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Review

Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity, and MRP2-mediated drug resistance

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Abstract

The membrane proteins mediating the ATP-dependent transport of lipophilic substances conjugated to glutathione, glucuronate, or sulfate have been identified as members of the multidrug resistance protein (MRP) family. Several isoforms of these conjugate export pumps with different kinetic properties and domain-specific localization in polarized human cells have been cloned and characterized. Orthologs of the human MRP isoforms have been detected in many different organisms. Studies in mutant rats lacking the apical isoform MRP2 (symbol ABCC2) indicate that anionic conjugates of endogenous and exogenous substances cannot exit from cells at a sufficient rate unless an export pump of the MRP family is present in the plasma membrane. Several mutations in the human *MRP2* gene have been identified which lead to the absence of the MRP2 protein from the hepatocyte canalicular membrane and to the conjugated hyperbilirubinemia of Dubin–Johnson syndrome. Overexpression of recombinant MRP2 confers resistance to multiple chemotherapeutic agents. Because of its function in the terminal excretion of cytotoxic and carcinogenic substances, MRP2 as well as other members of the MRP family, play an important role in detoxification and chemoprevention.

Keywords: ATP-dependent transport; Dubin–Johnson syndrome; Green fluorescent protein; Leukotriene C_4; MRP1,2,3; Multidrug resistance

Contents

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1. Introduction

The membrane proteins mediating the ATP-dependent unidirectional transport of conjugates of lipophilic substances with glutathione, glucuronate, or sulfate have been recognized as members of the multidrug resistance protein (MRP) family [1–12]. In addition to many anionic conjugates [13], a number of unconjugated amphiphilic anions can serve as substrates for MRP1, MRP2, and possibly for additional members of the MRP family of transport proteins [14–18]. Prior to the elucidation of the transport function of MRP1 in 1994 [1–3,19], ATP-dependent transport of conjugates was characterized by use of inside-out plasma membrane vesicles from many cell types including erythrocytes [20,21], hepatocytes [22–24], heart [25], and mastocytoma cells [26]. It has become apparent that anionic conjugates of endogenous and exogenous substances cannot exit from cells into the extracellular space at a sufficient rate unless a MRP-type transporter is present in the plasma membrane (Fig. 1). This is exemplified by studies in mutant rats selectively lacking the MRP2 protein in the hepatocyte canalicular membrane [27–30]. These mutant animals cannot release the endogenous glutathione *S*-conjugate leukotriene C_4 (LTC_4) and its metabolites across the hepatocyte canalicular membrane into bile [31,32]. This leads to an accumulation of the conjugates within the hepatocytes and to their subsequent exit across the basolateral membrane into blood [32] in a transport step probably mediated by MRP3 [33]. Finally, the LTC_4 metabolites undergo renal elimination into the urine [31,32].

The localization of MRP2 in the apical membrane of polarized epithelia [27–29,34,35] favors a particular role of this MRP isoform in detoxification by the terminal excretion of conjugation products of drug metabolism into bile or urine (Fig. 1). Moreover, since both MRP2 and MRP1 export oxidized glutathione [36], these MRP family members contribute to the control of the GSSG/GSH ratio and to the defense against oxidative stress under conditions of enhanced GSSG formation. In cancer chemotherapy, MRP1 [37,38], MRP2 [8], and MRP3 [39] have the capacity to confer resistance to chemotherapeutic agents. Thus, knowledge of the substrate specificity, the expression level, the plasma membrane domain-specific localization, and the cell type-specific expression of the MRP isoforms contributes to our understanding of detoxification pathways and drug resistance mechanisms, and suggests approaches to the development of inhibitors interfering with MRP-mediated export of cytotoxic agents and drugs.

2. The MRP family of conjugate export pumps

ABC transporters belonging to the MRP family and mediating the export of anionic conjugates out of cells have been identified in a variety of different organisms including yeast, nematodes, and plants [12,40,41] (Table 1). The family of the human multidrug resistance proteins (MRPs) consists of at least six members known as MRP1 (symbol ABCC1), MRP2 (ABCC2; also known as cMRP or cMOAT), and the more recently identified family members MRP3, MRP4, MRP5, and MRP6 (ABCC3, ABCC4, ABCC5, and ABCC6 [44,45]). The deduced amino acid sequence lengths range from 1325 amino acids for MRP4 to 1545 amino acids for MRP2. The membrane topology, predicted by several computational methods, is a notable feature common to at least four of the six members. In contrast to the typical six plus six transmembrane segment organization described for the members of the P-glycoprotein family [46,47], the MRPs exhibit an additional

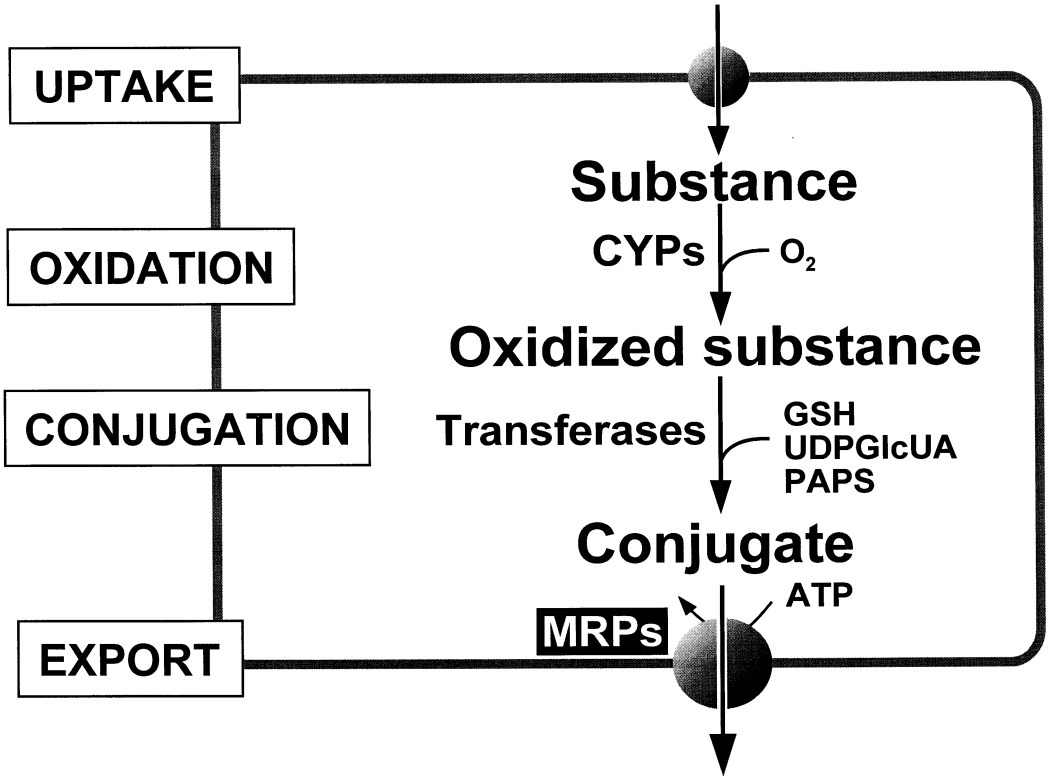

Fig. 1. Uptake, biotransformation, and MRP-mediated export of endogenous substances, drugs, and carcinogens.

