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Role of two adjacent cytoplasmic tyrosine residues in MRP1 (ABCC1) transport activity and sensitivity to sulfonylureas

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Abstract

The human ATP-binding cassette (ABC) protein MRP1 causes resistance to many anticancer drugs and is also a primary active transporter of conjugated metabolites and endogenous organic anions, including leukotriene C_4 (LTC_4) and glutathione (GSH). The sulfonylurea receptors SUR1 and SUR2 are related ABC proteins with the same domain structure as MRP1, but serve as regulators of the K^+ channel Kir6.2. Despite their functional differences, the activity of both SUR1/2 and MRP1 can be blocked by glibenclamide, a sulfonylurea used to treat diabetes. Residues in the cytoplasmic loop connecting transmembrane helices 15 and 16 of the SUR proteins have been implicated as molecular determinants of their sensitivity to glibenclamide and other sulfonylureas. We have now investigated the effect of mutating Tyr^{1189} and Tyr^{1190} in the comparable region of MRP1 on its transport activity and sulfonylurea sensitivity. Ala and Ser substitutions of Tyr^{1189} and Tyr^{1190} caused a $\geq$50% decrease in the ability of MRP1 to transport different organic anions, and a decrease in LTC_4 photolabeling. Kinetic analyses showed the decrease in GSH transport was attributable primarily to a 10-fold increase in K_m. In contrast, mutations of these Tyr residues had no major effect on the catalytic activity of MRP1. Furthermore, the mutant proteins showed no substantial differences in their sensitivity to glibenclamide and tolbutamide. We conclude that MRP1 Tyr^{1189} and Tyr^{1190}, unlike the corresponding residues in SUR1, are not involved in its differential sensitivity to sulfonylureas, but nevertheless, may be involved in the transport activity of MRP1, especially with respect to GSH.

Keywords: MRP1; Organic anion transport; Glutathione transport; Sulfonylurea; ATP-binding cassette proteins; Site-directed mutagenesis

1. Introduction

During chemotherapy, some tumors exhibit cross-resistance to a broad range of amphiphilic but structurally unrelated drugs. This phenomenon is known as 'multidrug resistance' and is a serious impediment to the effective treatment of malignant disease. Multidrug resistance in cultured tumor cells has been associated with the increased expression of several energy dependent transporter proteins including P-glycoprotein (ABCB1), MRP1 (ABCC1) and BCRP (ABCG2) [1–5]. These three drug transporters all belong to the superfamily of ATP-binding cassette (ABC) proteins that is present in all species from bacteria to man. In humans, 49 members of the ABC superfamily have been reported and classified into one of 7 subfamilies (ABCA to ABCG), according to the HGNC Gene Family Nomenclature (http://www.nutrigene.4t.com/humanabc.htm). Most mammalian ABC proteins are capable of translocating a variety of molecules across membranes and some are involved in physiological processes that can lead to serious disorders when the cognate gene is mutated or underexpressed [4–10].

In addition to MRP1, the human ABC 'C' subfamily contains 11 other proteins: the MRP-related drug and/or organic anion transporters (ABCC2-6/MRP2-6 and ABCC10-12/MRP7-9) [5], the cystic fibrosis transmembrane conductance regulator (CFTR/ABCC7) chloride channel [6], and the sulfonylurea receptors, SUR1 (ABCC8) and SUR2A/B (ABCC9) [10,11]. Mutations of SUR1 are associated with the genetic disorder known as persistent hyperinsulinemic hypoglycemia of infancy

Abbreviations: ABC, ATP-binding cassette; MRP, multidrug resistance protein; BCRP, breast cancer resistance protein; LTC_4, leukotriene C4; $E_2$17βG, 17β-estradiol-17-β-(D-glucuronide); NBD, nucleotide binding domain; MSD, membrane spanning domain; TM, transmembrane; CL, cytoplasmic loop; SUR, sulfonylurea receptor; MTX, methotrexate; HEK, human embryonic kidney; $E_1$3SO_4, estrone 3-sulfate; WT, wild-type

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[10,11]. Like most other mammalian ABC transporters, the 'C' subfamily proteins have a core structure composed of two membrane spanning domains (MSDs) each containing six transmembrane (TM) helices, alternating with two nucleotide binding domains (NBDs) responsible for ATP binding and hydrolysis. A subset of ABC 'C' family members, including MRP1, SUR1 and SUR2A/B, contain an additional NH_2-terminal MSD (MSD1) composed of just five TMs. Thus, these latter ABC 'C' proteins have 17 TM helices and are arranged in a MSD1-MSD2-NBD1-MSD3-NBD2 fashion [5].

In addition to anticancer drugs, MRP1 (ABCC1) transports a wide variety of organic anions, many of which are conjugated to GSH, glucuronate or sulfate [5,12–17]. The most well characterized organic anion substrate of MRP1 is the cysteinyl leukotriene C_4 (LTC_4); other substrates include GSH and GSSG, folic acid, estrone 3-sulfate (E_13SO_4) and 17β-estradiol 17-(β-D-glucuronide) ($E_217\beta G$). SUR1 (ABCC8) and SUR2A/B (ABCC9) (which are 33–34% homologous to MRP1) do not transport organic anions but rather, they function as regulators of the K^+ channel Kir6.2 [11,18–20]. Thus, four subunits of SUR1 are assembled with four subunits of Kir6.2, and the resulting pore-forming octameric complex is known as the pancreatic β-cell ATP sensitive potassium (K_{ATP}) channel. When Kir6.2 is assembled with SUR2A and SUR2B, the complexes are known as the cardiac and smooth muscle K_{ATP} channels, respectively.

The SUR proteins contain binding sites for blockers (such as the sulfonylureas) and openers that can regulate K^+ channel activity, and the relative sensitivity of the various K_{ATP} channels to these agents is determined by the SUR homolog that is present in the complex [18,20–23]. Thus, sulfonylurea blockers such as glibenclamide and tolbutamide (Fig. 1) that are widely used in the treatment of type 2 diabetes bind to SUR1 with high affinity and stimulate insulin secretion from pancreatic β-cells [10]. Glibenclamide also binds to the highly homologous (88%) SUR2A but to a lesser extent than to SUR1. Similarly, Kir6.2-SUR1 but not Kir6.2-SUR2A channels are blocked by tolbutamide with high affinity [22]. Recently, it has been reported that MRP1 drug efflux activity is also inhibited by glibenclamide but tolbutamide had no effect [24].

