Evidence for the Role of Glycosylation in Accessibility of the Extracellular Domains of Human MRP1 (ABCC1)

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ABSTRACT: To enable cell surface localization of the human multidrug resistance protein (MRP1, ABCC1) and to assess the role of the extracellular domains of this transporter, the FLAG epitope tag was introduced into different extracellular loops of the three membrane-spanning domains (MSDs) of the transporter. We constructed and expressed various partially and fully glycosylation-deficient, FLAG-tagged MRP1 proteins in a Vaccinia virus-based transient expression system, and the cell surface expression level of MRP1 on intact cells was followed by flow cytometry, using the FLAG tag specific monoclonal antibody M2. We also expressed the wild-type MRP1 protein and some of the FLAG-tagged mutants in stably transfected HEK293 cells, and followed the cell surface expression and the transport function of MRP1 both by monitoring the efflux of fluorescent substrate and by their ability to confer resistance to HEK293 transfectants to anticancer agents such as daunorubicin and etoposide. When we inserted the FLAG epitope in extracellular loops of the MSD1 or MSD3, the tag was accessible upon removal of N-glycosylation sites (N → Q at positions 17, 23, and 1006, respectively), whereas the FLAG epitope placed in the MSD2 was not accessible even after removal of all three N-glycosylation sites, indicating that MSD2 region is deeply buried in the plasma membrane. However, all FLAG tagged MRP1 mutants were expressed at the cell surface to the same extent as the wild-type protein and also exhibited normal transport function. Our results demonstrate that the accessibility of the external FLAG epitope is strongly dependent on the position of the tag and the glycosylation state of the different FLAG-tagged MRP1s, and the conformation of extracellular loops in MSD1 and MDS3 does not appear to contribute to the functional status of MRP1.

One of the major limitations to cancer chemotherapy is the unique ability of tumor cells to develop resistance to a wide range of cytotoxic anticancer agents. Human P-glycoprotein (Pgp)[1] and members of the multidrug resistance protein family (MRPs) are overexpressed in various drug resistant cell lines and different tumors, contributing to the phenomenon known as multidrug resistance (MDR). MDR is characterized by cellular resistance to natural product and cytotoxic agents with diverse structures and unrelated mechanisms of action (*1*, *2*). MRP1, a 190-kDa integral membrane protein, mediates the efflux of anticancer drugs from the cell in an ATP-dependent manner. MRP1 is a member of the ABC (ATP binding cassette) superfamily of transporters (*3*), and contains three membrane-spanning domains (MSD1, 2, 3) and two ATP-binding sites. The topological model based on hydropathy analysis of the primary sequence indicates that it consists of two highly homologous halves, each containing putative six transmembrane helices and one nucleotide-binding domain, and an extra membrane-spanning domain of five transmembrane helices at the N-terminus (*4*). Both nucleotide-binding domains contain highly conserved regions, termed the Walker A and B motifs, which are commonly involved in ATP hydrolysis and the C region (also called the signature peptide or linker region) unique to ABC transporters, which may be involved in the coupling of ATP hydrolysis to drug transport (*1*, *3*). There is experimental evidence that the first transmembrane region (MSD1) is not required for transport activity, while the linker region (L_0) between MSD1 and MSD2 is essential for function (*5*). Since the cloning of the human *MRP*1 gene in 1992 (*6*), eight more members of the MRP family (ABCC subfamily) have been discovered and some of them (MRP 4, 5, 8 and 9) lack the MSD1 domain (*7*−*9*).

MRP1 is expressed in many cell types of the human body (*10*). Speculations about its physiological function suggest a similar role to that of Pgp, helping to protect the human body against toxic compounds (*7*). In immunohistochemical studies, it was localized at the basolateral side of epithelial cells, playing an important role in protection of the choroid plexus and the testicular epithelium. MRP1 also appears to be the major leukotriene C_4 transporter (*11*, *12*).

The substrate specificity of MRP1 initially seemed to be similar to that of Pgp, transporting *Vinca* alkaloids and anthracyclines, but it can also transport hydrophobic anionic

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[1] Abbreviations: ABC, ATP binding cassette; DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence activated cell sorter; FBS, fetal bovine serum; FLAG epitope, octapeptide DYKDDDDK; GSH, glutathione; IMDM, Iscove's modified Dulbecco's medium; mAB, monoclonal antibody; MDR, multidrug resistance; MRP1 (ABCC1), multidrug resistance protein 1; MSD, membrane spanning domain; Pgp (ABCB1), P-glycoprotein; WT, wild type.

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Table 1: Primers Used for Introducing the FLAG Epitope Sequence into Human *MRP*1 (ABCC1) cDNA

construct	PCR primers to insert FLAG tag (5′ → 3′)[a]
MRP1−Flag1−His10	F: GAC TAC AAG GAC GAC GAC GAT AAG TTC GTG AAC GAC ACG AAG
	R: ATC GTC GTC GTC CTT GTA GTC GAT GAG CAA CTT TAA GAT CTG
MRP1−Flag3-His10	F: GAC TAC AAG GAC GAC GAC GAT AAG GGG ACT CAG GAG CAC ACG
	R: CTT ATC GTC GTC GTC CTT GTA GTC GAC GAT GGT GTC ATC AGT
MRP1−Flag4−His10	F: GAC TAC AAG GAC GAC GAC GAT AAG GGG ACT CAG GAG CAC
	R: CTT ATC GTC GTC GTC CTT GTA GTC GAC GAT GGG GTC ATC
MRP1−Flag5NNN−His10	F: GAC TAC AAG GAC GAC GAC GAT AAG TGG AAT GTC ACG TGG
	R: CTT ATC GTC GTC GTC CTT GTA GTC CCA GAG CGG GTC GGA
MRP1−Flag5QQQ−His10	F: GAC TAC AAG GAC GAC GAC GAT AAG ACC AGC AAC CCC GAC TTC
	R: CTT ATC GTC GTC GTC CTT GTA GTC CTG CCA CGT GAC CTG CCA

[a] F: forward primer, R: reverse primer.

compounds. Depletion of cellular glutathione abolished MRP1-mediated resistance against *Vinca* alkaloids and anthracyclines (*13*). MRP1 prefers organic anions as substrates, e.g., drugs either conjugated to or cotransported with glutathione (GSH), glucuronide, or sulfate. Other known substrates are organic anions such as methotrexate, aflatoxin B, and arsenate (*14*, *15*).

MRP1 has six potential N-linked glycosylation sites, but it is N-glycosylated in its mature form only at three asparagine (N) residues, 19, 23, and 1006, respectively, as demonstrated by site-directed mutagenesis (*10*). Many studies suggest for both Pgp and MRP1 that glycosylation is not required for the drug transport function, and the lack of this posttranslational modification does not cause any gross alterations in substrate specificity (*4*, *10*, *16*, *17*). Therefore, glycosylation possibly plays some role in intracellular sorting and/or routing of the transporters to the cell surface.