amino-proximal membrane-spanning domain represented by an extension of approximately 200 amino acids [48]. This membrane topology distinguishes the MRP family from other ABC-transporters [48]. Best characterized members of the MRP family are the founding member MRP1 [49] and the apically localized member MRP2 [29,50]. MRP1 was cloned from a drug-selected human lung cancer cell line and consists of 1531 amino acids. The *MRP1* gene, located on chromosome 16p13.12–13, spans approximately 200 kb and contains 31 exons and a high proportion of class 0 introns [51]. Elucidation of the sequence [49] and the function [1,2] of MRP1 suggested the presence of functionally related transporters in other tissues. Comparison of transport characteristics obtained with normal rat liver canalicular membrane vesicles and vesicles from GY/TR$^-$ and EHBR mutant rat strain livers resulted in the characterization and identification of MRP2 (cMRP, cMOAT), the apical isoform of MRP1 [27–29]. This protein is mainly localized to the canalicular membrane of hepatocytes [27,29] and, in addition, in several other apical membrane domains including the apical membrane of kidney proximal tubule epithelia [34,35]. Immunofluorescence studies demonstrated, that the MRP2 protein is absent in livers of GY/TR$^-$ [27–29] and EHBR rats [29,30] and in the liver of patients with Dubin–Johnson syndrome [52,53].

The close relationship between MRP1 and MRP2 is also evident from the comparison of the topology of both transporters. Several methods have been used to predict the transmembrane organization of human MRP1 and MRP2. These methods include computational analyses, mutational analyses and limited proteolysis experiments [54], as well as epitope insertion studies [55–57]. Most studies focused on the topology prediction of MRP1, but several hydrophobicity and transmembrane predicting programs, such as the transmembrane analysis program (TMAP) [58], demonstrated the close topological relationship of MRP1 and MRP2. Both transporters are predicted to consist of a MDR-like core structure of two transmembrane regions and two ATP-binding domains and, in addition, of a large third amino-proximal transmembrane region. A remarkable topological feature of both proteins was found in studies

Table 1
Amino acid sequence identity of human MRP2 with related transporters of the MRP family

Species	Protein	Symbol	AA	Identity (%)	Accession number	Conjugate export
Human	MRP1	ABCC1	1531	48	NM_004996	++
	MRP2	**ABCC2**	**1545**	**100**	**FX96395**	++
	MRP3	ABCC3	1527	46	Y17151	++
	MRP4	ABCC4	1325	37	AF071202	
	MRP5	ABCC5	1437	35	AF104942	++
	MRP6	ABCC6	1503	38	AF076622	
	CFTR	ABCC7	1480	27	P13569	
	SUR	ABCC8	1581	30	U63421	
Rabbit	MRP2		1564	82	Z49144	++
Rat	MRP2		1541	78	X96393	++
	MRP3		1523	46	AB010467	++
	MRP6		1502	36	AB010466	
Mouse	MRP1		1528	48	AF022908	++
	MRP5		1436	35	AB019003	
Caenorhabditis elegans	MRP1		1540	42	U66260	+
	MRP2		1525	42	U66261	+
Arabidopsis thaliana	MRP1		1622	36	AF008124	++
	MRP2		1623	35	AF020288	++
	MRP3		1515	33	U92650	++
	MRP4		1516	35	AJ002584	+
	MRP5		1514	32	U11250	
Yeast	YDR135C		1515	39	L35237	++
	YLL015W		1559	34	Z73120	

Amino acid sequence identities are given in relation to human MRP2 (boldface). Transporters for which the conjugate transport function has been shown directly are indicated by ++, transporters for which the conjugate export has been demonstrated indirectly, e.g. by determination of toxin resistance, are indicated by +. Computer analysis was performed using tree alignment from Husar program [42] based on the Wisconsin Genetics Computer Group program package [43].

on the localization of the amino terminus. The amino terminus was predicted to be extracytosolic since most predictions favored an odd number of transmembrane domains. This was described first for MRP2 on the basis of TMAP analyses [29], and subsequently established by epitope insertion experiments, performed on MRP1 [59] and by immunofluorescence studies for MRP2 [8]. These studies demonstrate experimentally the extracellular location of the amino terminus. Elucidation of structural features, genomic organization, and transport properties of MRP1 and MRP2 provided a basis for the comparison with additional members of this family.

The identification of MRP3, MRP4, and MRP5 was mainly based on expressed sequence tags (EST) database analyses [44] followed by the cloning of cDNA fragments [45]. All three proteins are encoded by different genes located on different chromosomes, *MRP3* on chromosome 17q21.3, *MRP4* on chromosome 13q31–32, and *MRP5* on chromosome 3q27 [45]. Among these, MRP3 is best characterized with respect to its localization and in part with respect to its transport function [9,33,39]. The tissue expression pattern of MRP3 exhibits similarities with the tissue distribution of MRP2. Both proteins are highly expressed in liver and colon and on a lower level in kidney as demonstrated by Northern blotting [33]. MRP3 was localized exclusively to the basolateral membrane of hepatocytes as studied by immunofluorescence microscopy [33,39]. A comparison of *MRP3* mRNA expression in normal rat liver and liver from bile duct-ligated or MRP2-deficient GY/TR$^-$ and EHBR mutant rats demonstrated that *MRP3* is expressed at a low level under normal conditions but upregulated under conditions when the canalicular secretion of conjugates is impaired [9]. This upregulation of MRP3 was also observed in studies on the protein expression in human liver.

Liver from a patient with Dubin–Johnson syndrome and liver from a patient with primary biliary cirrhosis exhibited a strong MRP3 expression in the basolateral hepatocyte membrane when compared with apparently normal liver [33]. MRP3 may serve as an overflow mechanism mediating the export of conjugates across the basolateral membrane under conditions when the canalicular secretion of MRP2 substrates is impaired. The genomic organization of MRP3 has been studied in part [60] and revealed similarities to the known genomic structures of human MRP1 [51] and MRP2 [53] as indicated by many identical splice junction sites on amino acid level.

Little is presently known about localization and transport characteristics from MRP4, MRP5, and MRP6. MRP6 was first reported to be a short, MRP-related protein with only one nucleotide binding fold. The protein was cloned from anthracycline-selected cells [61] and was thus called ARA (anthracycline resistance-associated). Further analyses suggested that this short MRP isoform is a cell selection or cloning artefact and that ARA is in fact the carboxyl terminal half of human MRP6 [62]. The *MRP6* gene is located on chromosome 16 next to the *MRP1* gene with which it is coamplified during overexpression [62].

