUSTALX(1.64b). The protein sequences used (with GenBank accession numbers) were: human MRP (hMRP1, L05628); human MOAT/MRP2 (U63970); human MRP3 (hMRP3, AB010887); human MRP4 (hMRP4, AF071202); human MRP5 (hMRP5, AB019002); human MRP6 (hMRP6, AF076622); human CFTR (hCFTR, P13569); human SUR1 (hSUR1, L78207); *Saccharomyces cerevisiae* YCF1 (L35327); *Arabidopsis thaliana* MRP1 (AtMRP1, AF008124); *Arabidopsis thaliana* MRP4 (AtMRP4, AJ002584); *Arabidopsis thaliana* MRP2 (AtMRP2, AF020288); *Arabidopsis thaliana* MRP3 (AtMRP3, U92650); *Leishmania tarentolae* P-glycoprotein (LtPGPA, X17154); human P-glycoprotein (hMDR1, AF016535).

teins and in both NBDs of P-glycoprotein and most other eukaryotic ABC transporters [10]. This highly conserved deletion alters the spacing between the Walker A and B motifs in NBD1 and recent studies in which the 13 amino acids of NBD1 of human P-glycoprotein were inserted into NBD1 of MRP1 have demonstrated that this deletion affects the folding and activity of this domain [46]. It is present in most of the MRP1-related proteins, including the murine mrp1 ortholog and all five of the more recently identified human MRPs as well as CFTR (Fig. 2). The absence of these 13 amino acids in CFTR and in the majority of MRP1-related proteins contributes to the relatively low sequence identity ($\sim$30%) between the two NBDs within each of these proteins. There is strong evidence that the two NBDs of CFTR are functionally dissimilar [47,48]. In light of the shared structural features of the NBDs of CFTR and the MRP1-like transporters, it appears likely that the two NBDs of the latter proteins will also be functionally distinct, and recent analyses of purified recombinant MRP1 suggest that this may be true [49].

A second characteristic feature of MRP1-related proteins is that they are typically larger than other full-length ABC proteins. Hydropathy profiles of the MRP1-related proteins can be aligned remarkably well with those of other eukaryotic ABC transporters through the four structural domains characteristic of these proteins [43–45,50]. However, most of the MRP1-related proteins contain up to approximately 250 additional amino acids at their NH_2-termini for which there is no comparable region in other ABC transporters, including CFTR. The exceptions are human MRP4 (which is more related to CFTR) and human MRP5, both of which appear to lack the third MSD. The hydropathy profiles of the MRP1-related proteins with a third MSD indicate that these regions are comprised of an odd number of transmembrane helices and a predicted extracytosolic NH_2-terminus. In the case of MRP1 and MRP2, this has been experimentally verified (see below) [51,52]. Thus, the MRP1-related proteins typically have five domains with a MSD1-MSD2-NBD1-MSD3-NBD2 configuration. The amino acid sequences of the NH_2-terminal MSDs are more variable than those of the other two MSDs of these transporters. This could be because functional constraints on this region are low [53]. Alternatively, it is possible that certain members of the MRP branch of the superfamily may have arisen by a series of independent gene fusion events involving a common ancestral CFTR-like ABC transporter and a number of shorter polytopic membrane proteins [50,53].

3. Topology studies of MRP1

Newly synthesized MRP1 migrates as a polypep-

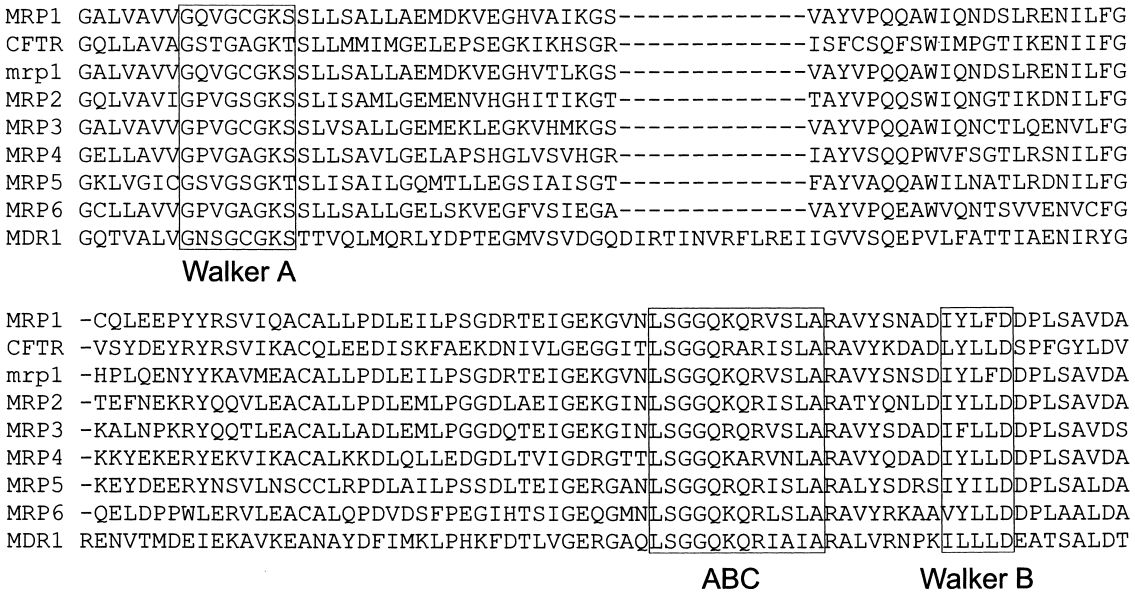

Fig. 2. Alignments of the amino acid sequences of the first nucleotide binding domain of human MRP1 with the comparable regions of other members of the MRP branch of the ABC transporter superfamily. Amino acids 671–790 of MRP1 comprising NBD1 are shown aligned with the comparable sequences in its murine ortholog mrp1 and other human MRP-related transporters. Multiple sequence alignment was performed using CLUSTALX(1.64b). The Walker A, Walker B and ABC signature motifs characteristic of all ABC proteins are boxed. The protein sequences used (with their GenBank accession numbers) were: human MRP1 (L05628); human CFTR (P13569); mouse mrp1 (AF022908); human MRP2/MOAT (MRP2, U63970); human MRP3 (AB010887); human MRP4 (AF071202); human MRP5 (AB019002); human MRP6 (AF076622); and human P-glycoprotein (MDR1, AF016535).

