Cysteine-scanning mutagenesis provides no evidence for the extracellular accessibility of the nucleotide-binding domains of the multidrug resistance transporter P-glycoprotein

Emma J.Blott, Christopher F.Higgins and Kenneth J.Linton

MRC Clinical Sciences Centre, Imperial College School of Medicine, Hammersmith Hospital, Du Cane Road, London W12 ONN and CRC Drug Resistance Group, Nuffield Department of Clinical Biochemistry, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DU, UK

Multidrug resistance of cancer cells is, at least in part, conferred by overexpression of P-glycoprotein (P-gp), a member of the ATP-binding cassette (ABC) superfamily of active transporters. P-gp actively extrudes chemotherapeutic drugs from cells, thus reducing their efficacy. As a typical ABC transporter, P-gp has four domains: two transmembrane domains, which form a pathway through the membrane through which substrates are transported, and two hydrophilic nucleotide-binding domains (NBDs), located on the cytoplasmic side of the membrane, which couple the energy of ATP hydrolysis to substrate translocation. It has been proposed that the NBDs of ABC transporters, including the histidine permease of Salmonella typhimurium and the cystic fibrosis transmembrane conductance regulator, are accessible from the extracellular surface of the cell, spanning the membrane directly or potentially contributing to the transmembrane pore. Such organization would have significant implications for the transport mechanism. We determined to establish whether the NBDs of P-gp are exposed extracellularly and which amino acids are accessible, using cysteine-scanning mutagenesis and limited proteolysis. In contrast to other transporters, the data provided no evidence that the P-gp NBDs are exposed to the cell surface. The implications for the structure and mechanism of P-gp and other ABC transporters are discussed.

Keywords: ABC transporter/cysteine-scanning mutagenesis/multidrug resistance/nucleotide-binding domain/P-glycoprotein

Introduction

Treatment with cytotoxic drugs is an important aspect of cancer therapy, complementing surgery and radiotherapy. A major impediment to successful chemotherapy is the occurrence of multidrug resistance (MDR), which causes the malignant cells to become refractory to a wide range of cytotoxic drugs. Multidrug resistance is commonly due to the active extrusion of these drugs from the cell by a 180 kDa plasma membrane protein, P-glycoprotein (P-gp; reviewed by Gottesman and Pastan, 1993). P-gp, encoded by the MDRI gene in humans, is a member of the ATP-binding cassette (ABC) transporter superfamily (Higgins et al., 1986; Hyde et al., 1990). The protein comprises 1280 amino acids organized into four domains: the two transmembrane domains (TMDs), which span the membrane multiple times and form the pathway through which solute crosses the membrane, and the two nucleotide-binding domains (NBDs), which bind and hydrolyse ATP.

However, little is known about the structure of P-gp or any other ABC transporter. Although high resolution structures of the NBDs of two bacterial ABC transporters have been determined (Hung et al., 1998; M.Hermodsen and C.Stauffacher, personal communication), these shed little light on the overall organization of the transporter or how it might work. Current models for the transport cycle suggest an intimate interaction between the NBDs and TMDs: substrate binding by the TMDs induces ATP hydrolysis by the NBDs, and the energy of ATP hydrolysis is transduced to the TMDs to drive solute translocation across the membrane. A low-resolution electron microscopy structure of P-gp is consistent with this model, showing a large hydrophilic chamber through the lipid bilayer, which is closed at the cytoplasmic face of the membrane, and two cytoplasmic lobes, which probably correspond to the NBDs, closely associated with the TMDs (Rosenberg et al., 1997; M.F.Rosenberg, R.Callaghan, I.D.Kerr, K.J.Linton, G.Velarde, R.C.Ford and C.F.Higgins, submitted).