3. Substrates and kinetic properties of MRP1, MRP2, and MRP3

Following the demonstration in 1994 that MRP1 functions as an unidirectional ATP-dependent export pump for the endogenous glutathione *S*-conjugate leukotriene C_4 [1–3], the substrate specificity of this transport protein was studied intensively using inside-out membrane vesicles from cells expressing the recombinant protein at a high level [4–6,36,63–71]. The substrate spectrum comprises amphiphilic anions, particularly conjugates of lipophilic compounds with glutathione, glucuronate, or sulfate, including cysteinyl leukotrienes, bilirubin glucuronosides, 17β-glucuronosyl estradiol, and sulfatolithocholyl-taurine as endogenous compounds (Table 2). The glutathione *S*-conjugate leukotriene C_4 is the substrate with the highest affinity identified so far (K_m value 0.1 μM). Glutathione disulfide is also transported by MRP1, however, with a relatively low affinity (K_m value 100 μM) [36], indicating a role of this export pump in oxidative stress associated with increasing concentrations of glutathione disulfide. MRP1-mediated ATP-dependent transport of reduced glutathione was not detectable in these studies with isolated membrane vesicles [36]. However, reduced glutathione can serve as a cosubstrate with neutral or cationic substances. This is indicated by the ATP-dependent transport of the cytostatic drugs vincristine and daunorubicin in the presence of reduced glutathione [6,65,71]. Both drugs are also substrates for MDR1 P-glycoprotein, which, in contrast to MRP1, transports these compounds unmodified and independently of glutathione [47]. For some cytostatic drugs (Table 2) their glutathione and glucuronate conjugates have been identified as MRP1 substrates. The anionic drug methotrexate was identified as a cytostatic agent transported in its native form [70]. This transport of drugs is in line with the multidrug resistance conferred by MRP1. In addition to methotrexate, several additional amphiphilic organic anions which are not conjugated have been shown to be transported by MRP1 directly as exemplified by the penta-anionic fluorescent dye Fluo-3 taken up into inside-out membrane vesicles [16,18].

The spectrum of substrates for MRP1 is similar to that of the ATP-dependent hepatocyte canalicular conjugate export pump functionally known for many years [22,24,75] and molecularly identified as MRP2 [27–30,50]. Previously, ATP-dependent transport measurements into inside-out hepatocyte canalicular membrane vesicles from normal rats in comparison with vesicles from GY/TR$^-$ and EHBR mutant rats selectively lacking MRP2 [27,29] have been valuable in estimating the substrate specificity of this transport protein [24,75,77,89], which is similar to that of MRP1 (Table 2). The availability of cell lines stably expressing the recombinant protein enabled the characterization of the substrate specificity of human and rat MRP2 under defined conditions [7,8,10,74] and to identify kinetic differences between MRP2 and MRP1 [8]. MRP1 exhibits a 10-fold higher affinity to leukotriene C_4 and a 5-fold higher affinity to 17β-glucuronosyl estradiol in comparison to MRP2 (see K_m values Table 2) [8]

Table 2
Substrates for MRP1, MRP2, and MRP3

Substrate	K_m value (μM)	References
MRP1 (human, recombinant)		
Leukotriene C_4	0.1	[2,5,67]
Leukotriene D_4		[2,4]
Leukotriene E_4		[2,4]
N-Acetyl-leukotriene E_4		[4]
S-Glutathionyl aflatoxin B1	0.2	[64]
S-Glutathionyl 2,4-dinitrobenzene (DNP-SG)	3.6	[4]
S-Glutathionyl prostaglandin A_2		[68]
S-Glutathionyl ethacrynic acid	28	[66]
S-Glutathionyl *N*-ethylmaleimide		[72]
Chlorambucil		
– monochloro-monoglutathionyl		[69]
– monohydroxy-monoglutathionyl		[69]
– bisglutathionyl		[69]
Melphalan		
– monochloro-monoglutathionyl		[4,69]
– monohydroxy-monoglutathionyl		[69]
Glutathione disulfide	93	[36]
Bilirubin		
– monoglucuronosyl		[63]
– bisglucuronosyl		[63]
17β-Glucuronosyl estradiol	1.5	[4]
	2.5	[6]
	2.9	[67]
6α-Glucuronosyl hyodeoxycholate		[4]
Glucuronosyl etoposide		[4]
3α-Sulfatolithocholyltaurine		[4]
Chlorambucil		[69]
Folate		[13]
Methotrexate	50	[70] Leier et al., 1999[a]
Fluo-3	12	[18]
p-Aminohippurate	372	Leier et al., 1999[a]
Vincristine+reduced glutathione		[5,65,67,71]
Daunorubicin+reduced glutathione		[71]
Aflatoxin B1+reduced glutathione		[64]
MRP2 (human, recombinant)		
Leukotriene C_4	1.0	[8]
S-Glutathionyl 2,4-dinitrobenzene (DNP-SG)	6.5	[7]
	70	[73]
S-Glutathionyl ethacrynic acid		[7]
Bilirubin		
– monoglucuronosyl	0.7	[74]
– bisglucuronosyl	0.9	[74]
17β-Glucuronosyl estradiol	7.2	[8]
Methotrexate		[70]
p-Aminohippurate	880	Leier et al., 1999[a]
Ochratoxin A		Leier et al., 1999[a]
MRP2 (rat, recombinant)		
Leukotriene C_4	1.1	[8,10]
S-Glutathionyl 2,4-dinitrobenzene (DNP-SG)	0.2	[10]

Table 2 (*continued*)

Substrate	K_m value (μM)	References
Bilirubin		
– monoglucuronosyl	0.8	[74]
– bisglucuronosyl	0.5	[74]
17β-Glucuronosyl estradiol	6.9	[8]
MRP2 (rat; normal/mutant BCM)		
Leukotriene C_4	0.3	[24]
Leukotriene D_4	1.5	[24]
Leukotriene E_4		[24]
N-Acetyl leukotriene E_4	5.2	[24]
S-Glutathionyl 2,4-dinitrobenzene (DNP-SG)	18	[76]
S-Glutathionyl sulfobromophthalein		[77]
Glutathione disulfide		[75,78]
Bilirubin		
– monoglucuronosyl		[63]
– bisglucuronosyl		[63,78]
Glucuronosyl nafenopin		[79]
Glucuronosyl E3040	5.7	[76]
Glucuronosyl grepafloxacin	7.2	[80]
Glucuronosyl SN38 carboxylate		[81]
Glucuronosyl SN38 lactone		[81]
SN38 carboxylate		[81]
CPT11 carboxylate		[81]
Sulfobromophthalein	31	[82]
Fluo-3	3.7	[16]
5-Methyltetrahydrofolate	126	[83]
Methotrexate	295	[84]
Temocaprilat	93	[85]
Pravastatin	220	[86]
BQ123	98	[87,88]
BQ485	6.5	[88]
BQ518	15	[88]
MRP3 (human, recombinant)		
Bilirubin		
– monoglucuronosyl		Kamisako et al., 1999[a]
MRP3 (rat, recombinant)		
Leukotriene C_4		[9]
S-Glutathionyl 2,4-dinitrobenzene (DNP-SG)		[9]
17β-Glucuronosyl estradiol	67	[9]
Glucuronosyl E3040		[9]
Methotrexate		[9]

Compounds listed have been identified as substrates by measurements of the ATP-dependent transport into inside-out membrane vesicles from cells expressing the respective recombinant multidrug resistance protein in comparison to membrane vesicles from control vector-transfected cells. In addition, measurements of the ATP-dependent transport into hepatocyte canalicular membranes vesicles from MRP2-deficient mutant rats (GY/TR$^-$ and EHBR) [28,29] in comparison to those from normal rats are presented.