tide of approximately 170 kDa in SDS-polyacrylamide gels, consistent with the molecular mass of 171 kDa predicted from its cDNA sequence. This immature form of the protein is rapidly processed to the larger 190 kDa mature form by the addition of *N*-linked glycosylation [15]. When MRP1 was first identified, it was proposed that the protein had eight and four transmembrane segments in the NH_2- and COOH-proximal halves, respectively [10]. One of the most important pieces of experimental data that led to a substantial revision of the originally proposed topology was obtained by studying post-translational modification of the protein by limited proteolysis and site-directed mutagenesis [43,44,51,54]. These experiments revealed that MRP1 is glycosylated at Asn^{19} and Asn^{23} inferring an extracytosolic NH_2-terminus, and at Asn^{1006} in the COOH-proximal MSD3 [51]. One topological model of MRP1, generated using the PredictProtein algorithm, is depicted in Fig. 3A [51]. According to this model, MRP1 possesses five, six, and four transmembrane segments in MSD1, MSD2, and MSD3, respectively. This model is consistent with almost all of the available experimental data. Thus, it correctly predicts that the NH_2-terminus of the protein is extracytoplasmic and correctly localizes the glycosylated Asn^{1006} residue to the extracytoplasmic face of the membrane in the loop joining the first two transmembrane segments of MSD3. Two of the potential glycosylation acceptor sites that are not utilized (Asn^{354} and Asn^{1156}), are both predicted to be extracellular. Although this may appear to conflict with the PredictProtein model, both of these sites contain negatively charged amino acids in the +1 position of the glycosylation sequons, and are therefore not likely to be efficiently glycosylated [55]. Furthermore, Asn^{354} is predicted to be situated less than 14 amino acids from the start of a transmembrane segment, and may therefore not be accessible to the glycosyltransferase catalytic site [56]. The lack of glycosylation at these two sites is therefore uninformative with respect to MRP topology of MSD2 and MSD3. The model also correctly localizes three cytoplasmic peptide sequences identified by epitope mapping of the MRP1-specific monoclonal antibodies (mAbs) QCRL-1, MRPr1, and MRPm6 [51,57,58].