The fact that there is still no monoclonal antibody available to identify extracellular regions of MRP1 (*18*) led us to introduce a well-characterized affinity tag, called the FLAG epitope (peptide sequence DYKDDDDK (*19*)), to different extracellular locations of MRP1. Our aim was to engineer MRP1-tagged with an extracellular epitope for the assessment of cell surface expression and to explore the role of the extracellular loops in MSDs on the functional status of the transporter. In this study, glycosylation-deficient (Gly^-) MRP1 mutants with FLAG epitope tag inserted in various extracellular loops of the membrane-spanning domains have been generated to compare the accessibility of the FLAG tag in the presence and absence of glycosylation. The WT MRP1 and various mutant proteins were characterized in a Vaccinia virus-based transient expression system as well as in stably transfected HEK293 cells. Our data demonstrate that the glycosylation-deficient FLAG-tagged MRP1 variants are functionally similar to WT protein. Furthermore, we observed that the accessibility of the FLAG epitope depends on the location of the tag and the presence of glycosylation sites in MSD1 and MSD3. Cell surface glycosylation seems to play a primary role in masking the extracellular epitopes of human MRP1, and it remains to be explored whether other ABCC subfamily members share this feature.

EXPERIMENTAL PROCEDURES

Materials. Dulbecco's modified Eagle's medium (DMEM), Iscove's modified Dulbecco's medium (IMDM), L-glutamine, penicillin/streptomycin, Lipofectin, Lipofectamine TM 2000 reagent kit, and PCR primers were obtained from Life Technologies (Gaithersburg, MD). FBS was supplied by HyClone (Logan, UT). Calcein-AM was obtained from Molecular Probes (Eugene, OR). MK-571 was obtained from Alexis Corp. (San Diego, CA), and the anti-FLAG M2 monoclonal antibody was from Sigma (St. Louis, MO). MRPr1 and mrpm6 antibodies were obtained from Chemicon (Temecula, CA). FITC conjugated anti-mouse IgG_1 secondary antibody was obtained from PharMingen (San Diego, CA). IntraPrep Permeabilization reagent kit was obtained from Immunotech (Marseille, France). ECL reagents were obtained from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). Human MRP1 cDNA was provided by Dr. Gary Kruh (Fox Chase Cancer Center, Philadelphia, PA). Restriction enzymes, calf intestinal phosphatase, and T4 DNA ligase were from New England Biolabs (Beverly, MA). Expand High Fidelity PCR system was from Boehringer Mannheim (Roche Diagnostics Corp., Indianapolis, IN). Recombinant Vaccinia virus encoding bacteriophage T7 RNA polymerase (vTF7-3) was obtained from Dr. B. Moss (NIAID, NIH, Bethesda, MD).

Construction of Mutant MRP1 cDNAs. Initially, the pTM1-*MRP*1 vector was constructed using pGEM-*MRP*1 vector, a gift from Dr. Gary Kruh. The first 840 bp cDNA fragment was obtained by PCR using pGEM-*MRP*1 as template, and the PCR fragment was subcloned into the linearized pTM1 vector prepared by digestion with *Nde*I and *BamH*I. Subsequently, the second fragment of the *MRP1* cDNA was removed from the pGEM-*MRP*1 construct by *BamH*I digestion and ligated into the pTM1-*MRP*1 (1−840) plasmid linearized with *BamH*I. The 10×Histidine tag was inserted at the carboxy terminus by ligation of a polynucleotide linker, using restriction enzyme sites *NgoMIV* and *Pvu*I. The FLAG epitope (Eastman Kodak, New Haven, CT) was cloned into pTM1-*MRP*1-His10 at different positions by two-step PCR gene fusion technique using Expand High Fidelity PCR kit. Fragments generated contained restriction sites *Nde*I, *BamH*I, *EcoR*I, and *Sph*I in which the FLAG tag was inserted at various locations. The primers for the insertion of the FLAG epitope tag are listed in Table 1. Single point mutations were introduced at asparagine (N) residues 19, 23, and 1006 and changed to glutamine (Q), then cloned into plasmid pTM1-*MRP*1-His10, which has been used for the transfection of HeLa cells in a Vaccinia virus expression system. To create stably transfected HEK293 cells, the wild-type *MRP*1-His10, and the mutant *MRP*1-QQFlag4-His10 and *MRP*1-Flag5QQQ-His10 constructs were cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA) by using restriction enzymes *Nhe*I and *Not*I. All construct sequences were verified in both directions by automated sequencing with the PRISM Big Dye

Terminator Sequencing kit (Perkin-Elmer Corporation, Norwalk, CT).

Cell Lines and Viruses. HeLa cells were propagated as a monolayer at 37 °C, 5% CO_2, in DMEM supplemented with 4.5 g/L glucose, 5 mM L-glutamine, 50 units/mL penicillin, 50 μg/mL streptomycin, and 10% FBS. Recombinant Vaccinia virus encoding bacteriophage T7 RNA polymerase (vTF7−3) was propagated and purified as previously described (*20*). HEK293 cells were cultured under the same conditions as HeLa cells, and the recombinant pcDNA3.1 plasmids carrying the *MRP*1-His10, *MRP*1-QQFlag4-His10, and *MRP*1-Flag5QQQ-His10 cDNA constructs, respectively, were used to transfect HEK293 cells. Transfections were carried out using the Lipofectamine TM 2000 reagent kit (Life Sciences, Gaithersburg, MD), according to the manufacturer's instructions. The stable transfectants were selected in Geneticin (G418, 800 μg/mL), Mediatech Inc., Herndon, VA), and the selected clones were cultured in VP-16 (5 μM). The cells were grown in the presence of G418 without the selection agent (VP-16) for 21 days before flow cytometry or cell toxicology analysis.

Vaccinia Virus Expression. A 70−80% confluent monolayer of HeLa cells was co-infected/transfected with the vTF 7-3 Vaccinia virus (10 pfu/cell) and 15 μg of the control plasmid, pTM1; the WT plasmid, pTM1-MRP1-His10; or the different mutant FLAG-tagged constructs; using 45 μg of lipofectin (*21*). The cells were fed at 4 h post-infection with 12 mL of DMEM supplemented with 10% FBS and incubated for 24 h at 32 °C, 5% CO_2.

Fluorescence Activated Cell Sorting (FACS) Analysis. A FACSort flow cytometer equipped with Cell Quest software (Becton-Dickinson, San Jose, CA) was used for FACS analysis.

Cell Surface Staining with M2 Antibody. Cell surface accessibility of the FLAG tag in different extracellular positions in MRP1 was detected in HeLa cells by using anti-FLAG monoclonal antibody M2. The labeling was done using 5 μg of antibody per 300 000 cells at 37 °C for 30 min. The cells were washed with IMDM supplemented with 5% FBS. Anti-mouse IgG_1 FITC-conjugated secondary antibody was added at 1 μg/300 000 cells and incubated at 37 °C for 30 min; mouse IgG_1 monoclonal antibody was used as isotype control. Cell surface staining of the FLAG epitope was measured by FACS in intact infected/transfected HeLa cells. For staining with MRPr1 antibody with an internal epitope, permeabilization of the cells was done by using IntraPrep Permeabilization reagent kit, according to the manufacturer's instructions.