BCM, bile (hepatocyte) canalicular membranes; BQ123, cyclic pentapeptide, endothelin antagonist (cyclo[D-Trp-D-Asp-L-Pro-D-Val-L-Leu]); BQ485, linear peptide (perhydroazepino-*N*-carbonyl-L-Leu-D-Trp-D-Trp); BQ518, (cyclo[D-Trp-D-Asp-L-Pro-D-Thg-L-Leu]); CPT11, irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin); E3040, 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl)benzothiazole; Fluo-3, 1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxo-3*H*-xanthen-9-yl)]-2-(2′-amino-5′-methyl-phenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid penta ammonium salt; 5-methyltetrahydrofolate, 5-methyl-tetrahydropteridinyl L-glutamic acid; SN38, de-esterified metabolite of CPT11 (7-ethyl-10-hydroxycamptothecin); temocaprilat, pharmacologically active metabolite of temocapril hydrochloride, prodrug of angiotensin-converting enzyme inhibitor (α-(2*S*,6*R*)-6-[(1*S*)-1-ethoxy-carbonyl-3-phenyl-propyl]amino-5-oxo-2-(2-thienyl)perhydro-1,4-thiazepin-4-yl acetic acid hydrochloride).

[a] Unpublished data.

whereas bilirubin glucuronosides are preferentially transported by MRP2 [63,74].

MRP3, which exhibits a similar tissue distribution as MRP2, but is localized to the basolateral membrane domain of polarized cells [33,39], has been also shown to function as a conjugate export pump [9]. Studies with membrane vesicles from cells expressing recombinant rat MRP3 suggested a preference for glucuronosides and a low affinity for leukotriene C_4 [9]. However, the K_m value for 17β-glucuronosyl estradiol (Table 2) was almost 10-fold higher than that for MRP2. This may reflect the function of MRP3 as a basolateral overflow system in hepatocytes ensuring the elimination of compounds under conditions of impaired canalicular MRP2-mediated secretion.

In addition to the elucidation of the substrate specificity, the identification of potent and specific inhibitors of MRP-mediated transport is of importance for the characterization of the transport function as well as for the development of modulators of multidrug resistance mediated by MRP transporters. The most potent inhibitors reported so far and tested for inhibition of ATP-dependent MRP1-mediated transport in isolated membrane vesicles are structural analogs of the cysteinyl leukotrienes (summarized in [13] and [90]). For example, the LTD_4 analog MK571, a monoanionic quinoline derivative developed as a LTD_4 receptor antagonist, was shown to inhibit MRP1-mediated transport competitively in sub-micromolar concentrations [1,2,4,36]. This compound also inhibits MRP2-mediated transport, although with lesser potency [29]. A structural analog of leukotriene C_4, *S*-decylglutathione, also acts as a potent competitive inhibitor of MRP1-mediated transport [6]. Cyclosporin A and the non-immunosuppressive cyclosporin A derivative PSC 833, which are both known to inhibit several ATP-dependent export pumps at low concentration [91], are relatively weak inhibitors of MRP1- [2] and MRP2-mediated transport [74]. The K_i value of cyclosporin A was 5 μM in human MRP1-mediated leukotriene C_4 transport [2] and 21 μM in human MRP2-mediated transport of monoglucuronosyl bilirubin [74]. Compounds suggested as inhibitors on the basis of their effect on drug accumulation or on resistance reversal in MRP1-overexpressing cells include the tyrosine kinase inhibitor genistein [92], the bisindolylmaleimide protein kinase C inhibitor GF109203X [93], and the quinolone difloxacin [94]. An overview on inhibitors of MRP1-mediated transport has been published recently [90].

4. Drug resistance conferred by the apical conjugate export pump MRP2

MRP1 when overexpressed in cells confers resistance to many different antitumor drugs (reviewed in [37]). MRP2 and MRP1 share a similar substrate spectrum despite differences in cellular localization and kinetic properties [8,13]. This has suggested that MRP2 may also confer drug resistance by pumping drug conjugates or drug complexes with glutathione out of the cell. Northern blot analyses and RNase protection assays provided the first indications that MRP2 expression may correlate with multidrug resistance [45,50,95]. *MRP2* cDNA was cloned initially from rat liver [27–29], in addition human *MRP2* cDNA was cloned from liver [29,96] and from cisplatin-resistant human cancer cell lines KB-CDP4 and P-CDP5 [50]. In both drug-resistant cell lines *MRP2* mRNA was overexpressed in com-