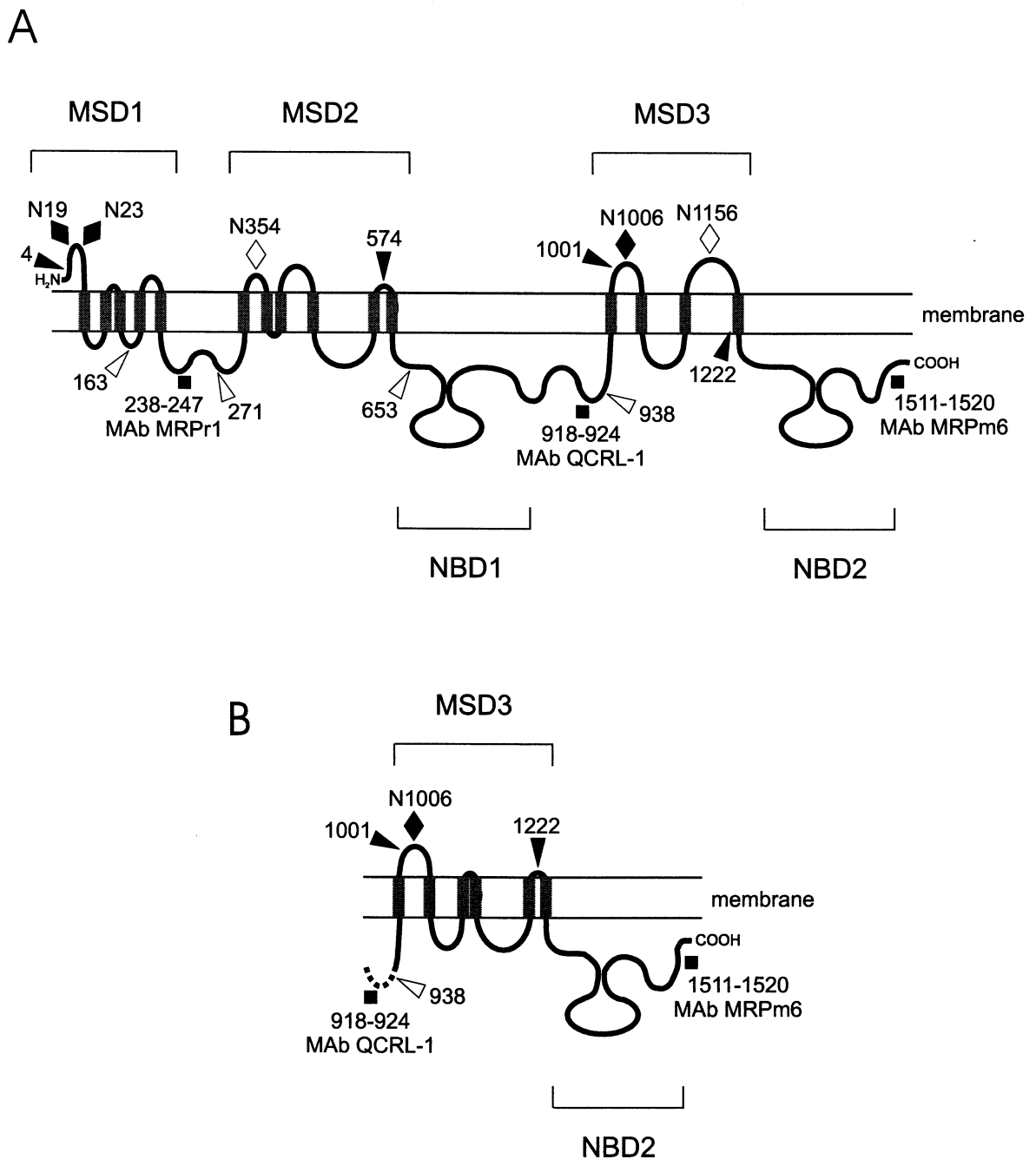

Fig. 3. Proposed membrane topologies of MRP1. Models of the membrane topology of MRP1 are shown with amino acids experimentally determined to be localized to the cytosolic or extracytosolic side of the membrane indicated. Six amino acids have been localized to the extracytoplasmic side of the membrane by *N*-glycosylation site utilization analysis (N19, N23 and N1006) (filled diamonds) or HA epitope insertion (amino acid positions 4, 574, and 1001) (filled triangles). Additional amino acids or regions have been localized to the cytosolic side of the membrane by epitope mapping of MRP1-specific mAbs MRPr1 (amino acids 238–248), QCRL-1 (amino acids 918–924) and MRPm6 (amino acids 1511–1520) (filled squares) or by HA epitope insertion (at amino acid positions 163, 271, 653, 938) (open triangles). (A) Topological model of MRP1 generated using the PredictProtein server as described in Hipfner et al. [51]. Glycosylation sites at Asn^{354} (N354) and Asn^{1156} (N1156) that are not utilized but predicted to be extracytosolic are indicated (open diamonds). (B) Topological model of MSD3 with a more conventional ABC transporter configuration of six transmembrane segments and supported by epitope insertion analysis at amino acid position 1222 [60] (see text for further details).

The topology of MRP1 has also been examined using epitope insertion methodology. In these studies, the membrane orientation of hemagglutinin (HA) epitope tags inserted at various locations throughout MRP1 was determined in permeabilized and non-permeabilized cells expressing the mutant molecules [59,60]. With the exception of a molecule containing an HA tag at amino acid position 1222, the immunolocalization of the various HA-tagged MRP1 molecules tested is consistent with the PredictProtein-generated model shown in Fig. 3A. The extracytoplasmic localization of the HA tag inserted at amino acid 1222 indicates that MSD3 of MRP1 exists in a conformation with six transmembrane segments (Fig. 3B) rather than four (Fig. 3A), an arrangement that is more typical of MSDs of ABC transporters [22]. Interestingly, two HA tags at position 1222 were required for detectable immunoreactivity and even with two tags at this site, the reactivity was weak relative to other HA-tagged molecules. One interpretation of these findings is that the poor immunoreactivity is attributable to the proximity of the HA tags to the extracellular face of the plasma membrane. However, it is also possible that this region of MRP1 exists in two or more conformational states, such that the HA tags are accessible from the extracellular surface in only a fraction of the MRP1 molecules present. The topology of the comparable region of P-glycoprotein (MSD2) is still a matter of some debate. Although it is widely accepted that MSD2 of P-glycoprotein possesses six transmembrane segments, there is strong experimental evidence to suggest that it may also exist in mammalian cells in a conformation with only four transmembrane segments [46,51,61]. By analogy, MSD3 of MRP1 may also exist in two conformations similar to those shown in Fig. 3A,B, and could conceivably shift between the two states as part of its catalytic cycle [46].

4. Functions of MRP1

4.1. Transport of organic molecules by MRP1

In an interesting example of the convergence of two seemingly unrelated fields of research, MRP1 has been shown to transport a spectrum of substrates similar to that attributed to a transporter(s) known as the multispecific organic anion transporter (MOAT) [62]. Before its molecular identity was known, ATP-dependent MOAT-like activity had been identified in a number of different cell types, including hepatocytes, erythrocytes, mast cells, eosinophils and cardiac cells [63]. MOAT (sometimes referred to as GS-X pump) activity was considered responsible for so-called Phase III elimination of conjugated organic anions produced by Phase I and Phase II metabolism of many endo- and xenobiotics [64]. Most of these organic anion substrates are glutathione (GSH), glucuronide, or sulfate conjugated molecules.

One well characterized MOAT substrate is the cysteinyl leukotriene LTC_4 [65,66], which is derived from arachidonic acid in a series of reactions that culminate in conjugation to GSH. LTC_4 is an important chemical mediator of inflammatory responses in receptor-mediated signal transduction pathways that control vascular permeability and smooth muscle contraction. During the purification of protein(s) with MOAT-like activity from a LTC_4-secreting mouse mastocytoma cell line, a 190 kDa integral membrane glycoprotein which could be specifically photoaffinity labelled by LTC_4 was identified [67,68]. It was subsequently shown that this protein was MRP1 [69]. Vesicle transport studies with membranes from MRP1-transfected cells confirmed that MRP1 was a primary active transporter of LTC_4 with kinetic characteristics similar to those of the MOAT-like activity in membranes from the mastocytoma cell line [69,70]. Further support for the direct transport of LTC_4 by MRP1 was provided by studies with MRP1-specific mAbs. These mAbs, QCRL-2, QCRL-3 and QCRL-4, recognize non-overlapping conformation-dependent cytoplasmic epitopes in MRP1 and are all equally capable of inhibiting MRP1-mediated LTC_4 transport [13,46,71]. The epitopes recognized by mAbs QCRL-2 and QCRL-3 reside in NBD1 between amino acids 617–658 and 617–932, respectively. In contrast, the QCRL-4 epitope has been localized to NBD2 (amino acids 1294–1531) of MRP1 [46]. The precise mechanism by which these mAbs inhibit transport is unclear but none of them inhibit azido-ATP labelling of MRP1. However, mAb QCRL-3 has been shown to inhibit photolabelling of MRP1 by LTC_4, suggesting

that this mAb may interfere with substrate binding [71].