Whether or not MRP1 and SUR1 share a common sulfonylurea binding site is presently unknown. Previous studies have indicated that the SUR1 glibenclamide binding site is bipartite, with an NH_2-terminal component within the cytoplasmic loop CL3 that connects MSD1 to MSD2, and a COOH-terminal component in CL7 that connects TM15 to TM16 in MSD3 [25,26]. In addition, the tolbutamide binding site of SUR1 is reported to involve a Ser^{1237} in CL7, based on the observation that tolbutamide no longer blocks SUR1 when this residue is replaced with Tyr as is present in the less sensitive SUR2A/B (Tyr^{1206}) [22]. Binding of [^{3}H]glibenclamide to membranes expressing SUR1-S1237Y is abolished concomitantly with the loss of high-affinity tolbutamide block. Conversely, mutation of SUR2B-Tyr^{1206} to Ser (as it is in SUR1) increases the affinity of SUR2B for glibenclamide seven-fold; however, the effect on tolbutamide sensitivity was not reported [23]. Sequence alignments indicate that CL7 of MRP1 contains two adjacent Tyr residues (Tyr^{1189} and Tyr^{1190}) located at a position that aligns closely with SUR1-Ser^{1237} and SUR2A/B-Tyr^{1206} (Fig. 2). In the present study, we

	^{1181}															^{1198}
Hum/MRP1	K	V	D	E	N	Q	K	A	**Y**	**Y**	P	S	I	V	A	N	R	W
Hum/SUR1	Y	T	D	S	N	N	I	A	**S**	**L**	F	L	T	A	A	N	R	W
Hum/SUR2	L	T	D	T	N	N	I	A	**Y**	**L**	F	L	S	A	A	N	R	W
Hum/MRP2	R	I	D	T	N	Q	K	C	**V**	**F**	S	W	I	T	S	N	R	W
Hum/MRP3	K	V	D	A	N	Q	R	S	**C**	**Y**	P	Y	I	I	S	N	R	W
Hum/MRP4	H	Q	D	L	H	S	E	A	**W**	**F**	L	F	L	T	T	S	R	W
Hum/MRP5	L	L	D	D	N	Q	A	P	**F**	**F**	L	F	T	C	A	M	R	W
Hum/MRP6	R	V	D	E	S	Q	R	I	**S**	**F**	P	R	L	V	A	D	R	W
Hum/CFTR	A	L	N	L	H	T	A	N	**W**	**F**	L	Y	L	S	T	L	R	W
can/Mrp1	K	V	D	E	N	Q	K	A	**Y**	**Y**	P	S	I	V	A	N	R	W
mus/Mrp1	K	V	D	E	N	Q	K	A	**Y**	**Y**	P	S	I	V	A	N	R	W
rat/Mrp1	K	V	D	E	N	Q	K	A	**Y**	**Y**	P	S	I	V	A	N	R	W
Mon/MRP1	K	V	D	E	N	Q	K	A	**Y**	**Y**	P	S	I	V	A	N	R	W
Tau/Mrp1	K	V	D	E	N	Q	K	A	**Y**	**Y**	P	S	I	V	A	N	R	W

Fig. 2. Sequence alignments of human MRP1 and related ABC 'C' subfamily proteins in the region of CL7 containing Tyr^{1189} and Tyr^{1190}. The alignments show the relative conservation of MRP1 Tyr^{1189} and Tyr^{1190} and the corresponding residues (in boldface) in other ABC subfamily 'C' member proteins. Hum, human; can, canine; mus, mouse; Mon, monkey; Tau, bovine; SUR, sulfonylurea receptor; CFTR, cystic fibrosis transmembrane conductance regulator.

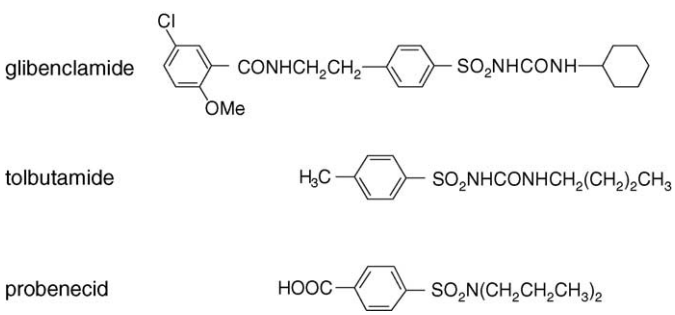

Fig. 1. Chemical structures of glibenclamide, tolbutamide, and probenecid.

have investigated the consequences of mutating Tyr^{1189} and Tyr^{1190} of MRP1 to determine whether these residues are involved in the transport function of MRP1 and its sensitivity to glibenclamide.

2. 2. Materials and methods

2.1. Materials

[14,15,19,20-^{3}H]LTC_4 (115.3 Ci mmol^{-1}) was purchased from Amersham Pharmacia Biotech. [6,7–^{3}H]$E_2$17βG (55 Ci mmol^{-1}), [6,7(*n*)-^{3}H]$E_1$3SO_4 (53 Ci mmol^{-1}), and [glycine-2-^{3}H]GSH (40–44.8 Ci mmol^{-1}) were from Perkin Elmer Life Sciences. [3′,5′,7,-^{3}H(*n*)]Methotrexate sodium salt (17 Ci mmol^{-1}) was from Moravek Inc. 8-Azido-α-[^{32}P]ATP (16.2 Ci mmol^{-1}) was from Affinity Labeling Technologies Inc. LTC_4 was purchased from Calbiochem and nucleotides, GSH, apigenin, acivicin, $E_2$17βG, $E_1$3SO_4, and 2-mercaptoethanol were purchased from Sigma Chemical Co.

2.2. Sequence alignments

Fourteen sequences homologous (MRP2-6, SUR1, SUR2, CFTR) or orthologous (dog, mouse, rat, monkey, bovine Mrp1) to a stretch of 18 amino acids in CL7 of human MRP1 that contains Tyr^{1189} and Tyr^{1190} were aligned using ClustalW (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html).