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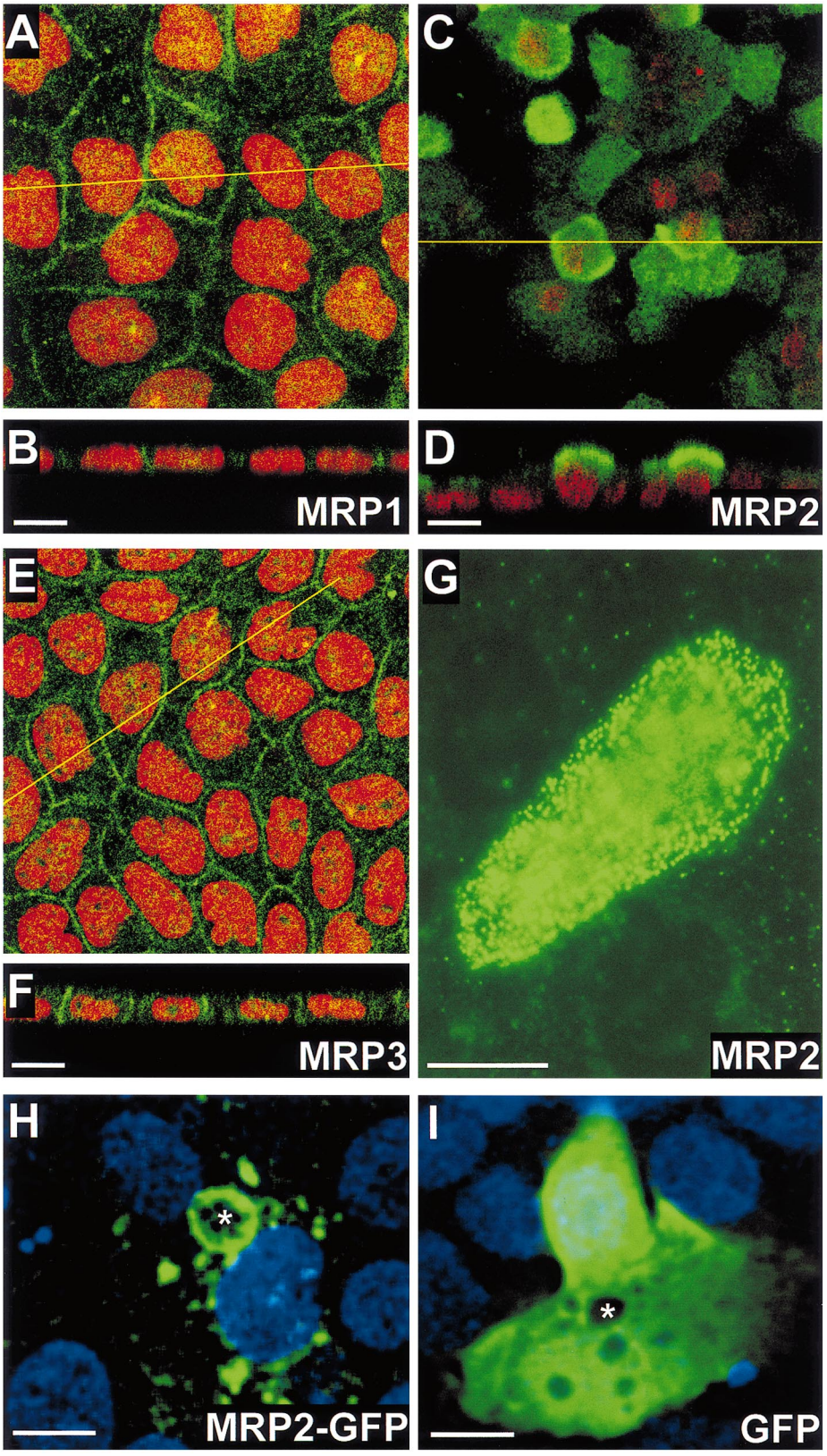
Fig. 2. Confocal laser scanning micrographs of MRP isoforms stably expressed in polarized MDCK cells using isoform-specific antibodies (green fluorescence in A–G), and fluorescence micrographs of MRP2, C-terminally tagged with GFP, and GFP alone in live, polarized HepG2 cells (green fluorescence in H,I). Nuclei were stained with either propidium iodide (red fluorescence in A–F) or Hoechst 33258 (blue fluorescence in H,I). (A,C,E) *xy*-planes and (B,D,F) corresponding optical vertical sections (*xz*-planes) are shown. (A,B) Human MRP1 was detected with the MRP1-specific QCRL1 antibody [55]. (C,D) MDCK cells expressing human MRP2 were stained with an antibody directed against the carboxyl terminus (EAG) [8,29,63]. (E,F) Localization of human MRP3 in MDCK cells was visualized with an antibody directed against the carboxyl terminus of the MRP3 isoform (FDS) [33]. (G) Non-permeabilized MDCK cells expressing rat MRP2 were stained with the MDK antibody directed against the amino terminus of rat MRP2 demonstrating the extracellular location of the amino terminus [8]. (H,I) Live, polarized HepG2 cells form apical vacuoles between adjacent cells (asterisks). (H) MRP2-GFP localized to the apical membrane and, additionally, to intracellular vesicular structures. (I) GFP alone was expressed throughout the cell, including the cytoplasm and the nucleus, but excluding apical vacuoles. Scale bars: 10 μm.


A
B
MRP1
C
D
MRP2
E
F
MRP3
G
MRP2
H
MRP2-GFP
I
GFP

parison to non-resistant parental cell lines. A correlation between *MRP2* expression and cisplatin-resistance was also demonstrated for several additional cell lines by RNase protection assays and Northern blot analyses [45,95]. Moreover, antisense cDNA was used to reduce the expression of MRP2 protein in the human hepatoma cell line HepG2 [97]. Upon transfection of the antisense *MRP2* cDNA HepG2 cells showed elevated intracellular glutathione levels and enhanced sensitivity to antitumor drugs including cisplatin, vincristine, doxorubicin, and the camptothecin derivatives CPT11 and SN38, but not to etoposide, 3-[4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea (ACNU), 5-fluorouracil, and mitomycin C [97]. Direct evidence for MRP2-mediated multidrug resistance [8] as well as resistance to antifolates [70] was obtained from transfection studies with *MRP2* cDNA. A prerequisite for measuring drug resistance mediated by MRP2 is the plasma membrane localization of MRP2 in transfected cells. Both in MDCKII cells and in HEK293 cells recombinant MRP2 was localized to the plasma membrane, though a large proportion of recombinant MRP2 was also found intracellularly in HEK293 transfectants [8]. Expression of both human and rat MRP2 in MDCKII cells confers significant resistance to etoposide and vincristine (Table 3). In transfected HEK293 cells recombinant human MRP2 confers resistance to etoposide, cisplatin, epirubicin, and doxorubicin (Table 4). The mechanism of MRP2-conferred resistance to these drugs is still under investigation. One may expect that the same mechanisms are involved as shown earlier for MRP1 [65,71]. MRP2 as well as MRP1 were also shown to confer resistance to the antifolate methotrexate, a substrate for MRP1 and MRP2, in transfected human ovarian carcinoma 2008 cells [70]. Clinical relevance of MRP2-mediated drug resistance has been emphasized by the finding that MRP2 was detected in 95% renal clear-cell carcinomas both at the mRNA and at the protein level [35]. MRP2 expression was also detected by RT-PCR, immunoblotting, and immunofluorescence microscopy in lung, gastric, and colorectal cancer cells, and in hepatocellular carcinomas ([98]; Nies et al., unpublished). Taken together, overexpression of MRP2 was found in different epithelial malignant tumors and may contribute to their resistance to antitumor drugs.

Table 3
MRP2-mediated resistance to etoposide and vincristine in transfected MDCKII cells

	Etoposide		Vincristine	
	IC_{50} (μM)	RR	IC_{50} (μM)	RR
MDCK-Co	163	1.0	8.2	1.0
MDCK-MRP2 human	612	3.8[a]	49	6.0[a]
MDCK-MRP2 rat	809	5.0[a]	19	2.3[a]

[a] $P < 0.01$. Sensitivity to etoposide and vincristine was determined by the MTT cell viability assay. The relative resistance factor (RR) was calculated by dividing the IC_{50} value of cells transfected with rat or human *MRP2* by the IC_{50} value of cells transfected with control vector (MDCK-Co). Modified from [8].

Table 4
MRP2-mediated resistance to antitumor drugs in transfected HEK293 cells

	HEK-Co	HEK-MRP2 human	
	IC_{50}	IC_{50}	RR
Cisplatin (μM)	2.4	24	10.0[a]
Etoposide (μM)	0.3	1.2	4.0[a]
Doxorubicin (nM)	44	346	7.8[a]
Epirubicin (nM)	3.8	19	5.0[a]

[a] $P < 0.01$. Sensitivity to antitumor drugs was determined by the MTT cell viability assay. The relative resistance factor (RR) was calculated by dividing the IC_{50} value of cells transfected with human *MRP2* (HEK-MRP2) by the IC_{50} value of cells transfected with control vector (HEK-Co). Modified from [8].

5. Expression and localization of MRP2 in polarized cells

5.1. Immunofluorescence localization of MRP isoforms in polarized cells

Antibodies of high affinity and selectivity have been useful for the identification and localization of individual MRP isoforms in different cell types. The isoforms MRP1, MRP2, and MRP3 have related functions, but differ in their membrane localization. Studies on the domain-specific sorting of individual isoforms have become possible by the permanent expression of the respective recombinant MRP isoform in polarized cells (Fig. 2).