Since the initial discovery that MRP1 transports LTC_4, there have been reports of ATP- dependent uptake of numerous endogenous and exogenous conjugated organic anions into inside-out membrane vesicles isolated from many different cell types overexpressing this (or other MRP1-related) protein (reviewed elsewhere in this volume) [63,72]. Recent studies suggest that although MRP1, MRP2 and MRP3 have many substrates in common, the three transporters may differ in their relative affinities for individual compounds [38,52,63,73] (reviewed elsewhere in this volume). LTC_4 remains the highest affinity substrate known for MRP1, with a K_m of approximately 100 nM. In addition to LTC_4, many of the identified endogenous MRP1 substrates, such as bilirubin glucuronides [74], glucuronide and sulfate conjugated bile salts [75], and glutathione disulfide (GSSG) [76,77], are well characterized MOAT substrates. MRP1 also transports the 17β-estradiol 17-(β-D-glucuronide) [75,78] and the GSH conjugates of prostaglandin A_2 [79], and appears to play a role in the translocation of certain lipid analogs [80–82].

Among the metabolites of exogenous molecules transported by MRP1, GSH-conjugated aflatoxin B_1 is the highest affinity substrate identified to date [83]. Aflatoxin B_1 is a mycotoxin produced by certain *Aspergillus* species that is bioactivated by a cytochrome P450-mediated Phase I reaction to a mutagenic and carcinogenic intermediate, aflatoxin B_1-8,9-epoxide [84]. The *endo*- and *exo*-epoxides formed are detoxified by glutathione *S*-transferase (GST)-catalyzed conjugation to GSH and, once formed, both conjugates are actively extruded from the cell [64]. Using a mixture of both stereoisomers, measurement of aflatoxin B_1-SG uptake into inside-out membrane vesicles from MRP1-transfected HeLa cells yielded an apparent K_m for transport of approximately 200 nM [83]. As observed in studies of MRP1-mediated LTC_4 and 17β-estradiol 17-(β-D-glucuronide) transport, aflatoxin B_1-SG transport could be inhibited by the conformation-dependent MRP1-specific mAb QCRL-3, confirming that aflatoxin B_1 is a high affinity MRP1 substrate [71,78,83]. Identification of aflatoxin B_1-SG as a substrate was the first direct indication that MRP1 may have a role in the elimination of naturally occurring toxins in addition to its role in clinical chemotherapy resistance. Interestingly, MRP1 transported the individual *endo*- and *exo*-isomers of aflatoxin B_1-SG with similar efficiency [83]. This indicates that MRP1 may not have rigid stereospecificity requirements of its substrates. In contrast, other studies suggest that the site of conjugation within the parent molecule is probably quite critical for substrate/inhibitor recognition by MRP1 [78].

Other exogenous molecules that are transported by MRP1 include the GSH or glucuronide conjugates of several drugs that are used in cancer chemotherapy. For example, MRP1 transports synthetic GSH conjugates of the alkylating agents melphalan [75] and chlorambucil [85], as well as VP-16-glucuronide [75] and doxorubicin-SG [86]. While MRP1 is known to confer resistance to VP-16 and doxorubicin, it does not confer resistance to alkylating agents [85,87,88], and the relevance of in vitro MRP1-mediated transport of such chemically synthesized conjugates of chemotherapeutic agents to resistance in vivo is not clear.

4.2. MRP1-mediated drug resistance phenotype

Since its discovery in the highly resistant H69AR lung cancer cell line, MRP1 has been found to be overexpressed in multidrug-resistant cell lines derived from many different tissue and tumor types, including both small cell and large cell lung cancers, carcinomas of the colon, breast, bladder, prostate, thyroid, and cervix, glioma, neuroblastoma, fibrosarcoma, and various forms of leukemia [89–92]. While most of these cell lines were selected for resistance in doxorubicin, MRP1-mediated resistance is clearly not restricted to cells selected in this agent, as other selecting drugs such as epirubicin [93], VP-16 [94], VM-26 [95], and vincristine [96] have also given rise to overexpression of MRP1.

In general, the resistance profiles of the various drug-selected, MRP1-overexpressing cell lines are similar, and typically include moderate to high level resistance to various natural product drugs. However, the relative levels of resistance to these drugs varies from cell line to cell line. While the resistance phenotype conferred by MRP1 expression may be influenced by the type of cell in which it is expressed, much of this variability is undoubtedly the result of

the complexity of cellular responses to drug selection. Interestingly, overexpression of MRP1 and class I P-glycoprotein are not mutually exclusive [94,97–100]. It is not clear what factors determine which mechanism(s) of resistance will predominate after selection. However, analysis of several sets of related cell lines selected for sequentially higher levels of drug resistance suggest that MRP1 (and possibly topoisomerase II) may be involved at lower drug concentrations, whereas P-glycoprotein overexpression often emerges at higher levels of resistance [94,97,100,101]. On the other hand, resistant variants of the same parental cell line exclusively overexpressing either MRP1 (e.g. H69AR [5,10]) or P-glycoprotein (e.g. H69/LX4 [102]), have been isolated using similar selection protocols, indicating that this model may be overly simple.