SDS−PAGE and Immunoblot Analysis. Immunoblot analysis, with the monoclonal antibodies M2 and MRPr1, was used to determine the total cellular content of the WT and mutant MRP1s. SDS−PAGE samples were separated on 6% Tris-glycine gel and transferred to a 0.45 μm nitrocellulose membrane at 400 mA for 1 h using a Tris (25 mM)-glycine (192 mM) buffer with 10% (v/v) methanol. The membranes were blocked for 30 min with 5% dry milk. The blots were incubated with M2 (1:2000) or MRPr1 (1:3000) monoclonal antibodies. HRP-conjugated anti-mouse (for M2) or anti-rat (for MRPr1) secondary antibody was added at 1:10 000 dilution, and the blots were developed with ECL reagents by following the manufacturer's protocol.

Calcein-AM Accumulation Assay. Cells were harvested after trypsinization by centrifugation at 500*g* and resuspended in 1 mL of IMDM supplemented with 5% FBS. Calcein-AM (0.25 μM) was added to 500 000 cells in 4.5 mL of PBS in the presence or absence of reversing agent MK-571 (5 μM). The cells were incubated at 37 °C for 10 min in the dark and pelleted by centrifugation at 500*g*. The cell pellet was resuspended in 300 μL of PBS prior to FACS analysis.

In Vitro Toxicology Assay. The relative resistance of HEK293 cells, transfected with the WT or FLAG-tagged mutant MRP1 constructs, to etoposide and daunorubicin was determined by using an MTT-based toxicology assay kit (Sigma), according to the manufacturer's instructions.

RESULTS

Construction of Plasmids to Express FLAG-tagged MRP1s. To express various FLAG-tagged, glycosylation-deficient MRP1s in HeLa cells, asparagines at positions 19, 23, and 1006 in the WT human MRP1 cDNA were mutated to glutamine (N → Q) in different combinations, and the FLAG sequence was introduced into MSD1, 2, or 3 of MRP1, respectively (see Figure 1 for the localization of the FLAG tag in different constructs, and Table 2 for the summary of all constructs and their glycosylation status). The WT and mutant *MRP*1 cDNA's, containing a 10×Histidine tag at the carboxyl terminus, were placed under the control of a T7 promoter and were downstream of an internal ribosome entry site in the pTM1 vector. It has been demonstrated previously for Pgp that high levels of expression of WT and mutant proteins could be obtained in mammalian cells upon infection with the vTF7-3 recombinant Vaccinia virus encoding bacteriophage T7 RNA polymerase followed by transfection with the pTM1-*MDR*1 vector (*17*, *22*).

Transient Expression of the Wild-Type and Glycosylation-Deficient FLAG-Tagged MRP1s Using a Vaccinia Virus System. The advantage of transient expression of multidrug transporter proteins with a Vaccinia virus expression system is that the long-term pleiotropic effects of drug selection on stable transfectants could be avoided (*21*, *22*). All constructs, the WT and the different FLAG-tagged Gly^- MRP1s, were expressed in HeLa cells using a Vaccinia virus expression system. On the basis of previous experience with expressing human Pgp, we chose HeLa cells (cervical epidermoid carcinoma) for our transfection−infection experiments, due to their low level of expression of endogenous Pgp and MRP1, ability to express a high level of Pgp, and relative ease of transfection (*21*, *23*).

Detection of FLAG Epitope Tagged MRP1 Mutant Proteins on the Cell Surface of HeLa Cells. The expression of WT and FLAG-tagged MRP1 mutants at the cell surface was detected at 24 h post infection−transfection by FACS analysis, using the monoclonal antibody M2 (The time-course studies showed that the maximum level of expression of WT and mutant MRP1 proteins was observed at 24 h post infection−transfection, data not shown). Figure 2 shows the histogram plots of the FACS analysis for the WT and various FLAG-tagged MRP1 proteins. The WT protein (without a FLAG tag) as well as the Flag1 and Flag3 MRP1 variants was not recognized on the cell surface by anti-FLAG monoclonal antibody M2. Cells expressing Flag4 and Flag5

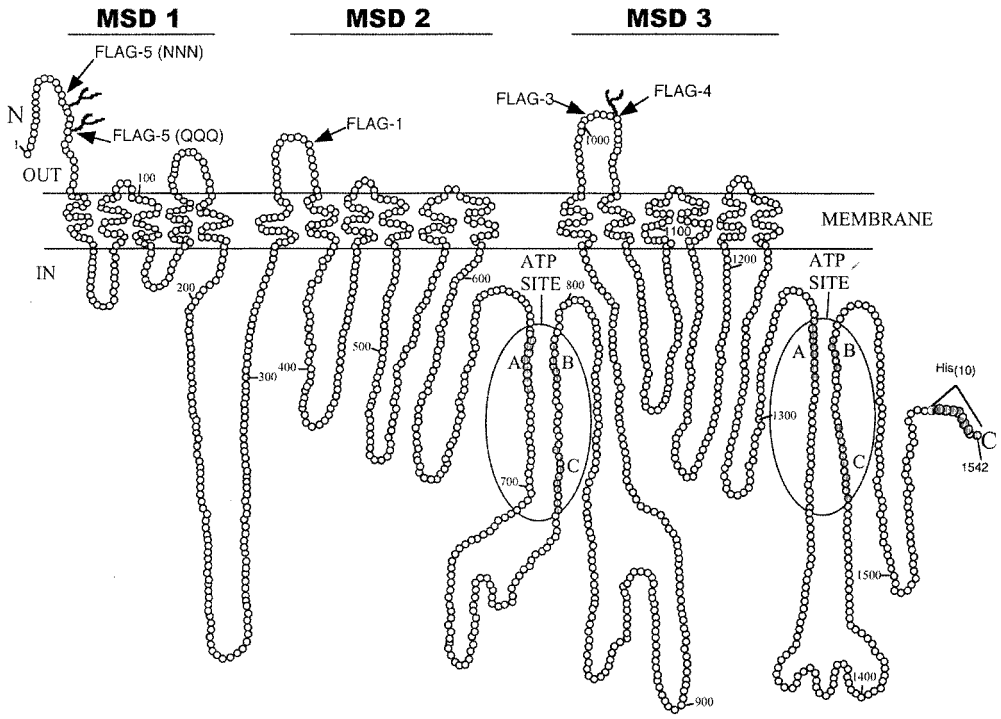

FIGURE 1: Two-dimensional hypothetical model of MRP1 (ABCC1) structure. A hypothetical two-dimensional model of human MRP1, based on prediction described in refs *4* and *10*. The glycosylation sites at the N-terminal end (N19 and N23) and in the sixth extracellular loop (N1006), and the FLAG tag insertions (at amino acid positions 18, 24, 363, 1003, or 1007) are shown. The two nucleotide binding sites with the conserved Walker A and B sequences and the C or linker region are marked with oval. The MSD 1, 2, and 3 domains are indicated at the top. In addition, a 10×Histidine tag at the C-terminus is also indicated.