MRP2 is the apical MRP isoform and is localized to the canalicular membrane of rat and human hepatocytes [29,96,99,100], as well as to the apical mem-

brane of rat hepatoma cells [16]. MRP2 expression has also been shown by immunoblotting in mouse [101] and rabbit liver [102], and in human hepatoma HepG2 cells [63]. In addition to liver, *MRP2* mRNA was detected by Northern blotting in kidney, duodenum and ileum [28,30]. Moreover, MRP2 expression in human colon-derived Caco-2 cells was shown by immunoblotting [103]. Furthermore, the apical localization of MRP2 was demonstrated in rat and human kidney proximal tubules [34,35]. After transfection, rat [8] and human [7,8] MRP2 localized to the apical membrane of polarized MDCK cells. Furthermore, MRP2-expressing MDCK cells and antibodies specific for the amino terminus of MRP2 were useful in identifying the extracellular location of the amino terminus of MRP2 [8].

In addition to the apical conjugate export pump MRP2, hepatocytes express basolateral conjugate export pumps which have been considered to be MRP isoforms [27,99]. Antibodies specific for MRP1 showed expression of this isoform in the plasma membrane of many different cell types [104–106] and in the basolateral membrane of polarized epithelia [107,108]. Transfection of pig kidney cells resulted in basolateral localization of MRP1 [109]. Significant amounts of *MRP1* mRNA, however, were not detected in human liver [45,49]. Instead, the isoform MRP3 was recently cloned from liver by several groups [33,39,110–112] and localized to the basolateral membrane of human hepatocytes [33,39] and human cholangiocytes [39] by using MRP3-specific antibodies. mRNA encoding MRP3 was also detected in colon, small intestine, pancreas, prostate and kidney [33,111,112]. It will be of interest to analyze the membrane localization of MRP3 in these tissues. Transfection of *MRP3* cDNA resulted in basolateral membrane expression of this isoform in MDCK cells (Fig. 2, [39]).

Thus, expression of proteins with similar function, but different membrane localization in a single cell type provides a unique tool for studying distinct sorting motifs of these related proteins of the MRP family.

5.2. MRP2-GFP construct expression in polarized HepG2 cells

MRP2 is predominantly found in the apical membrane of polarized cells; however, cholestasis, hyperosmotic stress, or disruption of cell junctions cause partial redistribution of this conjugate export pump into intracellular vesicles by endocytic retrieval [100,113,114]. For detailed studies on this process, visualization of intracellular transport of MRP2 provides a useful tool. Tagging of proteins with the green fluorescent protein (GFP) has been valuable for studies on molecular mechanisms of intracellular protein trafficking (reviewed in [115,116]). Human hepatoma HepG2 cells polarize after several days in culture and form apical vacuoles between adjacent cells [117–119]. The expression pattern of MRP2, C-terminally tagged with GFP, was completely different from that seen for GFP alone (Fig. 2). GFP was distributed throughout the cells, excluding the apical vacuoles. MRP2-GFP predominantly localized to the apical membrane which appeared as a ring-like structure along the circumference of apical vacuoles. In addition, MRP2-GFP was associated with the membrane surrounding intracellular vesicles of varying size. Immunofluorescence studies with antibodies specific for the amino terminus of MRP2 demonstrated that the observed GFP fluorescence correctly reflected the localization of MRP2. Because of the autofluorescence of GFP-tagged proteins, intracellular trafficking of MRP2-GFP can be visualized by real-time video imaging. In addition, the effect of substances, such as microtubule or microfilament inhibitors on trafficking can be analyzed.

5.3. Secretion of fluorescent substrates by MRP2

Fluorescent substrates are often used to analyze MRP activity by measuring their efflux out of intact cells. Anionic fluorescent dyes, such as carboxyfluorescein [120] and carboxy-2′,7′-dichlorofluorescein [121,122], have been suggested as MRP1 substrates because the rate of dye extrusion directly correlated with MRP1 expression levels. A similar correlation was observed between MRP1 expression levels and the release rate of the fluorescent Ca^{2+} indicators calcein [14,15] and Fluo-3 [17] from intact cells. Fluorescent glutathione *S*-conjugates, such as glutathione bimane [123,124] and glutathione methylfluorescein [114], are likely transported by MRP2 as indicated by lack of their transport in hepatocytes from MRP2-deficient mutant rats. Fluo-3 has been