To circumvent many of the problems associated with investigating the multifactorial forms of drug resistance that often emerge in drug-selected cell lines, the features of MRP1-mediated drug resistance have been examined extensively in cell lines transfected with MRP1 expression vectors. The resistance profiles of MRP1- and P-glycoprotein-transfected cells are similar, and typically include moderate levels of resistance to the anthracyclines, *Vinca* alkaloids, and VP-16 [18,20,21,103]. Neither protein confers resistance to platinum-based drugs or to heavy metals such as Cd^{2+}. On the other hand, overexpression of MRP1 is associated with low level resistance to arsenical and antimonial oxyanions [18,104]. The murine ortholog of MRP1 also confers multidrug resistance and resistance to arsenical and antimonial oxyanions when expressed in human cells [105]. However, there is a remarkable difference between the resistance phenotypes conferred by expression of human MRP1 and mouse mrp1 in the same cell type. The murine protein does not confer resistance to the anthracyclines, nor does it transport the organic anion conjugate 17β-estradiol 17-(β-D-glucuronide) with comparable efficiency [105]. Recent studies with hybrid murine/human proteins indicate that the structural causes of these functional differences reside in the COOH-proximal third of the proteins [106].

As previously observed with P-glycoprotein, MRP1-mediated resistance is usually associated with a decreased steady-state cellular accumulation of drugs. MRP1-transfected cells accumulate less vincristine and daunorubicin than control transfectants [18,20]. MRP1 overexpression in certain cell lines is also associated with altered intracellular distribution of the intrinsically fluorescent drugs doxorubicin or daunorubicin, often with the accumulation of drug in cytoplasmic vesicles or a perinuclear, Golgi-like region [96,107–110]. Interestingly, functional MRP1 has been detected in intracellular vesicles and the Golgi apparatus of drug-selected GLC4/ADR lung cancer cells. It has been suggested that, in addition to efflux of drugs across the plasma membrane, intracellular sequestration and vesicular export of drug may be an important component of MRP1-mediated resistance in some cell lines [10,110]. However, no significant differences in the intracellular distribution of daunorubicin could be demonstrated in MRP1-transfected HeLa cells [54]. Furthermore, significant levels of intracellular P-glycoprotein and altered subcellular drug distribution have also been observed in several different P-glycoprotein overexpressing cell lines [108,111–114]. Thus, this phenomenon may be cell type-specific, or may result from secondary adaptive changes that occur in both MRP1 and P-glycoprotein-expressing cells in response to selective pressure from drug exposure.

4.3. Role of GSH in MRP1-mediated drug resistance and oxidative stress

In vitro transport studies using MRP1-enriched membrane vesicles under conditions similar to those used to study drug transport by P-glycoprotein have failed to demonstrate that MRP1 directly transports natural product chemotherapeutic agents [71,75, 77,115], and reports to the contrary have been withdrawn [116,117]. Early clues to the reason why transport of these drugs was not detectable came from studies using buthionine sulfoximine (BSO). BSO is a potent irreversible inhibitor of γ-glutamylcysteine synthase, the enzyme that catalyzes the first, rate-limiting step in de novo GSH biosynthesis. Treatment of cells with this agent results in reduction of intracellular GSH levels by as much as 90% within 24 h, depending on the cell line examined. Several studies showed that BSO treatment could enhance drug accumulation and toxicity in MRP1-overexpressing cell lines, although the extent of chemosensitization varies to some degree with respect to the

individual cell line and drug being tested [54,118–122]. In some cases (but not in others), BSO had similar effects on drug-sensitive parental or P-glycoprotein-overexpressing cell lines [54,118–120,123,124]. Several lines of evidence suggest that modulation of MRP1 activity by BSO is the direct result of GSH depletion. First, acute exposure of cells to BSO had no effect on drug accumulation, suggesting that BSO itself does not inhibit MRP1-mediated transport [119]. In addition, treatment with the membrane-permeable GSH ethyl ester increased cytoplasmic GSH levels and concomitantly decreased daunorubicin accumulation in two BSO-treated, MRP1-overexpressing cell lines [120]. Although the effects of BSO do not appear to be entirely MRP1-specific, these observations taken together provided strong evidence that the involvement of GSH is an important feature of MRP1-mediated resistance [125].

Given that MRP1 apparently does not directly transport unmodified natural product chemotherapeutic drugs but can transport anionic Phase II biotransformation products conjugated to GSH or other small molecules in vitro, it was speculated that MRP1 confers resistance by eliminating drug conjugates from cells [125]. In support of this idea, transfection studies demonstrated that GSTs and MRP1 may act synergistically to confer resistance to certain drugs. For example, co-expression of a π class GST and MRP1 in MCF7-derived breast cancer cells resulted in moderately high levels of resistance to 4-nitroquinoline 1-oxide as well as cellular efflux of a GSH-conjugated metabolite of this drug, whereas expression of either protein alone did not [126]. Similarly, both MRP1 and GSTα overexpression were required for resistance to the alkylating agent chlorambucil in the same system [88]. Although it seems unlikely that increased elimination of drug metabolites (which are typically less toxic) by MRP1 would have major chemoprotective effects, efficient elimination of these biotransformation products may prevent end-product inhibition of the Phase II enzymes required for xenobiotic conjugation.

There are, however, a number of problems with this model of MRP1-mediated drug resistance. For example, although the GST/MRP1 co-expression studies cited above suggest that increased formation and elimination of drug conjugates should result in resistance to 4-nitroquinoline 1-oxide and chlorambucil, neither of these drugs is known to be part of the MRP1 resistance phenotype. Furthermore, cells co-expressing MRP1 with GSTs of the α, μ or π classes showed no increased resistance to the natural product oncolytics vincristine, doxorubicin, and VP-16, relative to cells expressing MRP1 alone [88]. These observations are not surprising in light of the fact that conjugation to GSH is not known to be a major pathway for metabolism of most of the drugs to which MRP1 confers resistance. In addition, there is no convincing evidence that drug-resistant tumor cell lines carry out Phase II conjugation reactions to any significant extent [123,127]. These reactions occur primarily in the liver, and it is highly improbable that cells of all of the tissue origins in which MRP1 overexpression has been shown to confer drug resistance would be capable of forming Phase II conjugates at levels sufficient for resistance. Consistent with the view that MRP1-mediated resistance to natural product drugs is not dependent upon Phase II biotransformation, only unconjugated forms of vincristine and daunorubicin were found in the culture medium of MRP1-transfected drug-resistant cells exposed to these drugs [123].