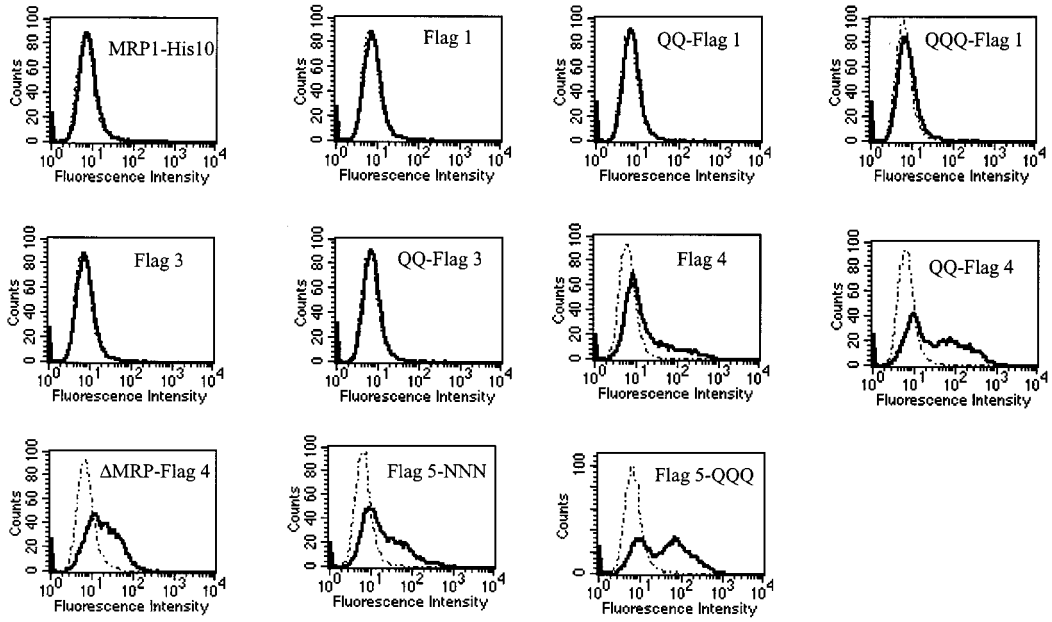

FIGURE 2: Cell surface detection of WT and mutant MRP1 variants by anti-FLAG M2 antibody. Infected-transfected HeLa cells were stained with the FLAG epitope specific monoclonal antibody M2, and subjected to FACS analysis, as described in Experimental Procedures. HeLa cells were incubated with an isotype primary antibody $IgG1_b$ (- - -), or M2 antibody (−); curves are superimposed for each construct. The name of each construct is given in each histogram, and the detailed description of various constructs is given in Table 2.

constructs showed significant staining with M2 antibody, which was considerably enhanced by the removal of glycosylation sites in QQ-Flag4, ΔMRP1-Flag4 and Flag5-QQQ proteins. A quantitative assessment of these data (Table 3) shows a direct correlation between the length of the putative externally exposed region in MSD1 (32 amino acids), MSD2 (19 amino acids), and MSD3 (27 amino acids), and the accessibility of the FLAG epitope, as judged by median fluorescence intensity, though the glycosylation status of the protein is an important "regulator" of the accessibility. Insertion of the FLAG epitope increases the length of the extracellular loops by 22% (MSD1/Flag5-NNN), 25% (MSD1/Flag5-QQQ), 42% (MSD2/Flag1), 30% (MSD3/Flag3), and 26% (MSD3/Flag4), respectively. Although insertion of two FLAG sequences, one into the first extracellular loop and the other into the MSD3 (construct QQ-Flag4-Flag5) in-

Table 2: Summary of the Construction of Various FLAG-Tagged MRP1 Mutants

construct	location of FLAG epitope	no. of glycosylation sites	glycosylation status
MRP1−His10	none	3	WT, all three sites (19, 23, and 1006) are available for glycosylation
MRP1−Flag1−His10	in third extracellular loop (MSD 2) between residues 362 and 363	3	all three glycosylation sites present (19, 23, and 1006)
MRP1QQ−Flag1−His10	in third extracellular loop (MSD 2) between residues 362 and 363	1	N → Q at residue 19 and 23; glycosylation only at residue 1006 is possible
MRP1QQQ−Flag1−His10	in third extracellular loop (MSD 2) between residues 362 and 363	0	N → Q at all three sites (19, 23, and 1006)
MRP1−Flag3−His10	in sixth extracellular loop (MSD 3) between residues 1002 and 1003	3	all glycosylation sites present
MRP1QQ−Flag3−His10	in sixth extracellular loop (MSD 3) between residues 1002 and 1003	1	N → Q at residue 19 and 23; glycosylation only at residue 1006 is possible
MRP1−Flag4−His10	in sixth extracellular loop (MSD 3) between residues 1006 and 1007	2	glycosylation at residue 19 and 23; residue N-1006 mutated to D (first residue of FLAG tag)
MRP1−QQFlag4−His10	in sixth extracellular loop (MSD 3) between residues 1006 and 1007	0	N → Q at residue 19 and 23; residue N-1006 mutated to D (first residue of FLAG tag)
MRP1−QQFlag4−Flag5−His10	2 Flags: Flag 5 in the N-terminus region between residues 23 and 24; and Flag 4: in sixth extracellular loop (MSD 3) between residues 1006 and 1007	0	N → Q at residue 19 and 23; residue N-1006 mutated to D (first residue of FLAG tag)
MRP1−Flag5NNN−His10	Flag 5 in the N-terminal region between residues 17 and 18	3	glycosylation at all three sites (19, 23 and 1006) is possible; residue 17 (D) served as the first amino acid of the FLAG tag
MRP1−Flag5QQQ−His10	Flag 5 in the N-terminal region between residues 23 and 24	0	N → Q at all three sites (19, 23, and 1006)
ΔMRP1−1−203−Flag4−His10	MRP1 with deletion of residues 1 → 203; contains Flag4 between residues 1006 and 1007	0	N → D at residue 1006 with Flag epitope insertion. The first two glycosylation sites are deleted, as a result of removal of residues 1 → 203

Table 3: Determination of the FLAG Epitope Accessibility in Various MRP1 Mutants Detected with Anti-FLAG Monoclonal Antibody M2[a]

construct	median fluorescence (arbitrary units)	% of control
MRPl-Hisl0 control	7.10	100
Flag1	7.10	100
QQ-Flagl	7.24	102
QQQ-Flagl	7.31	103
Flag3	7.31	103
QQ-Flag3	7.46	105
Flag4	9.10	128
QQ-Flag4	21.87	308
Flag5-NNN	13.70	193
Flag5-QQQ	33.98	479
QQ-Flag4 + Flag5	47.78	673
ΔMRP−Flag4	12.78	180

[a] Cell surface accessibility of the FLAG tag in different extracellular positions in MRP1 was measured by FACS, using M2 monoclonal antibody. The median fluorescence was calculated by using histogram statistics in Cell Quest program, and the values shown here represent the average of two independent experiments.

creased fluorescence intensity in M2 staining experiments (Figure 3A), the mutant protein in the calcein-AM efflux assay shows total loss of function (data not shown), and for this reason the experiments with MRP1 tagged with two FLAG tags was not pursued further. The Figure 3A compares the M2 staining results for the various Flag-4 constructs (FLAG tag inserted in MSD 3), while in Figure 3B we overlapped the M2 staining data of the fully glycosylated and totally unglycosylated Flag5 constructs with the original, glycosylated Flag1 construct. The data suggest that the removal of the glycosylation sites significantly improves the recognition of FLAG epitope by the M2 antibody in both Flag4 and Flag5 constructs.