2.3. Vector construction and site-directed mutagenesis

Tyr mutations in MRP1 were generated using the QuikChange™ site-directed mutagenesis kit (Stratagene). The template for mutagenesis was prepared by cloning the 2-kb *XmaI* fragment (containing nucleotides 2337–4322 of the MRP1 sequence encoding amino acids 780–1440) from pcDNA3.1(−)-$MRP1_k$ into pGEM-3Z (Promega) [27]. Tyr substitutions were generated in the pGEM-3Z plasmids according to the manufacturer's instructions with the following mutagenic primers (substituted nucleotides are in bold) obtained from Integrated DNA Technologies: Y1189A (5′-GAT GCT GGG GAT **CGC** GGC CTT CTG GTT CTC-3′), Y1189S (5′-GAT GCT GGG GTA A**CT** GGC CTT CTG G-3′), Y1190A (5′-C CAC GAT GCT **C**GG G**GC** ATA GGC CTT C-3′), Y1190S (5′-C GAT GCT GGG **ACT** ATA GGC CTT C-3′). After confirming all mutations by diagnostic restriction enzyme digests, a 0.5-kb BstEII fragment containing the desired mutation was subcloned back into pcDNA-3.1(−)-$MRP1_k$ and the fragments in the full length constructs were sequenced to confirm the presence of the mutation.

2.4. Transfection of MRP1 expression vectors in human embryonic kidney cells

Constructs containing wild-type and Tyr^{1189} and Tyr^{1190} mutant pcDNA3.1(−)-MRP1 expression vectors were transfected into SV40-transformed human embryonic kidney cells (HEK293T) [27]. Briefly, ∼10×10^6 cells were seeded in 150-mm plates, and transfected 24 h later (at 50–80% confluency) with plasmid DNA (16 μg) using FuGENE™6 (Roche Diagnostics) according to the manufacturer's instructions. After 72 h, the HEK293T cells were harvested, and membrane vesicles were prepared as described previously [15,27]. Empty vector and vector containing the wild-type cDNAs were included as controls in all experiments. Levels of wild-type and Tyr mutant MRP1 proteins were determined by immunoblot or dot blot analysis as described below.

2.5. Measurements of MRP1 protein levels in transfected cells

The expression levels of wild-type and Tyr mutant MRP1 proteins were determined by immunoblot or dot blot analysis of membrane protein fractions from transfected cells essentially as described [28]. For immunoblots, proteins were resolved on a 7% polyacrylamide gel and electrotransferred to an Immobilon™-P membrane (Millipore). For dot blots, samples were loaded in a 96-well vacuum manifold and slowly drained by gravity. Blots were blocked with 4% (w/v) skim milk powder in TBS-T for 1 h followed by incubation with the human MRP1-specific murine monoclonal antibody QCRL-1 (diluted 1:5,000 to 1:10,000), which recognizes a linear epitope consisting of amino acids 918–924 [29], for 2 h at room temperature or overnight at 4 °C. After washing, the immunoreaction was visualized using a horseradish peroxidase-conjugated goat anti-mouse antibody (Pierce) (1:10,000) followed by application of Renaissance® chemiluminescence blotting substrate (NEN™ Life Sciences Products). Relative levels of MRP1 expression were estimated by densitometric analysis using a ChemiImager™ 4000 (Alpha Innotech). To ascertain relative equal loading of protein in the wells, blots were stained with amido black.

2.6. MRP1-mediated transport of ^{3}H-labeled substrates by membrane vesicles

Membrane vesicles from transiently transfected HEK293T cells were prepared as described previously [15,27], and ATP-dependent uptake of ^{3}H-labeled substrates by the membrane vesicles was measured using a rapid filtration technique as before [15]. Briefly, LTC_4 transport assays were performed at 23 °C in a 50 μl reaction containing 50 nM [^{3}H]LTC_4 (40 nCi per reaction), 4 mM AMP or ATP, 10 mM $MgCl_2$, 10 mM creatine phosphate, 100 μg ml^{-1} creatine kinase, and 1.8 μg of

vesicle protein in transport buffer (50 mM Tris–HCl, 250 mM sucrose, pH 7.4). Uptake was stopped at selected times by rapid dilution in ice-cold transport buffer, and then the reaction was filtered through glass fiber (Type A/E) filters presoaked in transport buffer. Radioactivity was quantitated by liquid scintillation counting. All data were corrected for the amount of [^{3}H]LTC_4 that remained bound to the filter, which was usually <10% of the total radioactivity. Transport in the presence of AMP was subtracted from transport in the presence of ATP to determine ATP-dependent LTC_4 uptake. All transport assays were carried out in triplicate, and results were expressed as means (±S.D.).

Uptake of [^{3}H]$E_2$17βG was measured in a similar fashion except that membrane vesicles (1.8 μg of protein) were incubated at 37 °C in a total reaction volume of 50 μl containing 400 nM [^{3}H]$E_2$17βG (40 nCi per reaction) and the components as described for [^{3}H]LTC_4. [^{3}H]E_13SO_4 uptake was performed at 37 °C in a 50 μl total reaction volume containing membrane vesicles (1.8 μg of protein), 300 nM [^{3}H]E_13SO_4 (100 nCi), in the presence of 3 mM GSH (or *S*-MeGSH or *S*-EtGSH) and 10 mM DTT and the same components described above [17,30,31]. Apigenin-stimulated [^{3}H]GSH uptake was also measured by rapid filtration with membrane vesicles (20 μg of protein) incubated at 37 °C for 20 min in a 60 μl reaction volume with 100 μM [^{3}H]GSH (120 nCi per reaction) and in the presence of apigenin (30 μM). To minimize GSH catabolism by γ-glutamyltranspeptidase during transport, membranes were preincubated with 0.5 mM acivicin for 10 min at 37 °C prior to measuring [^{3}H]GSH uptake [16,32]. [^{3}H]MTX uptake was also performed as described previously [27]. Assays were carried out at 37 °C for 20 min in a 60 μl reaction volume containing membrane vesicles (10 μg of protein), 50 μM [^{3}H]MTX (200 nCi per reaction), and other components as above.

[^{3}H]$E_2$17βG uptake was also measured in the presence of the sulfonylureas tolbutamide (100 μM) and glibenclamide (10 μM and 100 μM) as well as probenecid (100 μM). Sulfonylureas and probenecid were dissolved in DMSO (final concentration <1%). Final concentrations of tolbutamide >100 μM could not be achieved under these conditions due to solubility limitation. Uptake in the presence of vehicle alone showed no effect on the efficiency compared to uptake in the absence of vehicle and was set as the 100% reference value.