An understanding of how the NBDs interact with the TMDs is crucial to understanding the process of energy transduction and substrate translocation within the transport complex. Although there is no doubt that the bulk of the NBDs is peripherally located at the cytoplasmic face of the membrane, studies on several ABC transporters have led to the proposal that part of the NBD may span the membrane and be exposed to the extracellular surface, either directly through the lipid bilayer or through a pore formed by the TMDs. Specifically, labelling with biotinylated probes and protease accessibility studies have led to the suggestion that a portion of the NBDs of the histidine permease of Salmonella typhimurium (Baichwal et al., 1993), the maltose transporter of Escherichia coli (Schneider et al., 1995), a mutant form of the polysialic acid transporter of E.coli K1 (Bliss and Silver, 1997) and human cystic fibrosis transmembrane conductance regulator (CFTR) (Gruis and Price, 1997; Ko et al., 1997; Clancy et al., 1998) are exposed to the extracellular face of the membrane.

The hypothesis that the NBDs of ABC transporters are exposed extracellularly has become the accepted dogma and has implications for the mechanisms of signal and energy transduction between the TMDs and NBDs. It has also been proposed that residues within the NBD might contribute to the substrate translocation pathway,
potentially lining the transmembrane pore and contributing to substrate specificity (Baichwal et al., 1993; Clancy et al., 1998). As the data leading to the hypothesis were obtained for bacterial ABC transporters, and an isolated domain of a eukaryotic ABC transporter (CFTR), we set out to test this hypothesis rigorously for P-gp and to identify those regions of the NBD that are exposed extracellularly, using minimally invasive, cysteine-scanning mutagenesis. The accessibility of a single cysteine introduced at defined locations in the NBD was assessed using a membrane-impermeant, thiol-reactive probe. In addition, limited proteolysis was used to determine the accessibility of the NBDs to proteases at the extracellular surface of the membrane. Unexpectedly, and in apparent contrast to studies on bacterial ABC transporters and CFTR, we obtained no evidence that the NBDs of P-gp are exposed to the extracellular face of the membrane. Thus, at least for this ABC transporter, the NBDs cannot traverse the membrane or line the transmembrane pore. The possibility that this reflects the organization of all ABC transporters, and the implication for the mechanism of transport, are discussed.

Results

Introduction of single cysteines into P-gp by site-directed mutagenesis

Human P-gp contains seven cysteine residues. These seven cysteines were replaced by serine residues by site-directed mutagenesis of the MDR1 gene. The cysteine-less version of the MDR1 gene (MDR1-cys–), when expressed in insect cells using a baculovirus vector (Clontech), encoded a protein with similar drug-binding and ATPase characteristics to wild-type P-gp (L.Soceneantu, R.Callaghan, K.J.Linton and C.F.Higgins, unpublished results). It has previously been shown that replacement of these cysteines by alanines also has minimal effect on P-gp function (Loo and Clarke, 1995).

Twenty-eight cysteine residues were then introduced, individually, throughout the N-terminal NBD (NBD1) of cysteine-less P-gp (P-gp-cys–), by site-directed mutagenesis. The locations of the cysteines introduced were in regions of variable sequence predicted to be in surface loops based on structural modelling (Hyde et al., 1990) and the crystal structure of HisP (Hung et al., 1998) or RbsA (M.Hermodsen and C.Stauffacher, personal communication). The residues replaced by cysteine were amino acids considered unlikely to be essential for P-gp function based on comparison with non-human P-gp sequences and other ABC transporters. Each residue replaced by cysteine is indicated on the primary sequence of NBD1 (Figure 1A), and superimposed on the tertiary structure of HisP (Figure 1B). Two additional single-cysteine (SC) mutants (N280C and G324C), known to be located in an intracellular and extracellular loop of TMD1, respectively (Loo and Clarke, 1995), were also generated as controls.

Transient expression of single-cysteine P-gp mutants in a human cell line

The coding sequences for wild-type (wt) P-gp, P-gp-cys– and the 30 SC mutants were cloned into the mammalian expression vector, pCI-neo (Promega), and the resulting plasmids introduced into HEK293T cells by liposome-mediated transfection (lipofectin). P-gp-cys– and 21 of the 30 SC mutants (including the control mutants SC-N280C and SC-G324C) expressed full-length protein in both the lower molecular weight non-glycosylated and the higher molecular weight glycosylated forms (Figure 2). Four SC mutants (P549C, V569C, K578C and R580C) were expressed at significantly lower levels than wild-type P-gp, but use of an alternative transfection reagent (lipofectamine) allowed sufficient expression for further analysis. The five SC mutants that did not express any detectable protein product (see Figure 1A) were not assayed further.