It has previously been shown for Pgp that the reactivity of monoclonal antibody UIC2 with Pgp at the cell surface is increased at 37 °C in the presence of substrates or agents that induce ATP depletion (*24*, *25*). This enhanced antibody binding has been proposed to result from a conformational change induced by drug transport or by ATP depletion, resulting in greater UIC2 epitope accessibility. We addressed the possibility of a similar substrate/modulator-dependent change in conformation of MRP1 protein that is reflected in the accessibility of the FLAG tag. HeLa cells expressing the WT or QQ-Flag4 MRP1 were pretreated with MRP1 substrate VP-16 (50 μM) or modulator MK-571 (5 μM) prior to M2 staining at 37 °C. No change in M2 binding was observed in the presence of MRP1 substrate or modulator (data not shown).

We conducted the M2 staining experiments after permeabilizing the transfected−infected HeLa cells, and we also stained the permeabilized cells with MRPr1 antibody, which recognizes an intracellular epitope of human MRP1. These assays reflect on the overall cellular expression level of the protein, and showed similar level of MRP1 protein expression irrespective of introduction of the FLAG tag (data not shown).

The overall cellular level of MRP1 expression was detected in total cell extracts by Western blot analysis at 24

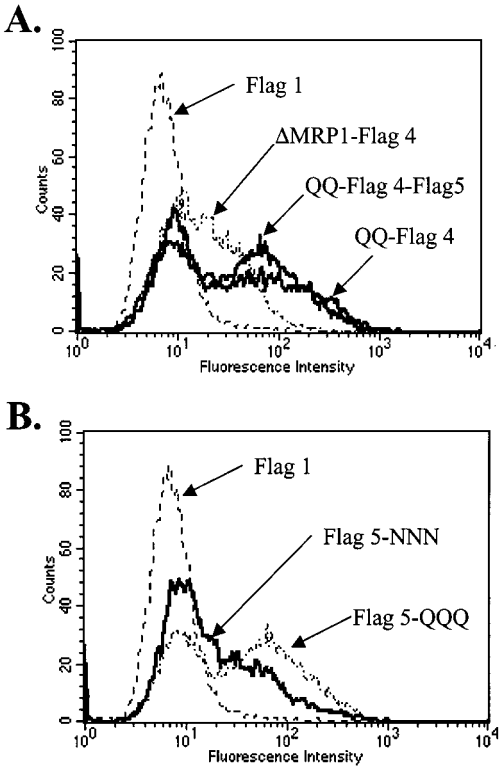

FIGURE 3: Comparison of the cell surface detection of WT and MRP1 variants by anti-FLAG M2 antibody. Cells were transfected with the indicated construct, and the M2 labeling of Vaccinia virus infected-transfected HeLa cells was determined by FACS analysis. The M2 staining of Flag1, QQ-Flag4, QQ-Flag4-Flag5, and ΔMRP1-Flag4 constructs is shown in panel A; and the staining of Flag1, Flag5-NNN, and Flag5-QQQ in panel B. After 24 h post-infection, cells were harvested, and labeled with M2 antibody as described under Experimental Procedures. The labeling with control isotype antibody as well as the M2 staining of MRP1-His10 is not shown for clarity, those were identical to Flag1.

h post-infection−transfection using the monoclonal antibody MRPr1, which recognizes an intracellular epitope (*4*) in the N-terminal region of MRP1 (Figure 4A). The same Western blots after stripping were probed with M2 monoclonal antibody to detect FLAG epitope expression (Figure 4B). HeLa cells infected with a Vaccinia virus and transfected with the control plasmid pTM1 show very low (undetectable) levels of endogenous MRP1 expression. As shown in Figure 4A, the FLAG-tagged, Gly^- mutant MRP1s are expressed at a comparable level to that of the WT protein. The WT MRP1 migrates in two separate bands, one represents the fully glycosylated mature form (~190 kDa), the other the core-glycosylated immature form (~150 kDa), attributed to incomplete glycosylation of overexpressed proteins (*22*). The unglycosylated mutant proteins migrated as a single band to the position similar to the immature form of WT protein.

Calcein-AM Efflux Assays in Transfected/Infected HeLa Cells. The functional status of the WT MRP1 and various mutants in HeLa cells was assessed by using calcein-AM efflux assay (see Experimental Procedures). HeLa cells, expressing pTM1 (vector alone), WT MRP1, and different Gly^-/FLAG MRP1s were incubated with calcein-AM (0.25 μM) for 10 min in the presence or absence of the MRP1 specific inhibitor MK-571 (5 μM). As shown in Figure 5, all mutant proteins exhibit the efflux of calcein-AM to the same extent as the WT protein, and this efflux of calcein-AM is blocked by the MRP1-specific inhibitor, MK-571 (5

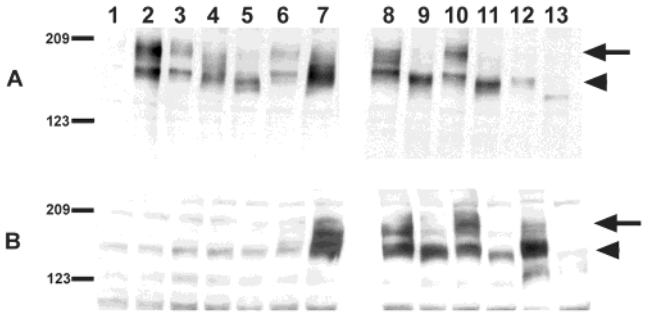

FIGURE 4: Total cellular expression of WT and glycosylation-deficient, FLAG-tagged MRP1 variants. Transfected-infected HeLa cells were harvested at 24 h post-infection, and Western blot samples were prepared as described in ref *17*. An immunoblot analysis of the cell extracts (2×10^4 cells/lane for mAb MRPr1 and 4×10^5 cells for mAb M2) was done. Panel A shows total cellular expression of the various mutants by antibody MRPr1; panel B, the expression of the FLAG-tag is followed by anti-FLAG monoclonal antibody M2. In both A and B panels, lane 1, pTM1 vector (mock-transfection); lane 2, WT-MRP1-His10; lane 3, Flag1; lane 4, QQ-Flag1; lane 5, QQQ-Flag1; lane 6, Flag3; lane 7, QQ-Flag3; lane 8, Flag4; lane 9, QQ-Flag4; lane 10, Flag5-NNN; lane 11, Flag5-QQQ; lane 12, QQ-Flag4-Flag5; and lane 13, ΔMRP-Flag4. The position of the glycosylated and unglycosylated MRP1 is shown by arrow and arrowhead, respectively.