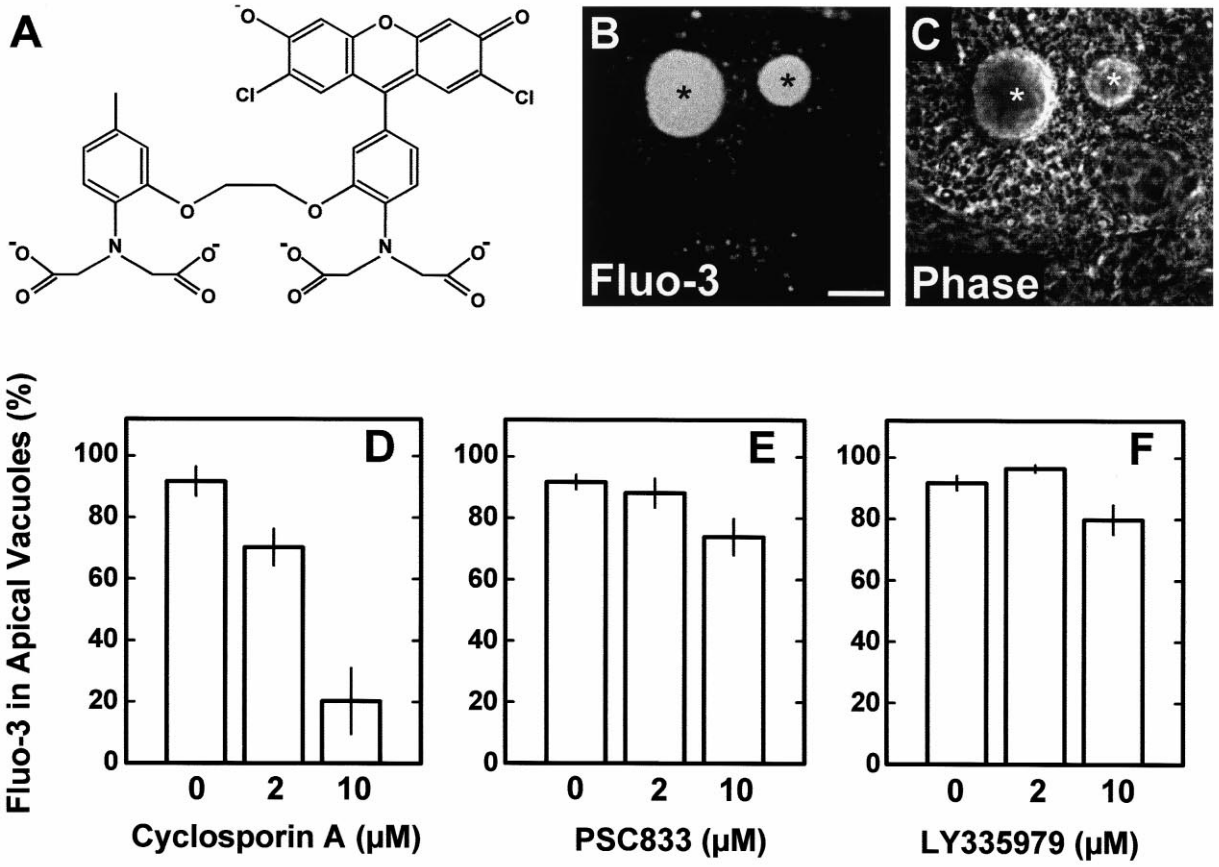


Fig. 3. Secretion of Fluo-3 into apical vacuoles of WIF-B cells and inhibition of Fluo-3 secretion by cyclosporin A and MDR1 P-glycoprotein inhibitors. (A) Structure of the fluorescent organic penta-anion Fluo-3 [127,128]. (B) WIF-B cells were incubated with the non-fluorescent acetoxymethylester of Fluo-3, Fluo-3-AM, which was taken up by the cells, and hydrolyzed to the fluorescent Fluo-3. Fluo-3 secretion into apical vacuoles was observed by fluorescence microscopy as described [16]. (C) Phase contrast micrograph corresponding to B. (D–F) Quantitative analysis of inhibition of Fluo-3 secretion into apical vacuoles of WIF-B cells was performed as described [16]. Whereas cyclosporin A inhibited Fluo-3 secretion (D), no significant inhibitory effects were observed at the same concentrations of the non-immunosuppressive cyclosporin derivative (and MDR1 P-glycoprotein inhibitor) PSC833 (E [129]), or with the selective MDR1 P-glycoprotein inhibitor LY335979 (F [130,131]). Scale bar: 10 μm.

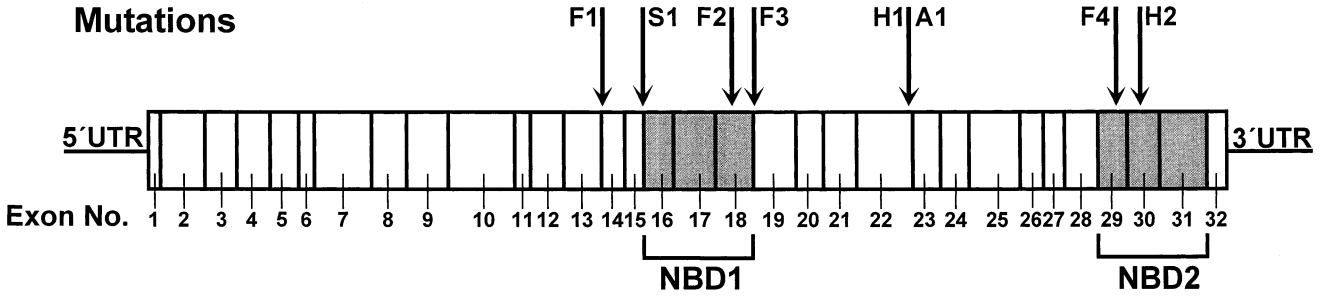


Fig. 4. Exon organization of the human *MRP2* gene and sites of mutations in Dubin–Johnson syndrome. The exon boundaries are represented by vertical lines, the exon numbers are indicated, and the nucleotide-binding domains (NBD1 and NBD2) are shaded. Arrows indicate the sites of mutations identified so far. H1 and H2 correspond to mutations identified in Heidelberg [53], A1 corresponds to a patient analyzed in Amsterdam [96], F1–4 correspond to mutations identified in Fukuoka [132,133], and S1 indicates the position of the mutation identified in Saga [130].