Functional characterization of SC-P-gp mutants

Each SC-P-gp mutant was tested for function following transient expression in HEK293T cells, using a dual-label, fluorescence-activated cell sorting (FACS) assay to correlate drug transport with the expression of P-gp at the cell surface. Transfected cells were incubated with the fluorescent P-gp substrate, rhodamine 123 (R123), and subsequently with the P-gp-specific monoclonal antibody UIC2 (conjugated to phycoerythrin, PE), and subjected to FACS analysis. Cells that express functional P-gp at the cell surface react with UIC2–PE and extrude R123. Untransfected cells fail to react with UIC2–PE and accumulate R123.

An example of one such dual-label FACS experiment is shown in Figure 3. Figure 3A and B shows data for mock-transfected cells (cells transfected with vector alone, therefore no P-gp expression). As expected, in the absence of R123 and UIC2–PE all cells exhibited low levels of fluorescence (Figure 3A). When incubated with R123 and UIC2–PE, the mock-transfected cells (Figure 3B) all showed low UIC2–PE fluorescence and high R123 fluorescence, indicating accumulation of R123 due to the absence of functional P-gp. In contrast, NIH 3T3–MDR cells (Shen et al., 1986) permanently transfected with human MDR1 (Figure 3C) all show high UIC2–PE fluorescence and low R123 fluorescence, indicative of cell surface expression of P-gp, which extrudes R123. The HEK293T cells, after transient transfection with pMDR-cys–, form two distinct populations of cells (Figure 3D). One population of cells showed little or no UIC2–PE fluorescence (indicating no surface expression of P-gp) and high R123 fluorescence (indicating no P-gp activity): these represent the untransfected fraction of cells and are indistinguishable from mock-transfected cells. The second population of cells represent those cells successfully transfected and they behave akin to NIH 3T3–MDR cells; the high UIC2–PE fluorescence demonstrating surface expression of P-gp and low R123 fluorescence indicating extrusion of R123 and, hence, P-gp function. Confirmation that the extrusion of R123 was due to P-gp activity was obtained by addition of the P-gp inhibitor verapamil, which increased the R123 fluorescence of the successfully transfected (UIC2–PE-reactive) population (Figure 3E). The reduction in R123 fluorescence, corrected for the level of cell surface expression of P-gp (UIC2–PE fluorescence), allowed a semi-quantitative assay of P-gp activity. Triplicate analysis showed that P-gp-cys– was more active than wild-type P-gp by ~30%; this is consistent with ATPase assays on the mutant protein (our unpublished data). The majority of the SC-P-gp mutants exhibited a similar activity to P-gp-cys– (within 20%), although...
mutations L554C and I488C resulted in proteins that had approximately half and twice the activity of P-gp-cys+, respectively. These activity data indicated that the folding of each of the SC-P-gp proteins must be close to that of the wild-type protein and therefore appropriate for structural analysis.

**Extracellular accessibility of the unique sulfydryl groups in NBD1 of P-gp**

The accessibility of the unique sulfydryl groups in the SC-P-gp mutants was assessed by a multi-step assay using biotin maleimide (BM), a membrane-impermeant, thiol-reactive probe. BM was added to intact HEK 293T cells transiently transfected to express wild-type or mutant P-gps, and the biotinylated proteins purified from cell lysates using neutravidin affinity chromatography. The retention of P-gp in the biotinylated fraction determined by Western blot analysis depends on its labelling by BM and, hence, the accessibility of the cysteine sulfydryl group to the extracellular face of the membrane. To establish the validity of the assay, SC-P-gp mutants G324C (known to be located in an intracellular loop of TMD1) and N280C (known to be located in an intracellular loop of TMD1) were used as positive and negative controls, respectively. In intact cells, the higher molecular weight, glycosylated P-gp-G324C was biotinylated while neither form of P-gp-N280C was biotinylated (Figure 4A), as expected. To demonstrate that P-gp-N280C was expressed and could be labelled by BM, the same assay was carried out in the presence of saponin, a gentle membrane permeabilizing agent. In this case, both the mature and immature (non-glycosylated) forms of G324C and N280C P-gp were labelled equivalently (Figure 4B), demonstrating that they are reactive once the membrane is permeabilized. Thus, none of the single cysteines introduced into NBD1 is exposed at the extracellular face of the membrane.