μM). Only the truncated ΔMRP1-F4 protein was found to exhibit reduced activity. These results demonstrate that introduction of the FLAG tag at different positions into the human MRP1 protein does not affect the function of the transporter significantly.

Analysis of the Stably Transfected HEK293 Cells. The expression of proteins in a Vaccinia virus-based system is of a transient nature and therefore not suitable for assessing the ability of multidrug transporters to confer resistance to anticancer agents. For this reason, we generated stable cell lines expressing the WT and FLAG-tagged mutant MRP1 proteins. HEK293 cells expressing the 10×His-tagged wild-type MRP1, QQ-Flag4-MRP1 or Flag5QQQ-MRP1 mutant constructs, cloned into pcDNA3.1, which confers resistance to G418, were selected in G418 (800 μg/mL). The selected clones were grown in the presence of G418 (800 μg/mL) and 5 μM VP-16, and only VP-16 was removed from the medium 21 days before the FACS and MTT assays (see below). The calcein-AM efflux assays were conducted under the same conditions as for the transiently transfected-infected HeLa cells, only the concentration of reversing agent MK-571 was increased to 20 μM. Figure 6 shows the cell surface expression of the FLAG epitope followed by M2 antibody staining in panel A, and the calcein-AM efflux experiments using the MRP1 specific inhibitor MK-571 in panel B. HEK293 transfectants expressing all three constructs show comparable activity in the functional assay, and the cell surface recognition of the FLAG-tagged constructs by M2 antibody is in good agreement with the results for the HeLa cells transiently transfected with the same constructs (Figure 2). We checked the overall cellular expression level of the three different MRP1 constructs by permeabilizing the cells and then staining with monoclonal antibody MRPr1, as we did for the proteins expressed in a Vaccinia virus expression system. We could not detect any substantial difference in the total cellular expression level of the mutant MRP1s compared to the WT in either of the expression systems (data not shown).

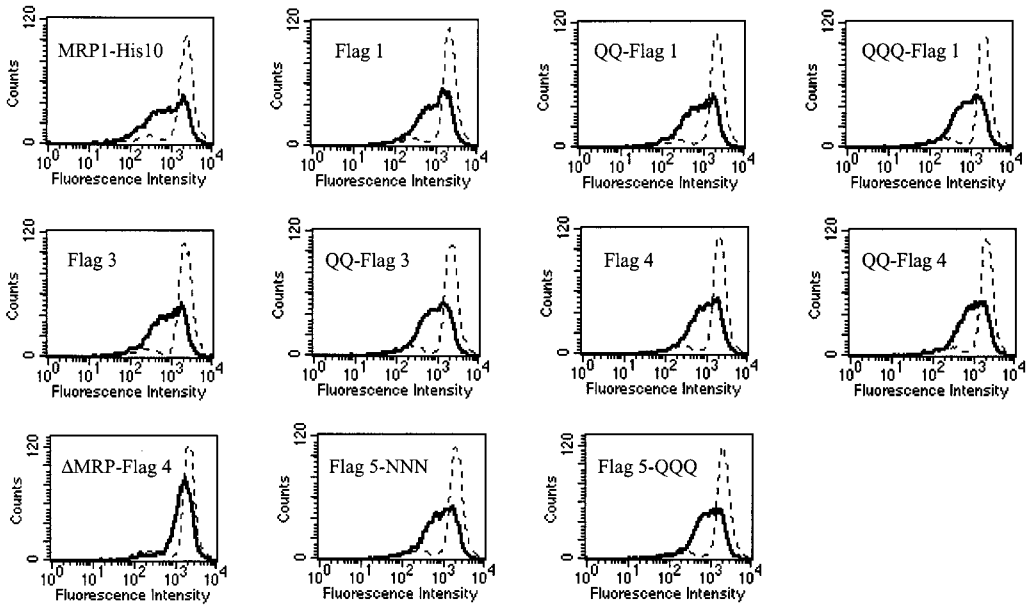

FIGURE 5: Calcein-AM accumulation in HeLa cells transfected with the WT and glycosylation-deficient FLAG-tagged MRP1 variants. The calcein-AM accumulation of Vaccinia virus infected−transfected HeLa cells was determined by FACS analysis. Cells were transfected with the WT and various mutant constructs as shown in each histogram. After 24 h post-infection, cells were harvested, washed, and loaded for 10 min with 0.25 μM calcein-AM, in the absence (−), or presence of an inhibitor, 5 μM MK-571 (- - -).

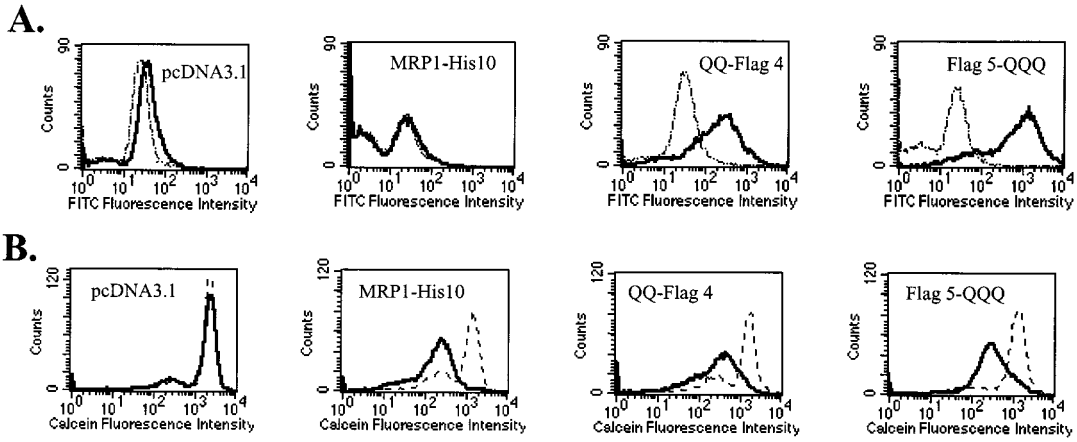

FIGURE 6: Cell surface detection and calcein-AM accumulation of WT and mutant MRP1 variants stably expressed in HEK293 cells. Panel A: Stably transfected HEK293 cells were stained with the FLAG epitope specific monoclonal antibody M2, and subjected to FACS analysis, as described under Experimental Procedures. HEK293 cells were incubated with an isotype primary antibody $IgG1_b$ (- - -), or M2 antibody (−); curves are overlaid for each construct. Panel B: The calcein-AM accumulation in stable transfected HEK293 cells was determined by FACS analysis. Cells expressing the WT or mutant constructs as shown in each histogram were subjected to the calcein-AM assay. For other details, see the legend to Figure 5.