4.4. Transport of unmodified drugs and GSH

The inability of MRP1 to directly transport natural product chemotherapeutic agents is in agreement with the observation that these xenobiotics are poor inhibitors of MRP1-mediated transport of other substrates. Even at extremely high drug concentrations (100 μM), little inhibition of LTC_4 transport was observed with doxorubicin, daunorubicin, vincristine, vinblastine, VP-16, or paclitaxel [70,71]. However, the inhibitory potency of some of these drugs was markedly increased in the presence of reduced GSH with the most dramatic enhancement observed with the *Vinca* alkaloids, vincristine and vinblastine [71]. It was subsequently demonstrated that uptake of unmodified vincristine into plasma membrane vesicles from MRP1-transfected HeLa cells was both ATP- and GSH-dependent and could be inhibited by the MRP1-specific mAb QCRL-3 [71,115]. Vincristine uptake in the absence of reduced GSH was extremely low and showed no ATP dependence, whereas active vincristine transport increased with

increasing concentrations of GSH in a physiologically relevant concentration range (up to 5 mM) [71,115]. These results have since been confirmed in other cell types [85,128]. Similarly, GSH stimulated the active transport of unmodified aflatoxin B_1 by MRP1 [83]. This transport was not the result of uptake of a spontaneously formed GSH conjugate of aflatoxin B_1 because evidence of aflatoxin B_1 metabolites other than the unmodified parent compound could not be found [83]. ATP- and GSH-dependent uptake of unmodified daunorubicin into membrane vesicles from MRP1-overexpressing cells has also been demonstrated and again, is inhibitable by mAb QCRL-3 [128].

The finding that MRP1-mediated vincristine, aflatoxin B_1, and daunorubicin transport could be stimulated by GSH suggests that either MRP1 co-transports these compounds with GSH or that GSH in some way activates MRP1, facilitating substrate binding and/or transport. Exposure of wild-type (*mrp1*$^{+/+}$) embryonic stem cells or resistant MRP1-overexpressing tumor cells to sodium arsenite or VP-16 stimulated GSH efflux to a greater extent than in *mrp1*$^{-/-}$ stem cells or drug-sensitive tumor cells [123,129]. However, GSH itself appears not to be a substrate of MRP1 [71,75,130]. Strong support for a co-transport mechanism was provided by studies examining the transport of both vincristine and GSH in vitro [115]. Thus, ATP-dependent uptake of [^{3}H]GSH into membrane vesicles from MRP1-transfected cells was stimulated by vincristine in a dose-dependent manner. Furthermore, as for other MRP1 substrates, this VCR-stimulated active transport of GSH could be inhibited by mAb QCRL-3 (and other conformation-dependent MRP1-specific mAbs) [115].

The mechanism of MRP1-mediated resistance to other chemotherapeutic agents remains unclear. For example, although vinblastine and VP-16 are both able to enhance GSH transport [115], GSH-stimulated transport of these drugs has not yet been demonstrated. Conversely, daunorubicin is transported by MRP1 in a GSH-dependent manner [128], yet neither daunorubicin nor doxorubicin stimulated GSH transport in MRP1-enriched membrane vesicles or GSH efflux from intact cells [115,120,123]. It is possible that these apparent discrepancies are the result of technical limitations of the transport assays. For example, the levels of GSH-stimulated unmodified vincristine transport are modest compared with many conjugated substrates, so the levels of GSH-dependent transport of some drugs may be below reliable detection limits. In order to measure GSH-stimulated daunorubicin uptake into membrane vesicles, for instance, it was first necessary to inhibit non-MRP1-mediated, ATP-dependent uptake with the vacuolar H^+-ATPase inhibitor bafilomycin A_1 to prevent proton gradient formation [128]. It also remains possible that the discrepancies may reflect real mechanistic differences in the way in which MRP1 confers resistance to different drugs. Resolution of this issue requires further investigation.

Because of its involvement in GSH-related cellular processes, MRP1 is a potentially important determinant of a cell's ability to tolerate oxidative stress. Expression of MRP1 protects cells directly against certain agents capable of generating reactive oxygen species (e.g. anthracyclines) by reducing cellular accumulation of the parent compound. MRP1 is also capable of transporting GSH conjugates formed in the defense against oxidative stress. In addition to Phase II biotransformation products, MRP1 can transport GSSG, a product of antioxidant action [76,77]. Under conditions of oxidative stress, when the rate of GSH oxidation exceeds the rate of GSSG reduction by GSH reductase, this activity of MRP1 may be important to prevent excess GSSG from reacting with protein thiol groups. Furthermore, it has been pointed out that two of the most harmful effects of reactive oxygen species, lipid peroxidation and oxidative DNA damage, result in the production of 4-hydroxyalkenals and base propenols. These compounds are established substrates for mammalian GSTs [131], and their Phase II metabolites are transported by mammalian GSH conjugate transporters [132], suggesting that they are likely substrates for MRP1 [70,133]. Thus, MRP1 is well suited for helping cells cope with the causes and consequences of oxidative stress.

Nevertheless, evidence for the general involvement of MRP1 in an oxidative stress response has been equivocal. Some studies have suggested that the expression of *MRP1* may be co-ordinately upregulated in certain cell lines with that of the γ-glutamylcysteine synthetase heavy chain gene in response to chemicals known to cause oxidative stress [134,135]. For example, MRP1 protein levels, intracellular

GSH levels, and LTC_4 transport activity were all increased by treatment with 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea in one human glioma cell line [135]. However, the effects were rather small and a similar upregulation of MRP1 and γ-glutamylcysteine synthetase expression was not observed in several additional cell lines in response to various oxidative stresses (E.U. Kurz, K. Sparks, R.G. Deeley, S.P.C. Cole, unpublished observation), indicating that this may represent a cell type-specific effect.