The data in Figure 4A show that none of the 23 single cysteines introduced into NBD1 of P-gp was accessible to BM from the extracellular cell surface. However, each SC-P-gp mutant was labelled by BM following permeabilization of the membrane with saponin (Figure 4B), demonstrating that they are reactive once the membrane is permeabilized. Thus, none of the single cysteines introduced into NBD1 is exposed at the extracellular face of the membrane.

Following membrane permeabilization, both the mature and immature forms of P-gp are expected to be accessible to BM. However, for some of the mutant P-gps (I469C, I488C, Y490C and I500C) only the immature form was labelled. As these mutant proteins are all active and expressed at the cell surface as mature, glycosylated protein, the cysteines located at positions 469, 488, 490 and 500 must become buried and inaccessible to BM during folding and maturation of the protein.

**Protease susceptibility of P-gp**

The biotinylation experiments above show that none of the cysteine residues introduced into NBD1 of P-gp are exposed to the extracellular face of the membrane. As these cysteines are scattered throughout NBD1, this excludes the possibility that any large part of the NBD traverses the membrane. However, it is conceivable that a small region of NBD1, or of NBD2, is exposed at the cell surface. As both NBDs of P-gp contain many putative trypsin and proteinase K cleavage sites, and because a proteolysis assay was used to conclude that the NBDs of bacterial ABC transporters are exposed at the extracellular face of the membrane, the sensitivity of P-gp to digestion by extracellular proteases was determined. NIH 3T3–MDR1 cells stably expressing wild-type P-gp were incubated with increasing concentrations of protease (trypsin or proteinase K) in the presence or absence of saponin. The amount of P-gp remaining after protease treatment was determined by Western blot analysis (Figure 5A and B). As a control for the integrity of the cell membrane, blots were also probed with an anti-β-tubulin mAb (Figure 5C and D).

In the absence of membrane permeabilization by saponin, P-gp was remarkably resistant to digestion by trypsin (Figure 5A). Digestion of P-gp by proteinase K was slight at 0.1 μg/ml of protease and complete at the highest level of protease used (1 μg/ml). However, at this high concentration of protease, β-tubulin was also degraded, indicating that membrane integrity had been disrupted. Following gentle permeabilization with saponin, more-or-less complete proteolysis of P-gp (and β-tubulin) was observed even at the lowest concentrations of protease used (Figure 5). Taken together, these results demonstrated that in intact cells, P-gp is not particularly susceptible to
extracellular proteolysis until sufficient protease is added to disrupt membrane integrity. Thus, none of the protease cleavage sites in the NBDs is exposed at the cell surface.

**P-gp is susceptible to deglycosylation**

The above observation that P-gp in intact cells is resistant to proteolysis could be a consequence of the experimental conditions preventing enzyme reaching the cell surface, rather than the inaccessibility of cleavage sites. To demonstrate that P-gp is accessible to extracellular enzymes in intact cells, the cells were treated with a deglycosylation enzyme, N-glycosidase F. The data in Figure 6 show that P-gp is readily susceptible to deglycosylation by this enzyme and, hence, is generally accessible to enzyme action.