2.7. Photolabeling of MRP1 by 8-azido-α-[^{32}P]ATP

Membrane vesicles (20 μg protein) were dispersed in 20 μl of transport buffer containing 5 mM $MgCl_2$ and 5 μM 8-azido-α-[^{32}P]ATP. After a 5 min incubation on ice, the samples were exposed in an open flexible 96-well plate to UV light at 302 nm on ice for 8 min at a distance of 8 cm. The reactions were stopped by the addition of 0.5 ml of ice-cold Tris–EGTA buffer (50 mM Tris–HCl, pH 7.4, 0.1 mM EGTA, 5 mM $MgCl_2$), and the membranes were centrifuged at 15,000 rpm for 15 min at 4 °C. The pellets were washed again and resuspended in 20 μl of the same buffer. Membrane proteins were then solubilized in Laemmli buffer and subjected to SDS-PAGE and the dried gel exposed to Kodak X-Omat film [33–35].

2.8. Orthovanadate-induced trapping of 8-azido-α-[^{32}P]ADP by MRP1

Membrane proteins (20 μg) were incubated in transport buffer (20 μl) containing 5 mM $MgCl_2$, 1 mM sodium orthovanadate, and 5 μM 8-azido-α-[^{32}P]ATP at 37 °C for 15 min. The reactions were stopped by the addition of 0.5 ml of ice-cold Tris–EGTA buffer (50 mM Tris–HCl, pH 7.4, 0.1 mM EGTA, 5 mM $MgCl_2$), and the membranes were centrifuged at 15,000 rpm for 15 min at 4 °C. The pellets were washed again and resuspended in 20 μl of the same buffer. The samples were transferred to a 96-well plate, exposed to UV light at 302 nm on ice for 8 min at a distance of 8 cm, solubilized in Laemmli buffer and subjected to SDS-PAGE and the dried gel was exposed to Kodak X-Omat film [33–35].

2.9. Photolabeling of MRP1 by [^{3}H]LTC_4

Wild-type and Tyr mutant MRP1 membrane proteins were photolabeled with [^{3}H]LTC_4 essentially as described [15,34]. Briefly, vesicles prepared from HEK293T cells transfected with wild-type and Tyr mutant MRP1 cDNAs (40 μg of protein in 50 μl) were incubated in transport buffer with [^{3}H]LTC_4 (200 nM; 0.12 μCi) and 10 mM $MgCl_2$ at room temperature for 30 min and then frozen in liquid nitrogen. Samples were then alternately irradiated at 302 nm for 1 min using a CL-1000 ultraviolet cross-linker (DiaMed) and snap-frozen in liquid N_2 10 times. Radiolabeled proteins (40 μg) were resolved by SDS-PAGE. The gel was fixed (isopropyl alcohol/water/acetic acid; 25:65:10) and then soaked in Amplify® (Amersham Biosciences Inc.) for 30 min. After drying for 2 h, the gel was exposed to Kodak X-Omat film for 1 week at −70 °C. Relative levels of photolabeling were estimated by densitometric analysis as before.

2.10. Kinetic analysis of [^{3}H]GSH transport

K_m and V_{max} values of ATP-dependent GSH transport by membrane vesicles (20 μg protein) were determined by measuring uptake at eight different [^{3}H]GSH concentrations (10–2500 μM for wild-type MRP1 and 50–10,000 μM for Y1189S mutant MRP1) for 20 min at 37 °C in 60 μl of transport buffer containing components as described above [16]. Data were analyzed using Graph Pad Prism™ software and kinetic parameters determined by nonlinear regression and Michaelis–Menten analyses.

3. Results

3.1. Expression levels and organic anion uptake by Tyr^{1189} and Tyr^{1190} mutant MRP1 proteins

Replacement of Tyr^{1189} and Tyr^{1190} with Ala and Ser was done by site-directed mutagenesis and then HEK293T cells were transfected to express the mutant and wild-type MRP1 proteins. Membrane vesicles were prepared, immunoblotted, and densitometric analysis showed that all mutants were expressed at levels comparable to those of wild-type MRP1 (60–110%) (Fig. 3A). This indicates that MRP1 biosynthesis or stability was not substantially affected by the replacement of Tyr^{1189} or Tyr^{1190}.

The ATP-dependent uptake of organic anions by the Tyr mutant-enriched vesicles was then examined. As shown in Fig. 3B, the ability of the mutants to support LTC_4 uptake after 3 min was reduced by approximately 55 and 25% for Y1189A and Y1189S, respectively, and by approximately 40 and 50% for Y1190A and Y1190S, compared to wild-type MRP1. The ability of the Tyr mutants to transport $E_2$17βG, $E_1$3SO_4, MTX and GSH was also tested and the results are summarized together with the LTC_4 transport data in Table 1. $E_2$17βG uptake by the Y1189A and Y1189S mutants was reduced by 30%, and by 55–65% for the Y1190A and Y1190S mutants. $E_1$3SO_4 uptake was reduced by 35% in the case of Y1189S and by 60–65% for the Y1189A, Y1190A and Y1190S mutants. MTX uptake by the four Tyr mutants was reduced by 65–75% while GSH uptake was reduced further still. Thus, the four Tyr^{1189} and Tyr^{1190} mutants showed levels of GSH transport that were just 10–30% those of wild-type MRP1. More conservatively substituted Y1189F and Y1189W mutant proteins were generated and these also exhibited levels of transport activity that were reduced by a similar extent (data not shown).