Toxicology Assays on HEK293 Cells Stably Expressing WT and FLAG-Tagged Mutant MRP1 Variants. Stably transfected HEK293 cells expressing the wild-type MRP1, QQ-Flag4-MRP1 or Flag5QQQ-MRP1 mutant constructs were exposed to increasing concentrations of two known MRP1 drug substrates, etoposide (VP-16) and daunorubicin (*7*), respectively, to determine the ability of the WT and FLAG-tagged MRP1 mutants to confer resistance to transfected HEK293 cells. The toxic effect of the anticancer agents was determined by using the MTT assay. We did not find any significant difference between the WT transporter and the two FLAG-tagged mutants, in terms of their ability to confer resistance to either of the chemotherapeutic drugs (Table 4). The concentration required for killing 50% of the cells (LD_{50}) of etoposide was in the range of 30−50 μM for each of the MRP1 constructs, which is about 100-times higher than the LD_{50} value for the mock transfected cells (cells transfected with pcDNA3.1 vector alone). For daunorubicin, the LD_{50} values for WT-MRP1 and the FLAG-

Table 4: Relative Resistance of the Wild-Type and Flag-Tagged MRP1 Variants-Transfected HEK293 Cells[a]

construct	VP-16 (μM)	daunorubicin (μM)
pcDNA3.1 vector alone	0.36 ± 0.15	0.06 ± 0.01
pcDNA3.1/MRP1−Hisl0	38.20 ± 11.39	0.80 ± 0.36
pcDNA3.1/MRP1−QQFlag4−Hisl0	40.80 ± 7.78	0.94 ± 0.30
pcDNA3.1/MRP1−Flag5QQQ−Hisl0	38.00 ± 10.88	0.85 ± 0.23

[a] The relative resistance of HEK293 cells, transfected with the WT or FLAG-tagged mutant MRP1 constructs, to etoposide (VP-16) and daunorubicin was determined by using an MTT based toxicology assay kit, as described in Experimental Procedures. LD_{50} values were calculated from each experiment run in triplicates, and the values represent the mean ± SD of three independent experiments for both cytotoxic drugs.

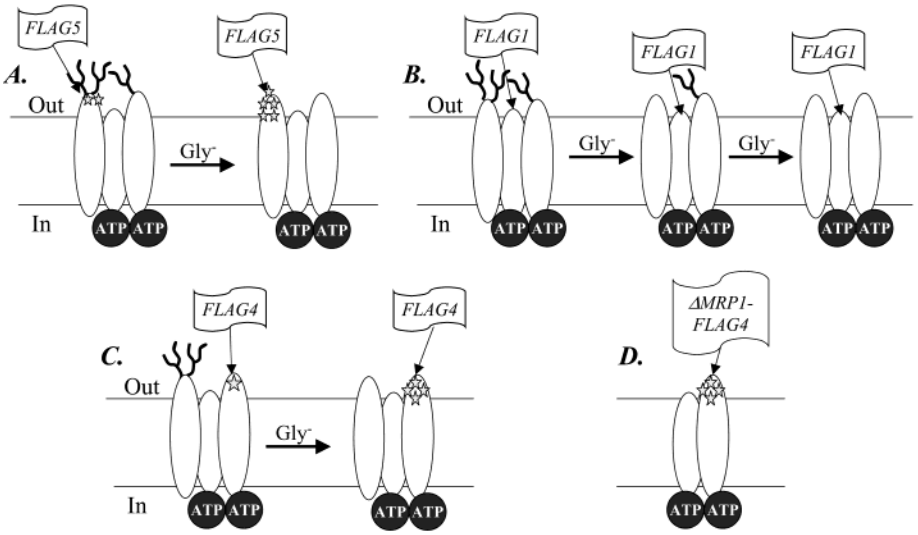

FIGURE 7: Schematic model for the effect of utilization of the glycosylation sites on the accessibility of FLAG epitope in MRP1. The membrane-spanning domains, MSD1, MSD2, and MSD3 are shown as oval structures in the membrane and filled circles represent two ATP-binding sites. The numbers of stars correspond to the level of accessibility of the FLAG tag assessed by the extent of staining with anti-FLAG monoclonal antibody, M2. Bold trees in the figure indicate the glycosylation sites. Panel A represents constructs where the FLAG sequence was introduced into the N-terminal region of MSD1 (Flag5 construct). The tag is marginally accessible even in the fully glycosylated protein, and removal of the glycosylation sites (indicated by Gly^-) increases the accessibility significantly. Panel B shows positioning of the FLAG tag in the third extracellular loop in MSD2 (Flag1 construct). This domain seems to be deeply buried in the cell membrane, as neither the partial nor the complete removal of glycosylation was sufficient to make the FLAG tag detectable. In panel C, the introduction of Flag4 sequence into the sixth extracellular loop (in MSD3) knocks out the glycosylation site at position 1006. The FLAG tag in this construct shows a slight accessibility, which dramatically increases with the removal of the N-terminal glycosylation sites (QQ-Flag4 construct). Panel D shows the case of the truncated protein where the entire first transmembrane domain, MSD1, is deleted (residues 1−203). Insertion of Flag4 sequence into the truncated mutant ensures excellent accessibility of the FLAG tag. In this construct insertion of FLAG4 results in removal of glycosylation site N1006 in MSD3, similar to that shown in panel C.

tagged mutants varied between 0.5 and 1.2 μM, 10−15 times higher than the LD_{50} concentration obtained for the mock transfected HEK293 cells.

DISCUSSION

Since the identification of the human *MRP*1 gene in 1992 (*6*), many efforts have been focused on understanding the structure and function of this drug transporter to improve the efficacy of chemotherapy for cancer patients. Although several monoclonal antibodies have been developed for the detection of MRP1, all of them recognize only an intracellular epitope. For the detection of MRP1 in intact cells, we constructed numerous recombinant variants of wild-type *MRP*1 with the insertion of a 10×Histidine tag at the C-terminus and the FLAG epitope in the first extracellular loop of the three transmembrane domains MSD1, MSD2, and MSD3, respectively. We found that recognition of the FLAG epitope was highly dependent on the location of the tag and the presence of glycosylation sites in extracellular regions of MRP1. In addition, the introduction of FLAG epitope in MSDs and removal of glycosylation does not affect the function of the multidrug transporter.

The FLAG sequence is an 8-amino acid peptide (DYKDDDDK), the only peptide tag designed as a tag (*19*) and not derived from any known protein. Originally, the FLAG sequence could be placed only at the amino terminus of the protein to have proper recognition by antibody M1. The availability of the M2 antibody makes it possible to detect the tag located internally within the protein or at the carboxyl terminus. A variety of FLAG-tagged proteins have been shown to retain full biochemical activity, e.g., transcription factors, growth factors, enzymes, and several membrane proteins. Positioning the FLAG sequence in extracellular loops of the epithelial sodium channel subunits did not change their trafficking to the cell membrane or ion permeation (*26*). To clarify the membrane topology of the Na^+-Ca^{2+} exchanger protein NCX1, the FLAG epitope was inserted into 10 putative extramembraneous segments of the exchanger protein. Four of the FLAG-tagged mutant proteins were targeted to the plasma membrane and retained their full activity (*27*).