**Discussion**

In this study we set out to test the hypothesis, proposed on the basis of studies on bacterial ABC transporters and CFTR, that the NBDs of P-gp are accessible from the extracellular face of the membrane and to identify those residues that are exposed. Unexpectedly, the data obtained using two independent experimental approaches, cysteine-scanning mutagenesis and protease accessibility, provided no evidence that the NBDs of P-gp are exposed to the extracellular face of the membrane. Although we cannot exclude the possibility that a very small number of residues in NBD1 are accessible from the extracellular face of the membrane through the pore of the transporter (i.e. a region where no cysteine was introduced and where there are no sites for trypsin and proteinase K), the data effectively exclude the possibility that the NBDs span the membrane or line the pore of the transporter.

The finding that the NBDs of P-gp are not accessible from the extracellular face of the membrane is apparently at odds with previous reports for other ABC transporters. It is, of course, possible that the overall architecture of P-gp differs from that of other ABC transporters: the other ABC transporters studied are bacterial uptake systems and CFTR, which is unique amongst known ABC transporters since it functions as an ion channel. However, the many sequence and mechanistic similarities between ABC transporters (Higgins, 1992) and their common evolutionary origin (Saier, 1996) makes it unlikely that their overall architecture is substantially different. Thus, in the light of the present data for P-gp, the data that led to the suggestion that the NBDs of other transporters are exposed extracellularly bear re-examination. The principal studies on the bacterial histidine and maltose transporters used protease sensitivity in membrane vesicle preparations to assess extracellular accessibility (Baichwal et al., 1993; Schneider et al., 1995). Under these conditions, the NBDs were actually remarkably resistant to proteolysis in right-side out vesicles: degradation was incomplete even at high concentrations of protease (up to 5 mg/ml) and following long incubation periods (up to 10 h), conditions considered extreme by others (Nikaido and Hall, 1998). Less extreme conditions were shown to be sufficient for cleavage of the TMDs of the maltose transporter (Schneider et al., 1995; B.Traxler, personal communication), and so it is not actually possible to conclude from the data published that...
the NBDs in the intact transport complex were indeed accessible to protease at the surface of the vesicles or whether prior degradation of the TMDs resulted in release and degradation of some of the NBDs.

In addition to protease accessibility studies, the NBD of the histidine permease (HisP) was also found to be accessible to biotinylation from the extracellular face of the membrane in vesicular preparations (Baichwal et al., 1993). It has been reported that primarily residue K204 is labelled in these experiments (Hung et al., 1998), although details of this study have not been published. We have shown that the equivalent residue in NBD1 of P-gp, mutated to a cysteine (R580C), is not accessible in whole cells. Furthermore, R580 of P-gp is a potential trypsin cleavage site but it was not recognized by extracellular protease. Thus, at least in P-gp, this residue does not appear to be exposed at the extracellular face of the membrane.

The protease accessibility studies on bacterial ABC transporters were carried out using membrane vesicles rather than intact cells. The aptness of membrane vesicles for such analyses is questioned by recent evidence that showed that the protease accessibility of SecA, a component of the protein translocase in E.coli, depends on how the membranes are prepared for assay. SecA is sensitive to proteolysis in right-side out membrane vesicles (Kim et al., 1994; van der Does et al., 1996; Ramamurthy and Oliver, 1997), but resistant when assayed in spheroplasts (Eichler and Wickner, 1998). Furthermore, the F1-ATPase used as a negative control in the histidine permease study, has been shown to reorient during membrane vesicle preparation (Wickner, 1976). It is also important that the protein is in the native conformation. Studies on NBD1 of CFTR using intact cell membranes, which led to the suggestion of extracellular accessibility (Gruis and Price, 1997; Ko et al., 1997; Clancy et al., 1998), relied on expression of a truncated NBD, which, perhaps surprisingly, located to the membrane even in the absence of the TMDs. As the function of this isolated domain could not be demonstrated (it did not bind ATP or 8-azido-ATP) there is no evidence that it is correctly folded and its properties may therefore not reflect the native state. In contrast, full-length functional P-gp expressed in mammalian cells is expected to be actively transporting during our biotinylation experiments: P-gp-dependent intracellular alkazation of multidrug-resistant cells (Thiebaut et al., 1990) and the high basal ATPase activity of P-gp-containing membranes (Sarkadi et al., 1992; Al-Shawi and Senior, 1993) both suggest that P-gp transports natural substrates found in biological systems.