4.5. Structure-function analyses of MRP1

In light of the structural and mechanistic differences between the MRP-related ABC transporters and other members of this superfamily, there has been considerable interest in structure-function analyses of MRP1. One approach that has proven to be quite informative has been to express intact MRP1 and its constituent domains in a baculovirus system. Although underglycosylated, full-length MRP1 is expressed at high levels in insect cells and has kinetic parameters for LTC_4 transport and competitive inhibition profiles that are similar to the protein expressed in mammalian cells [136,137]. MRP1 'half-molecules' composed of amino acids 1–932 or 932–1531 (i.e. separated in the poorly conserved linker region) had no transport activity when expressed independently. However, when co-expressed, the two halves formed a fully functional LTC_4 transporter, demonstrating that they do not need to be covalently attached to associate properly [136]. MRP1 half-molecules that are lacking most of the linker region (amino acids 1–858 and 932–1531) can also associate to form a functional transporter, indicating that this region is dispensable for LTC_4 transport activity [138].

An area of particular focus has been elucidation of the role of the unique NH_2-terminal MSD in the function of MRP1 since initial studies suggested that this region was essential for transport [138]. Thus, truncated MRP1 molecules containing amino acids 229–1531 or 281–1531 expressed in insect cells were inactive but LTC_4 transport activity could be reconstituted when either polypeptide was co-expressed with a fragment containing amino acids 1–281. On the other hand, co-expression of amino acids 1–227 and 281–1531 did not result in detectable transport activity, suggesting that at least a portion of the cytoplasmic region joining MSD1 and MSD2 was critical for activity [138]. In a separate study, it was reported that an NH_2-terminally truncated MRP1 molecule containing amino acids 204–1531 (which is lacking just the five transmembrane segments of MSD1) transported LTC_4 only slightly less efficiently than the intact protein when expressed in both mammalian and insect cells [137]. Taken together, these results suggest that determinants located between amino acids 204 and 229 in the third intracellular loop connecting MSD1 to MSD2, rather than the entire MSD1 region itself, are essential for transport. Unlike most of MSD1, this region contains a peptide segment that is relatively highly conserved among MRP1-like proteins [45,50,137] and may therefore be important for the transport activity of all proteins in this branch of the ABC superfamily. Interestingly, MRP1 lacking its extracytosolic NH_2-terminus and first transmembrane α-helix (amino acids 1–66) was inactive for reasons which are presently unclear [138]. On the other hand, MRP1 lacking its NH_2-terminal 203 amino acids properly localized to the basolateral membranes of polarized MDCKII cells, suggesting that MSD1 is also not essential for trafficking [137].

4.6. ATPase activity of MRP1

As demonstrated for P-glycoprotein and many other ABC superfamily transporters, it was presumed that ATP hydrolysis in the NBDs provides the energy required for transport by MRP1, because the protein can be labelled with azido-ATP, and MRP1-mediated transport is not supported by non-hydrolyzable ATP analogs [68,70,71,139]. Low level stimulation of ATPase activity by the GSH conjugated substrate dinitrophenyl-SG was reported in membrane vesicles isolated from MRP1-overexpressing lung cancer cells suggesting that MRP1 possesses substrate stimulatable ATPase activity [140]. MRP1 ATPase activity has been more extensively characterized with purified recombinant and native protein. Histidine-tagged recombinant MRP1 purified from transfected cells had a relatively high basal ATPase activity comparable to that of P-glycoprotein when assayed in buffer containing lipids in the absence of

detergents. This activity could be stimulated up to two-fold by the addition of known MRP1 substrates such as LTC_4 and GSSG. Somewhat surprisingly, ATPase activity was also stimulated by several anticancer drugs, including *Vinca* alkaloids and anthracyclines, that have not been shown to be directly transported by MRP1 in the absence of GSH [21]. The K_m of MRP1 for ATP measured in this study (3 mM), although similar to that of purified P-glycoprotein, is more than an order of magnitude higher than values determined in vesicle transport assays [69,71,77,78], an observation that remains unexplained. Native MRP1 isolated from multidrug-resistant H69AR cells using a mAb QCRL-1 affinity resin and then reconstituted into phospholipid vesicles also showed basal ATPase activity but much lower than that of the histidine-tagged MRP1. However, MRP1 purified from H69AR cells exhibited properties more consistent with those determined in membrane transport assays. Thus, the reconstituted MRP1 ATPase activity was not stimulated by anticancer drugs but was stimulated by the organic anion substrates LTC_4, 17β-estradiol 17-(β-D-glucuronide), and GSSG, over a concentration range consistent with their known K_m values. Furthermore, reconstituted MRP1 had a K_m value for ATP (100 μM) comparable to that measured in transport assays [166]. The reasons for discrepancies between the properties of soluble recombinant histidine-tagged MRP1 and reconstituted native MRP1 are not known, but may well be related to the influence of the lipid environment on activity. Consistent with the notion of substrate-stimulatable ATP hydrolysis, a number of MRP1 substrates have been shown to increase vanadate-induced trapping of a photoactivatable ATP analog by the protein [141].