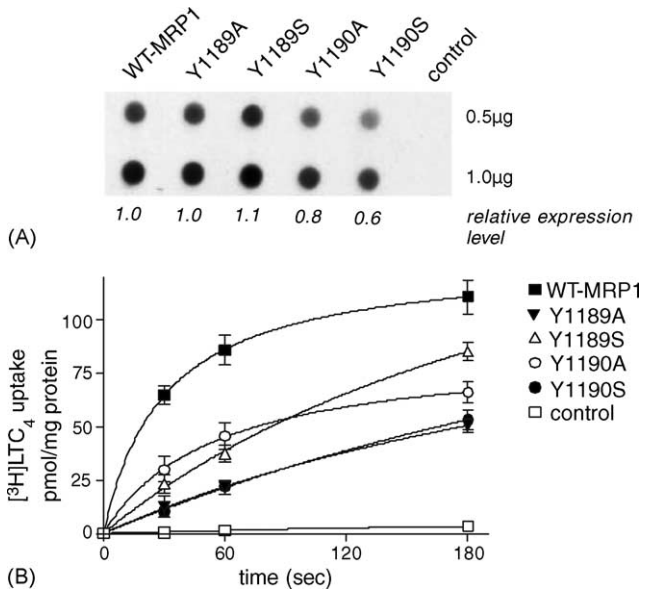

Fig. 3. MRP1 protein expression levels and [^{3}H]LTC_4 uptake by wild-type, Tyr^{1189} and Tyr^{1190} mutants of MRP1. (A) Immunodot blot of membrane vesicles (0.5 and 1 μg protein) prepared from HEK293T cells transfected with wild-type (WT-MRP1) and mutant (Y1189A, Y1189S, Y1190A, Y1190S) MRP1 cDNAs. Cells transfected with empty vector were used as a negative control (control). MRP1 proteins were detected with monoclonal antibody QCRL-1, and the average relative expression levels shown under the blot were estimated by densitometry. (B) Time course of ATP-dependent [^{3}H]LTC_4 uptake was measured in membrane vesicles prepared from HEK293T cells transfected with wild-type MRP1 (WT-MRP1, ■) and Tyr → Ala/Ser mutant MRP1 (Y1189A, ▼; Y1189S, △; Y1190A, ○; Y1190S, ●) cDNAs. Vesicles prepared from cells transfected with empty vector were used as a negative control (□). Membrane vesicles (shown in A) were incubated at 23 °C with 50 nM [^{3}H]LTC_4 and ATP or AMP and reactions stopped at the times indicated. Uptake values were normalized based on mutant MRP1 protein levels relative to wild-type MRP1 protein levels. The results shown are the means (±S.D.) of triplicate determinations in a single experiment; similar results were obtained in three additional experiments with independently prepared batches of vesicles from independent transfections.

Table 1
Summary of transport activities for Tyr^{1189} and Tyr^{1190} mutants of MRP1

Mutant	%Wild-type MRP1 uptake activity[a]				
	LTC_4[b]	$E_2$17βG	$E_1$3SO_4	MTX	GSH
Y1189A	45	70	40	35	10
Y1189S	75	70	65	35	30
Y1190A	60	45	40	35	20
Y1190S	50	35	35	25	25

[a] The values shown are means of triplicate determinations in a single experiment and are representative of results obtained in two to three independent experiments. Values have been corrected for different MRP1 expression levels according to the immunoblot shown Fig. 3A. For clarity, numbers have been rounded to the nearest 5% and S.D. values (which were typically 15% or less) were omitted.
[b] Data are from Fig. 3B.

3.2. Photolabeling of Tyr^{1189} and Tyr^{1190} mutant MRP1 proteins with 8-azido-α-[^{32}P]ATP and vanadate-induced 8-azido-α-[^{32}P]ADP trapping

Since the CL7 Tyr mutations caused a global decrease in transport activity, it was of interest to determine whether the mutations had altered the catalytic activity of MRP1. Consequently, experiments measuring azido-ATP photolabeling at 4 °C and vanadate-induced azido-ADP trapping at 37 °C were conducted using the photoactivatable radioactive nucleotide [α^{32}P]-8N_3 ATP (Fig. 4). The relative levels of labeling and trapping are indicated before and after correction for differences in relative MRP1 expression levels determined by immunoblotting (Fig. 4A). As shown in Fig. 4B, Y1189A, Y1189S, Y1190A and Y1190S exhibited a moderate (10–30%) decrease in azido-ATP labeling at 4 °C.

When the amount of azido-ADP trapped by vanadate under hydrolysis conditions at 37 °C was measured, the Ala-substituted mutants Y1189A and Y1190A exhibited a reduction of 30–40% compared to wild-type MRP1, whereas little or no (≤10%) decrease in trapping was observed for the Ser-substituted Y1189S and Y1190S mutants (Fig. 4C).

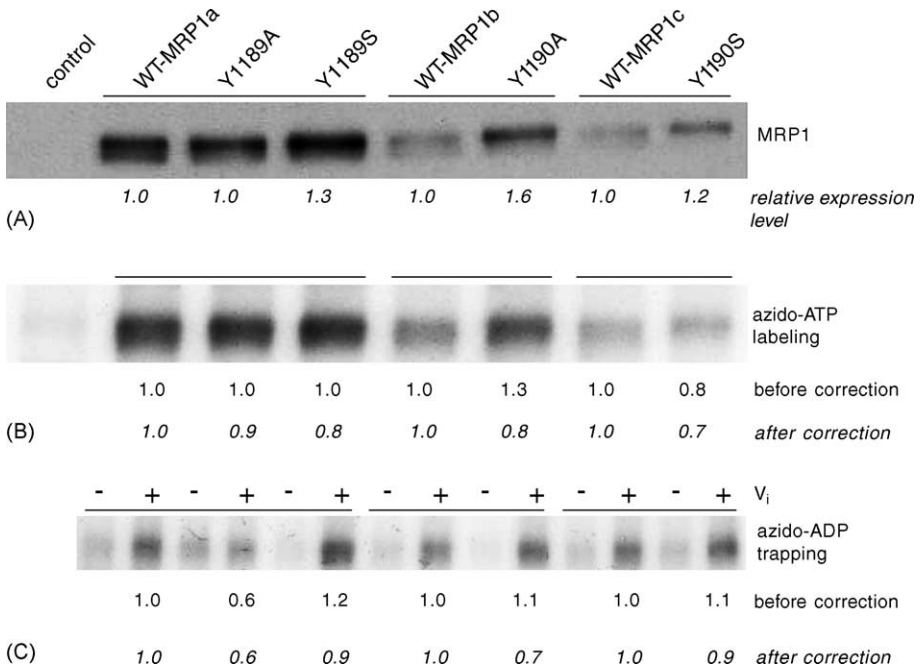

Fig. 4. Azido-ATP photolabeling and vanadate-induced azido-ADP trapping of wild-type, Tyr^{1189} and Tyr^{1190} mutants of MRP1. (A) Immunoblot of membrane vesicles (1 μg protein) prepared from HEK293T cells transfected with wild-type (WT-MRP1a, b, c), and mutant (Y1189A, Y1189S, Y1190A, Y1190S) MRP1 cDNAs as in Fig 3A. (B) Membrane vesicles (20 μg protein) were incubated with 5 μM [α-^{32}P]-8N_3-ATP at 4 °C for 5 min in transport buffer containing 5 mM $MgCl_2$. Samples were photo-crosslinked, resolved by SDS-PAGE and exposed to film. (C) Vanadate-induced trapping of nucleotide. Membrane vesicles (20 μg protein) were incubated at 37 °C with 5 μM [α-^{32}P]-8N_3-ATP in the absence (−) or presence (+) of 1 mM vanadate (Vi) for 15 min in transport buffer containing 5 mM $MgCl_2$. Samples were washed, photo-crosslinked, resolved by SDS-PAGE, and exposed to film for 1–24 h. In panels B and C, different preparations of wild-type MRP1 vesicles (a, b and c) with expression levels comparable to the Tyr mutants were used for comparison. Relative levels of MRP1 expression were estimated by densitometry and are indicated below the blot. Azido-ATP labeling and azido-ADP trapping have been quantified by densitometry and are expressed before and after correction for differences in MRP1 expression levels. Similar results were obtained in at least two additional independent experiments.