In case of the cystic fibrosis conductance regulator protein (CFTR, ABCC7), a close relative of MRP1 in the ABCC subfamily, the detection of the mature form of the protein on the cell surface requires an indirect method, labeling the cell surface proteins with a biotinylated reagent followed by immunoprecipitation (*28*). The introduction of the FLAG sequence into the fourth extracellular loop of CFTR resulted in a fully glycosylated protein, and the introduction of the tag sequence did not change the functional properties of the protein (*29*). The FLAG tag has been inserted into human Pgp in the first extracellular loop, between residues 94 and 95, and the recombinant protein was expressed in the same Vaccinia transient expression system. The FLAG-tagged Pgp showed identical characteristics to WT protein, based on FACS analysis and photoaffinity labeling experiments (*30*).

It has been thus far reported that all described monoclonal antibodies against MRP1 detect internal epitopes, and thus permeabilization is necessary before they can bind to their epitopes (*18*). To overcome this problem, we placed the FLAG epitope at different extracellular locations and detected it by using an anti-FLAG monoclonal antibody, M2, in intact cells. The insertion of the FLAG tag was also used to assess the effect of the utilization of glycosylation sites located at

amino acid positions 19, 23, and 1006. Either one, two, or all three glycosylation sites were mutated {N → Q} in *MRP*1, and both the WT and mutant MRP1 proteins were expressed in a Vaccinia-based transient expression system. This expression system was used for the characterization of multidrug resistance-linked ABC transporters: Pgp (*17*, *22*), MRP1 (this work), and mitoxantrone resistance protein (MXR; ABCG2) (*31*). Transiently expressed WT Pgp in this system is predominantly core glycosylated. Many lines of evidence show for Pgp that the core glycosylated or unglycosylated protein is fully functional (*17*, *23*, *32*−*34*). The unglycosylated form of the human CFTR protein also shows the same properties as the fully glycosylated protein. Upon removal of the two glycosylation sites in CFTR by in vitro mutagenesis, it is still transported to the cell surface and functions as a chloride channel (*35*, *36*). The probable role of glycosylation may be in targeting membrane proteins, such as Pgp, to the cell surface, in stabilizing the protein against proteolytic degradation at the cell surface (*16*), or in proper folding of the molecule within the endoplasmic reticulum during biosynthesis (*17*).

In studies on human MRP1, inhibition of N-linked glycosylation by tunicamycin did not inhibit the transport function of the protein (*4*). MRP1 expressed in baculovirus-infected insect cells is underglycosylated but functional and shows the properties of the fully glycosylated counterpart (*5*, *37*). In MRP1, three of the potential glycosylation sites are utilized (*10*), two of them in the first extracellular loop of MSD1, at amino acid position 19 and 23, and the third one in the first extracellular loop of MSD3, at position 1006. Upon removal of the glycosylation sites (mutation of residues 19, 23, and 1006, N → Q), MRP1 does not get glycosylated. The replacement of Asn with Gln did not result in a significant change in the expression of the glycosylation-deficient MRP1s in our studies. Expressing the WT protein in a Vaccinia virus-based expression system, we observed two separate bands on an immunoblot, one represents the fully glycosylated mature form (∼190 kDa), the other represents the core-glycosylated immature form (∼150 kDa), due to incomplete glycosylation of the overexpressed protein. The mobility of the completely unglycosylated QQ-Flag4 construct was altered compared to WT, and only a single band of unglycosylated protein was detected (see Figure 4). The FACS analysis of the cell surface expression experiments showed that MRP1, containing the FLAG epitope tag in the second MSD (third extracellular loop), MRP1-Flag1, was inaccessible to monoclonal antibody M2. The FLAG tag at this location remained inaccessible even in the partial or full absence of glycosylation (QQ-Flag1, QQQ-Flag1); see Figure 2. However, the calcein-AM efflux assays clearly demonstrate that the MRP1-Flag1 proteins are at the cell surface (Figure 5).

The results presented in Figures 2−4 and 6 and Table 3 suggest that the utilization of glycosylation sites at amino acid residues 19, 23, and 1006 masks the accessibility of extracellular loops in the second and third transmembrane domain of MRP1, and the extracellular regions within the three MSDs interact with each other, respectively. On the basis of our results, we propose a model for the effect of glycosylation on the accessibility of extracellular regions of human MRP1. Figure 7 shows a schematic model of interaction between the external loops of MSD1, 2, and 3 and the accessibility of FLAG epitope. In Flag5 constructs, where the FLAG sequence was introduced into the first extracellular loop of MSD1, the tag is accessible even in the fully glycosylated protein, and the removal of the glycosylation sites further increases the accessibility of the tag. This is not surprising as the extracellular N-terminal tail of the protein, which is comprised of 32 residues, is possibly very flexible. MSD2 appears to be buried, or much closer to the membrane, compared to MSD1 and MSD3. If the FLAG tag is placed here, neither the partial nor the full removal of glycosylation was sufficient to make it accessible to M2 antibody. The introduction of Flag4 sequence into the first extracellular loop of MSD3 removes the glycosylation site at position 1006. The FLAG tag in this construct shows a weak accessibility, which dramatically increases with the removal of the N-terminal glycosylation sites (QQ-Flag4 construct). In case of the truncated protein where the entire first transmembrane domain is deleted (residues 1−203), insertion of the Flag4 sequence ensures excellent accessibility for the FLAG tag. This model is supported by the results we obtained from the experiments done with the stably transfected HEK293 cells, using the WT MRP1 protein and two mutant FLAG constructs, MRP1-QQFlag4 and MRP1-Flag5QQQ. The FLAG epitope insertion did not alter the level of resistance conferred to etoposide or daunorubicin in the stably transfected HEK293 cells (see Table 4).

In conclusion, insertion of the FLAG epitope can be useful to assess the level of expression of MRP1 (only unglycosylated variants) or other membrane proteins at the cell surface without permeabilizing the cells. Our results clearly demonstrate that the unglycosylated MRP1 with and without the FLAG tag is fully functional, and the FLAG-tagged unglycosylated variants retain their ability to confer resistance to anticancer agents in stable cell lines. Thus, the unglycosylated FLAG-tagged MRP1 should prove useful for the structure/function analysis of this transporter. A number of attempts by various groups have failed to generate monoclonal antibodies detecting external epitopes of MRP1 or other MRP (ABCC) subfamily members (*18*, *38*). Similar studies based on the accessibility of Flag tag with other ABCC family members will be useful to assess the role the extracellular domains and the influence of glycosylation in presentation of extracellular antigenic epitopes.

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