5. MRP1 and clinical multidrug resistance

There is considerable interest in determining the potential involvement of MRP1 in clinical multidrug resistance and a number of different MRP1-specific mAbs, including mAbs QCRL-1, QCRL-3, MRPr1, and MRPm6, have been used in a wide variety of immunoassays for the analysis of MRP1 expression and localization in both normal and malignant tissues [13,14]. The use of these reagents has been facilitated by epitope mapping studies demonstrating that all of these mAbs bind to distinct regions of the protein [46,57,58]. The linear epitopes bound by mAbs QCRL-1, MRPr1, and MRPm6 have been mapped to a resolution of 10 amino acids or less, and have been shown to reside in the protease-sensitive cytoplasmic loops connecting MSD1 to MSD2 (MRPr1) and NBD1 to MSD3 (QCRL-1), or near the COOH-terminus of the protein (MRPm6) [57,58]. Alignment of the linear epitope sequences of mAbs

Table 1
Epitope sequences of MRP1-specific monoclonal antibodies

Protein	MAb MRPr1	MAb QCRL-1	MAb MRPm6
HumMRP1	238 G S D L W S L N K E 247	918 S S Y S G D I 924	1511 P S D L L Q Q R - G L 1520
HumCFTR	L S D I Y Q I P S V	V N Q G Q N I	I Q K L L N E R - S L
MusMRP1	S S D L W S L N K E	S S H S G D T	P S E L L Q Q R - G I
HumMRP2	L E D V W E V D E E	S R S N G R H	P E E L L Q I P - G P
HumMRP3	E K D L W S L K E E	S S D G E G Q	P A N L I A A R - G I
HumMRP4	E D D M Y S V L P E	S V W S Q Q S	P Y V L L Q N K E S L
HumMRP5	M E D V W S L S K H	Q K K S Q D K	P S V L L S N D S S R
HumMRP6	P K D L W S L G R E	S V P E K D R	P A Q L L A Q K - G L
MDR1	K K P T V S V F S M	S N D S R S S	H Q Q L L A Q K - G I

QCRL-1, MRPr1 and MRPm6 with the comparable regions in other MRP1-like proteins suggests that these mAbs are very unlikely to cross-react with the other human MRP1-related proteins (Table 1).

The expression of MRP1 protein and/or mRNA has been detected in almost every tumor type examined, including both solid tumors (lung, gastrointestinal and urothelial carcinomas, neuroblastoma, glioma, retinoblastoma, melanoma, cancers of the breast, endometrium, ovary, prostate, and thyroid) [89,142–154] and hematological malignancies [89,142,155–158]. Among the common tumor types, expression of high levels of MRP1 is particularly frequent in the major histologic forms of non-small cell lung cancer. These studies suggest that MRP1 may be involved in resistance of some tumor types or subgroups of patients, although no comprehensive picture of the general relevance of this protein to clinical resistance has yet emerged. The difficulty of clearly establishing a causative role for MRP1 (and other resistance-associated proteins, including P-glycoprotein) in clinical multidrug resistance can be attributed to a number of different factors. For example, the broad spectrum of drugs encompassed by clinical drug resistance indicates that multiple resistance mechanisms are likely involved. In addition, differences in the methods used to quantify MRP1 in clinical samples have almost certainly contributed to the sometimes discrepant results reported by different investigators. The design and execution of more informative studies to address the role of resistance proteins in chemotherapy failure has been hindered by difficulties in obtaining suitable patient samples (e.g. pre- and post-chemotherapy samples from the same patient) for practical and/or ethical reasons.

Despite these and other problems, several studies have found expression levels of MRP1 to be of prognostic significance. Amplification of the N-*myc* oncogene has been established to be a powerful indicator of poor response to chemotherapy and poor outcome in childhood neuroblastoma [159]. Analyses of neuroblastoma tumor specimens from 85 patients revealed levels of *MRP1* mRNA that were significantly higher in tumors with N-*myc* amplification. Furthermore, *MRP1* mRNA levels were a strong predictor of reduced survival independent of N-*myc* amplification. These results suggest that MRP1 expression may account in part for the association between N-*myc* amplification and reduced survival [160,161]. One particular subtype of acute myeloid leukemia, designated M4Eo, is associated with an inversion in chromosome 16, inv(16)(p13q22) [162]. The *MRP1* gene maps proximal to the short arm breakpoint this inversion [10,163] and in one study of a subset of 22 M4Eo patients, one *MRP1* allele was found to be disrupted by this inversion. Patients in this subset had a significantly increased time to death or relapse after diagnosis compared to patients in which both *MRP1* alleles were retained, suggesting that hemizygosity for *MRP1* rendered the leukemic cells of these patients more sensitive to chemotherapy [164]. MRP1 has also been associated with the rare chemotherapy failure in cases of retinoblastoma which were treated with anticancer drugs and the P-glycoprotein inhibitor, cyclosporin A, suggesting that MRP1-mediated resistance may emerge in cases where P-glycoprotein overexpression is prevented by the use of P-glycoprotein chemosensitizers [150]. However, both the M4Eo leukemia and retinoblastoma studies were based on very small numbers of patient samples, and further analysis will be required before any firm conclusions can be drawn about the involvement of MRP1 in drug sensitivity or resistance of these malignancies.

6. Conclusions

Since the discovery of MRP1 in 1992, there has been considerable progress in our understanding of the biology of this protein. Many of the features of multidrug resistance associated with overexpression of MRP1 have been well characterized, and have been shown to be very similar to, but clearly distinct from, those of P-glycoprotein-mediated drug resistance. One notable aspect of MRP1-mediated resistance, related to the role of this protein as a broad specificity transporter of conjugated organic anions, is the involvement of GSH. Current evidence suggests that MRP1 co-transports some natural products drugs with GSH, but this appears not to be true for all drugs to which this protein confers resistance [165]. MRP1 was the founding member of a subfamily of ABC transporters characterized in part by the presence of a unique NH_2-terminal

MSD. Structure-function analyses have begun to address the importance of this and other domains of the protein for its expression and activity. Further characterization of the mechanism by which MRP1 transports its substrates will be facilitated by recent developments, including improved structural models of the protein supported by biochemical and immunological evidence and the purification and reconstitution of the protein in an active, transport competent form.

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