3.3. Photolabeling of Tyr^{1189} and Tyr^{1190} mutant MRP1 proteins with [^{3}H]LTC_4

To ascertain whether the decrease in LTC_4 uptake observed for the Tyr^{1189} and Tyr^{1190} mutants was associated with a decrease in substrate binding, membrane protein labeling experiments with the photoactivatable [^{3}H]LTC_4 were performed. The relative levels of labeling are indicated before and after correction for differences in relative MRP1 expression levels determined by immunoblotting (Fig. 4A). As shown in Fig. 5, all four Tyr mutants exhibited a significant decrease (40–60%) in [^{3}H]LTC_4 photolabeling compared to wild-type MRP1. These results correlate with the observed decrease in LTC_4 transport and indicate that binding of this substrate is diminished by both Ala and Ser substitutions of Tyr^{1189} and Tyr^{1190}.

3.4. Kinetic analysis of [^{3}H]GSH transport

To better understand the basis for the markedly reduced levels of GSH uptake by the Tyr mutants, the kinetic parameters of apigenin-stimulated GSH uptake by the Y1189S mutant were determined and compared to those of wild-type MRP1 (Fig. 6). These experiments showed

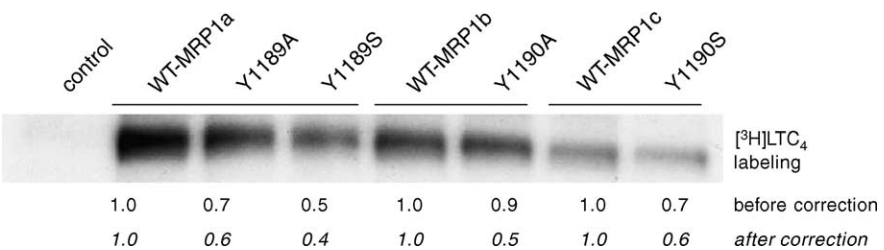

Fig. 5. [^{3}H]LTC_4 photolabeling of $Tyr^{1189}$and Tyr^{1190} mutant and wild-type MRP1 proteins. [^{3}H]LTC_4 photolabeling of membrane vesicle proteins prepared from HEK293T cells transfected with cDNAs encoding wild-type MRP1 (WT-MRP1) or Tyr → Ala/Ser MRP1 mutants (Y1189A, Y1189S, Y1190A, Y1190S). Cells transfected with empty vector were used as a control. Membrane vesicle proteins (40 μg protein) were incubated with [^{3}H]LTC_4 (200 nM, 0.12 μCi), irradiated at 302 nm, and then the radiolabeled proteins were resolved by SDS-PAGE and processed for fluorography and densitometry. Relative MRP1 protein expression levels were as shown in Fig. 4A. Different wild-type MRP1 vesicle preparations (a, b and c), with expression levels comparable to the expression levels of the Tyr mutants were used for comparison.

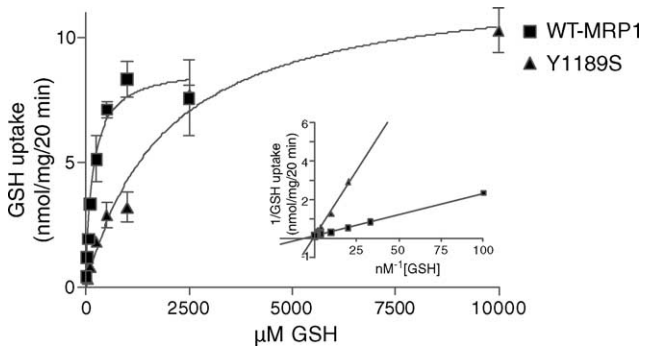

Fig. 6. Kinetic analysis of [^{3}H]GSH uptake by wild-type MRP1 and Tyr^{1189} → Ser mutant MRP1. Apigenin-stimulated GSH uptake by MRP1-enriched membrane vesicles was measured at different substrate concentrations (range 10–2500 μM for WT-MRP1 and 50–10,000 μM for Y1189S) in the presence of 30 μM apigenin. Eadie–Hofstee plots were generated and kinetic parameters determined. For WT-MRP1, the K_m and V_{max} values for GSH uptake were 162 μM and 8.9 nmol mg^{-1} 20 min^{-1}, respectively; for Y1189S, the K_m and V_{max} values were 1904 μM and 12.4 nmol mg^{-1} 20 min^{-1}, respectively. Data points are the means (±S.D.) of duplicate determinations in a typical experiment. Similar results were obtained in one additional independent experiment.

that the K_m (GSH) of Y1189S was increased 10-fold compared to that of wild-type MRP1 (1904 ± 420 μM versus 162 ± 29 μM). In addition, the V_{max} of GSH transport was also moderately increased (8.9 ± 0.4 versus 12.4 ± 1.0 nmol mg^{-1} protein after 20 min, respectively, for wild-type MRP1 and Y1189S). Thus, the overall transport efficiency of this substrate was decreased >8-fold by the Tyr^{1189} → Ser mutation ($V_{max}/K_m \times 10^3$ = 54.9 versus 6.5 for wild-type MRP1 and the Y1189S mutant, respectively). These results indicate that the mutation Y1189S altered mainly the uptake affinity of MRP1 for GSH and to a lesser extent the transport capacity of MRP1 for this tripeptide.