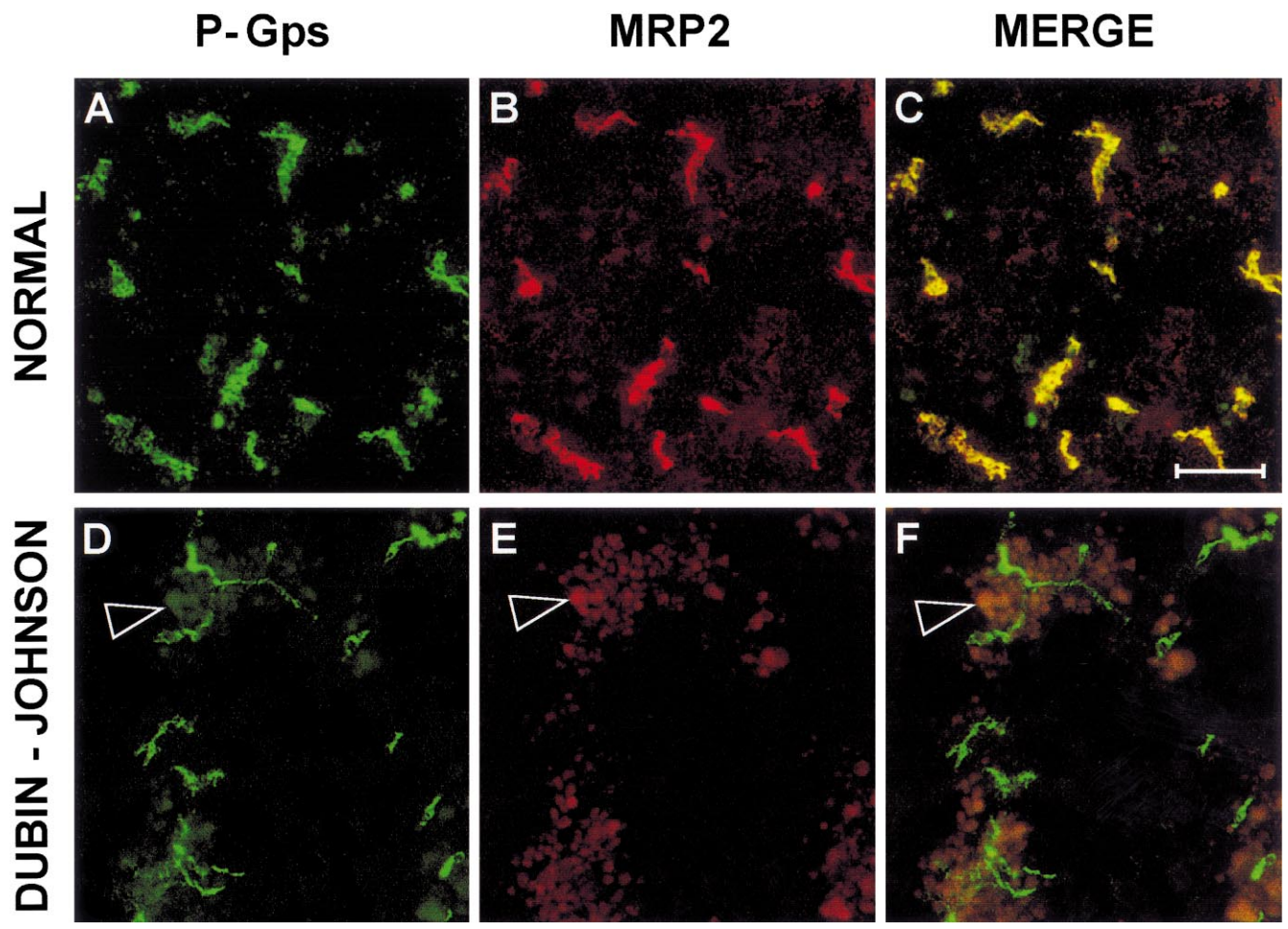


Fig. 5. Expression of MRP2 and P-glycoprotein (P-Gps) in (A–C) normal and (D–F) Dubin–Johnson syndrome liver. In normal liver, P-glycoprotein (A; green) and MRP2 (B; red) colocalize to the canalicular membrane domain, resulting in a yellow color (C). In patient H2 with Dubin–Johnson syndrome [53], immunofluorescence microscopy reveals the complete absence of MRP2 from the hepatocyte canalicular membrane as well as other membrane domains (E,F). Expression of P-glycoproteins, however, is not affected in the patient (D,F). Open arrowheads indicate the autofluorescence of pigment granules typically observed in Dubin–Johnson syndrome liver. These pigment granules do not contain MRP2 protein (scale bar in C: 10 μm; same original magnification in all panels). Reproduced with permission from Tsujii et al. [53].

shown to be a good substrate for MRP2 [16] and MRP1 [18]. Substrate properties of Fluo-3 were demonstrated by its ATP-dependent transport into inside-out membrane vesicles from normal, as opposed to MRP2-deficient membrane vesicles [16]. These results indicate that MRP2 is the predominant export pump which mediates the biliary excretion of Fluo-3. Polarized rat hepatoma cells form apical vacuoles between adjacent cells [125,126] and express MRP2 in the apical membrane [16]. Secretion of Fluo-3 [16] and other fluorescent substrates into the apical vacuoles can be readily observed by fluorescence microscopy (Fig. 3). Fluo-3 transport into apical vacuoles via MRP2 was inhibited by cyclosporin A, but not by the same concentration of the non-immunosuppressive cyclosporin A analog PSC833 or by the selective MDR1 P-glycoprotein inhibitor LY335979 (Fig. 3 [16]). Taken together, fluorescent anions and fluorescent glutathione *S*-conjugates may serve as useful substrates to study the function of apical MRP2 in intact polarized cells.

6. Hereditary deficiency of MRP2 in Dubin–Johnson syndrome

Mutations in the *MRP2* gene, some of which are associated with the absence of the MRP2 protein from the hepatocyte canalicular membrane (Figs. 4 and 5), have been discovered in humans [53,96,132–134] and rats [28,30]. The Dubin–Johnson syndrome in humans is an autosomal recessively inherited disorder characterized by conjugated hyperbilirubinemia and pigment deposition in the liver [135–137]. The deficient transport of monoglucuronosyl and bisglucuronosyl bilirubin as well as other anionic conjugates from hepatocytes into bile is caused by the absence of the MRP2 protein from the canalicular membrane [52,53,96,99]. So far, mutations in the *MRP2* gene leading to a functionally deficient protein inserted into the hepatocyte canalicular membrane have not been identified. Moreover, we have neither detected truncated MRP2 protein in the hepatocytes from a Dubin–Johnson syndrome patient with a stop-codon in exon 23 nor in a patient with a 6-nucleotide deletion in exon 30 [53]. Current knowledge on the sites of mutations in the coding sequence as well as in splice sites of the *MRP2* gene in Dubin–Johnson syndrome together with the exon organization of the human *MRP2* gene are depicted in Fig. 4. Determination of the exon–intron organization of the gene has been a prerequisite for the elucidation of mutations underlying Dubin–Johnson syndrome [53,133]. The human *MRP2* gene has been localized to chromosome 10q23–q24 [50], it contains 32 exons [53,133] and a high proportion of class 0 introns [53]. The comparison with the exon–intron organization of the human *MRP1* gene [51] displays some remarkable features. Both gene structures exhibit pronounced similarities as indicated by the number and size of the exons, by the high proportion of class 0 introns, and by 21 identical splice junction sites on the amino acid level [53]. Despite the fact that human MRP1 and MRP2 share only 48% amino acid sequence identity (Table 1), a close relationship of both transporters is indicated by their similar substrate specificity [8,13,40] and by their very similar genomic organization [53].

The mutations detected until now in the *MRP2* gene which are associated with Dubin–Johnson syndrome are scattered preferentially over the 3′-proximal half of the mRNA including the exons encoding both nucleotide-binding domains (Fig. 4). Established mutations include a nonsense mutation leading to a premature termination codon [53,96], a missense mutation affecting the first nucleotide-binding domain [132,133], a deletion mutation leading to the loss of two amino acids in the second nucleotide-binding domain [53], and splice junction mutations leading to exon deletions and premature termination codons [132–134]. Furthermore, mutations were identified in two well-characterized hyperbilirubinemic rat strains, which have been considered as animal models of the human Dubin–Johnson syndrome, the GY/TR$^-$ mutant rat [28] and the Eisai hyperbilirubinemic rat (EHBR) [30]. These mutations introduce premature termination codons at codon 401 and 855 in GY/TR$^-$ and EHBR mutant rats, respectively. In both mutant livers, however, no truncated proteins were detected [29] and the *MRP2* mRNA was below detectability as analyzed by Northern blotting [28–30]. The introduction of premature termination codons leads to a decrease in the level of some mRNAs by a mechanism termed 'nonsense mediated decay' [138]. In the case of a stop-codon 5′ of the last splice site, this is recognized during translation and the mRNA is subjected to decay [138]. It is likely that the absence of the MRP2 protein from the hepatocyte canalicular membrane, in some cases of Dubin–Johnson syndrome [52,53,99], is also a consequence of the rapid degradation of the mutated mRNA. Other mutations in the *MRP2* gene may lead to a reduced stability of the protein, may affect trafficking and apical localization of the protein, or may lead to a functionally deficient, but correctly localized, MRP2 protein.

7. Conclusions

Several members of the increasingly large MRP family mediate the primary-active ATP-dependent unidirectional transport of amphiphilic anions. Prototypic high-affinity substrates include the glutathione *S*-conjugate leukotriene C_4 and the glucuronosides bisglucuronosyl bilirubin and 17β-glucuronosyl estradiol. The human isoforms MRP1, MRP2, and MRP3 differ by their kinetic properties, by their domain-specific sorting and localization to

the plasma membrane, and by the regulation of their expression. Mutations in the human *MRP2* gene encoding the apical isoform have been identified which cause the absence of the MRP2 protein from the hepatocyte canalicular membrane and the conjugated hyperbilirubinemia of Dubin–Johnson syndrome. Expression of recombinant MRP2 at high levels has provided direct evidence that the apical isoform confers resistance to several chemotherapeutic agents including cisplatin. Elucidation of the function and localization of different MRP isoforms has contributed to our understanding of the role of these conjugate export pumps in terminal excretion of xenobiotic and endogenous substances, in detoxification, in chemoprevention, and in drug resistance.

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