3.5. Modulation of [^{3}H]$E_1$3SO_4 uptake by GSH derivatives

Because of the substantially reduced GSH transport activity of the Tyr mutants, we next examined the effect of the mutations on the relative ability of GSH and its

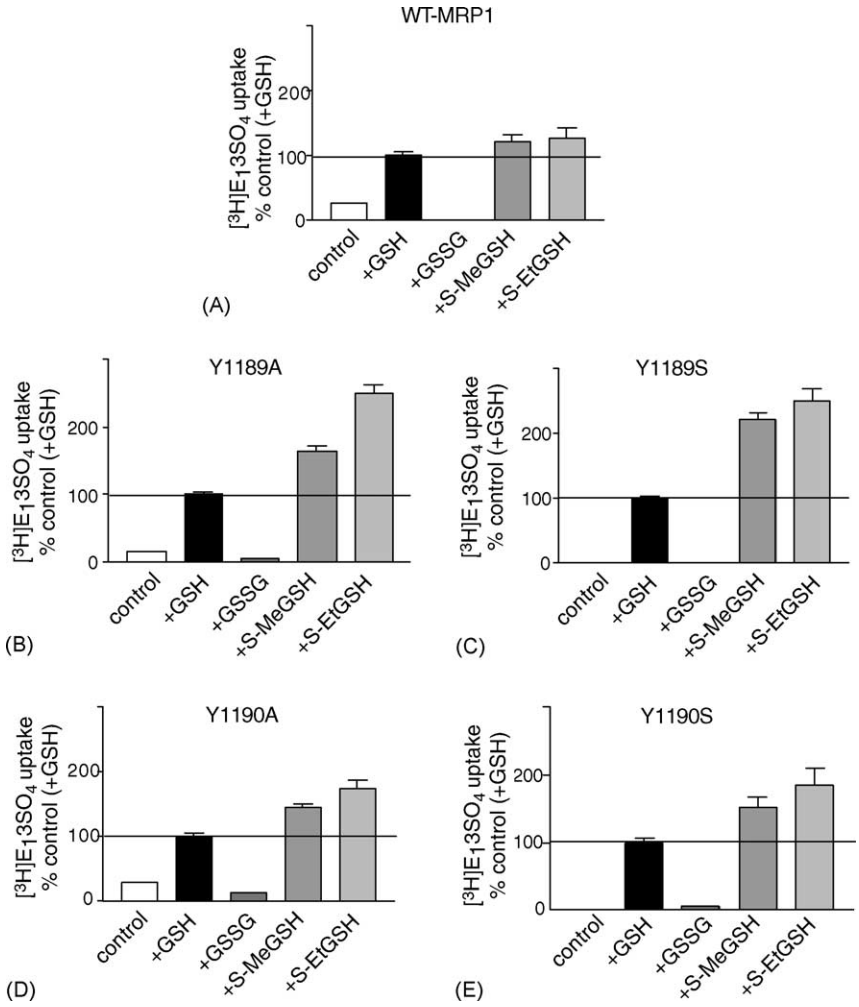

Fig. 7. Modulation of MRP1-mediated [^{3}H]$E_1$3SO_4 uptake by GSH analogs. Membrane vesicles (2.5 μg protein) prepared from transfected HEK293T cells expressing wild-type MRP1 (WT-MRP1) (A), Tyr^{1189} mutants Y1189A (B), and Y1189S (C), and Tyr^{1190} mutants Y1190A (D) and Y1190S (E) were incubated for 3 min at 37 °C with [^{3}H]$E_1$3SO_4 (300 nM, 100 nCi) and ATP or AMP. Uptake assays were carried out in the absence (control, *open bars*) or presence (*shaded bars*) of GSH, GSSG, *S*-methyl-GSH, or *S*-ethyl-GSH (all at 3 mM) as indicated. Results are expressed as a percentage of GSH-stimulated [^{3}H]$E_1$3SO_4 uptake.

Table 2
Effect of glibenclamide and tolbutamide on $E_2$17βG uptake by wild-type and Ser-substituted Tyr^{1189} and Tyr^{1190} mutant MRP1 proteins

Drug	Concentration (μM)	%Inhibition of $E_2$17βG uptake[a]		
		Wild-type	Y1189S	Y1190S
Glibenclamide	10	54 ± 7	36 ± 11	26 ± 16
Glibenclamide	100	67 ± 10	78 ± 10	88 ± 10
Tolbutamide	100	5 ± 7	0 ± 14	0 ± 7
Probenecid	100	61 ± 6	45 ± 9	20 ± 10

[a] The values shown are means of triplicate determinations in a single experiment and are representative of results obtained in two independent experiments. Values have been corrected for different MRP1 expression levels according to the immunoblot shown Fig. 3A.

derivatives GSSG, *S*-methyl-GSH and *S*-ethyl-GSH to stimulate E_13SO_4 uptake. As expected, GSSG (3 mM) had no stimulatory effect on E_13SO_4 uptake by either wild-type MRP1 or the Tyr^{1189} and Tyr^{1190} mutants. On the other hand, *S*-methyl-GSH and *S*-ethyl-GSH (also at 3 mM) stimulated E_13SO_4 uptake by wild-type MRP1 to the same extent or slightly more than the same concentration of GSH (Fig. 7). *S*-methyl-GSH and *S*-ethyl-GSH also stimulated transport by the Tyr mutants but to an even greater degree than was observed for wild-type MRP1. Thus, E_13SO_4 transport in the presence of *S*-methyl and *S*-ethyl-GSH was 1.5 to 2.5-fold higher than the GSH-stimulated uptake in the case of the Tyr mutants compared to only 1.2-fold for wild-type MRP1.

3.6. Effect of sulfonylurea drugs on [^{3}H]$E_2$17βG uptake

To determine whether the Tyr to Ser mutations affected the ability of sulfonylureas to modulate the vesicular organic anion transport activity of MRP1, $E_2$17βG uptake by the Y1189S and Y1190S mutants in the presence of glibenclamide and tolbutamide was examined. The general organic anion transport inhibitor probenecid was also included in these experiments as a positive control. As summarized in Table 2, glibenclamide inhibited $E_2$17βG uptake by Y1189S and Y1190S by 26–36% at a concentration of 10 μM and by 78–88% at 100 μM. In contrast, tolbutamide at 100 μM had no effect. $E_2$17βG transport by wild-type MRP1 was inhibited by 54–67% by 10 and 100 μM glibenclamide but not inhibited by tolbutamide. Probenecid (100 μM) inhibited $E_2$17βG transport by the Y1189S mutant by approximately 45%, the Y1190S mutant by 20%, and wild-type MRP1 by 61%. These results indicate that Ser substitution of Tyr^{1189} or Tyr^{1190} has no substantial effect on the sensitivity of MRP1 to sulfonylureas.

4. Discussion

In the present study, the consequences of mutating two adjacent Tyr residues located at positions 1189 and 1190 in CL7 of MRP1 were investigated. Our study was prompted by the previous demonstration that the amino acids in the analogous positions of SUR1 and SUR2 are critical for the different sensitivities of these K^+ channel regulatory subunits to tolbutamide and glibenclamide [22,23,25,26] and that the drug efflux activity of MRP1 was also differentially sensitive to these two sulfonylureas [24]. MRP1 and SUR1/SUR2 share 30% sequence identity overall which increases to 37% within CL7 (defined as amino acids 1141–1195 for MRP1) [36]. The residues Tyr^{1189} and Tyr^{1190} studied here (and the flanking eight amino acids on either side) are strictly conserved in MRP1 orthologs from a variety of different species but are only moderately conserved in other human MRP-related proteins (Fig. 2). With the exception of SUR1, however, at least one and often two aromatic amino acids are found in this position. Taken together, these observations suggested that Tyr^{1189} and/or Tyr^{1190} could be important for certain aspects of MRP1 function.

When Tyr^{1189} or Tyr^{1190} were individually replaced by either a neutral (Ala) or polar (Ser) residue, no major effects on MRP1 protein expression were observed. Routing of the transporter to the plasma membrane in the transfected cells was also unaffected (results not shown). On the other hand, vesicular membrane transport assays showed that these mutations caused a substantial reduction in the uptake levels of five different organic anion substrates of MRP1. The extent to which MRP1 transport activity was reduced was similar for both the Ala and Ser substituted mutants. This suggests that it is the loss of the aromatic polar Tyr residue itself that is responsible for the reduced activity rather than the introduction of either the Ser or Ala residue. Conservatively substituted mutants Y1189F and Y1189W also showed a comparable reduction in transport activity (not shown), suggesting that physical properties in addition to the aromaticity of Tyr^{1189} and Tyr^{1190} are important for their role in MRP1 transport activity.

In previous studies, we have observed that mutations of MRP1 that cause a global reduction in transport activity are sometimes associated with changes in the catalytic activity of the transporter [34,35,37]. However, none of the Tyr^{1189} or Tyr^{1190} mutants showed any substantial changes in azido-ATP binding at 4 °C. Changes in ATP hydrolysis at 37 °C as measured by vanadate-induced trapping of azido-ADP were observed but the decrease was relatively moderate (Fig. 4). On the other hand, the ability of all of the Tyr^{1189} and Tyr^{1190} mutants to be photolabeled with LTC_4 (Fig. 5) was markedly reduced, indicating that reduced substrate binding is responsible, at least in part, for the loss of transport activity observed.

Of the five organic anion substrates tested, apigenin-stimulated GSH transport was the most affected by the Tyr^{1189} and Tyr^{1190} substitutions. Indeed, the 10-fold difference in K_m (GSH) observed for the Y1189S mutant compared to the wild-type protein indicates a substantial

decrease in the uptake affinity of the mutant MRP1 for this tripeptide. In contrast, E_13SO_4 transport by the Tyr mutants appeared to be stimulated to a greater extent by *S*-methyl-GSH and *S*-ethyl-GSH than was wild-type MRP1 (Fig. 7). Taken together, these data are consistent with the idea that the Tyr^{1189} and Tyr^{1190} mutants have a diminished capacity to bind GSH but further studies are required to determine whether or not binding of the *S*-alkyl GSH derivatives is also affected. An additional possible explanation for these findings is that the Tyr mutations do not disrupt binding of either GSH or E_13SO_4 but rather, may disrupt the binding of apigenin which is required to reliably detect GSH transport activity by MRP1 in membrane vesicles [16]. However, this seems unlikely since apigenin inhibits $E_217\beta G$ transport by the Tyr mutants as effectively as transport by wild-type MRP1, indicating that the apigenin binding site of mutants is still intact (data not shown).

The starting point for this study was the observation that Ser^{1237} was important for the binding of glibenclamide and tolbutamide by SUR1 and when this residue was mutated to Tyr (as in SUR2A/B), the affinity of SUR1 for these sulfonylurea drugs was substantially diminished [22]. Conversely, mutation of Tyr^{1206} of SUR2B to Ser (as it is in SUR1) substantially increased the affinity of SUR2B for glibenclamide. Whether the converse is true (i.e., would tolbutamide sensitivity be conferred on SUR2A/B by mutation of Tyr^{1206} of SUR2A or SUR2B to Ser (as it is in SUR1)) is not known. However, when a portion of SUR2 containing CL7 is replaced with the analogous region of SUR1, the chimeric protein acquires a greater sensitivity to tolbutamide, indicating that at least some of the molecular determinants for tolbutamide sensitivity reside in this region of the SUR proteins [22]. With respect to MRP1, no substantial changes in either glibenclamide or tolbutamide sensitivity of $E_217\beta G$ transport were observed when either CL7 Tyr^{1189} or Tyr^{1190} were mutated to Ser (Table 2). Indeed, MRP1-mediated $E_217\beta G$ transport by both wild-type and Tyr mutant MRP1 was unaffected by 100 μM tolbutamide and while some differences in the sensitivity of the mutants to glibenclamide were observed, these were moderate.

In addition to Ser^{1237} in CL7, the bipartite sulfonylurea binding site of SUR1 is reported to involve a region of CL3 which connects MSD1 to MSD2 [25]. Whether or not the corresponding CL3 region of MRP1 is similarly involved in its sensitivity to glibenclamide remains to be determined. Interestingly, glibenclamide is also a potent inhibitor of CFTR which contains only two MSDs. The CFTR residues corresponding to Tyr^{1189} and Tyr^{1190} in MRP1 are Trp and Phe (Fig. 2) but it is not known if they are involved in the sulfonylurea sensitivity of CFTR. However, mutations within TM6 of CFTR (which corresponds to TM11 of MRP1 and SUR1/SUR2) significantly weaken glibenclamide block, whereas two mutations in TM12 (which corresponds to TM17 of MRP1 and SUR1/SUR2) strengthened glibenclamide block of CFTR [38]. Residues in TM11 and/or TM17 of MRP1 may also be involved in the ability of glibenclamide to inhibit MRP1-mediated transport but this remains to be determined.

In summary, we have shown that two adjacent Tyr residues in CL7 of MRP1 are important for its organic anion transport activity and in particular, its uptake affinity for GSH, but do not play a significant role in its sensitivity to sulfonylureas. Mutational analyses to date have mostly implicated residues in the TM helices as being critical for the substrate specificity and overall transport activity of MRP1 [27,34–37,39–45]. Recently, however, we have reported that mutation of Pro^{1150} in CL7 to Ala results in a complex phenotype in which transport of LTC_4, GSH and E_13SO_4 was reduced, but MTX and $E_217\beta G$ transport was markedly enhanced [34]. Thus, the present study identifies additional amino acids in CL7 that are important for the organic anion transport activity of MRP1. These and other studies are consistent with the notion that residues within the CLs of MRP1 and other ABC proteins play a complex role in their substrate specificity and transport activity [46,47].

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