In hindsight, therefore, data suggesting that the NBDs of other ABC transporters are exposed at the cell surface may be subject to alternative interpretations and, in the light of present data for P-gp, it is possible that the general architecture of ABC transporters does not include the exposure of a significant proportion of the NBD at the cell surface. This conclusion is consistent with other data. The low-resolution structure of P-gp (Rosenberg et al., 1997; M.F.Rosenberg, R.Callaghan, I.D.Kerr, K.J.Linton, G.Velarde, R.C.Ford and C.F.Higgins, submitted) shows a large transmembrane pore with no evidence of the NBD inserting through this pore. However, the NBDs are closely associated with the cytoplasmic face of the membrane and appear to be partially embedded in the plane of the lipid bilayer. The high-resolution structures of the NBDs of two bacterial ABC transporters, HisP (Hung et al., 1998) and RbsA (C.Stauffacher, personal communication) do not show a large loop or segment that might be expected to span the membrane. Finally, subcellular fractionation of bacterial ABC transporters, in which the NBDs and TMDs are encoded as separate polypeptides, showed that the NBDs are only peripherally associated with the membrane (Ames, 1986; Gallagher et al., 1989; Kerppola et al., 1991).

What are the implications of these findings for the mechanisms of transport by P-gp (and other ABC transporters)? It now seems unlikely that the NBDs contribute to the transmembrane pore of P-gp and, hence, to determining substrate specificity. Consistent with this is the absence of any significant evidence that the NBDs contribute to substrate specificity, in contrast to the TMDs, which can be labelled by photoaffinity substrate analogues and where mutations alter substrate specificity (reviewed by Gottesman and Pastan, 1992; Bosch and Croop, 1996).

Additionally, if the NBDs do not span the membrane at any point in the catalytic cycle, models in which the NBD, or part of the NBD, moves in and out of the membrane (or pore) to facilitate translocation of substrate would seem less likely. For bacterial periplasmic binding protein (PBP)-dependent transporters, it would now seem less likely that the NBDs contact the periplasmic protein directly. Indeed, the original genetic evidence that such contacts might be made (Ames and Spudich, 1976) have now been reinterpreted (Petronelli and Ames, 1991), and
Site-directed mutagenesis

The coding sequence of NB1 of the human MDR1-cys gene was excised from pMDR-cys using the EcoRI and NolI sites and sub-cloned into pALTER (Promega) to yield plasmid pEB1, which was used for all subsequent mutagenesis steps. Single-cysteine codons were introduced into the coding sequence of NB1 by oligonucleotide-directed mutagenesis (‘Altered sites’ II; Promega). The mutagenic oligonucleotides used included both the desired codon change and a silent diagnostic restriction site with which to screen putative mutants (Table I). The nucleotide sequence of the mutated DNA was verified by automated DNA sequencing (Perkin Elmer), before subcloning the mutated DNA fragment back into pMDR-cys to give the pSC plasmids.

Transient expression in mammalian HEK 293T cells

HEK 293T human epithelial kidney cells (Imperial Cancer Research Fund, cell production unit) were maintained by regular passaging in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 2 mM l-glutamine and 10% (v/v) fetal calf serum (FCS, Sigma) under 5% CO₂ at 37°C. Typically, 5 × 10⁶ cells were seeded in a 35 mm dish pre-coated with 0.01% poly-L-lysine (Sigma) and incubated for 18 h to give ~80% confluency. The cells were transfected with 5 or 5 µg DNA using the cationic lip (lipofectamine or lipofectin, Gibco-BRL), respectively, as described by the manufacturer. Twenty-four hours post-transfection the medium was replaced with fresh DMEM plus 2 mM butyric acid (Sigma) to enhance expression of P-gp. The cells were analysed a further 24 h later.

Western blot analysis

Whole cell lysates were prepared from transiently transfected cells by adding to the cell monolayer 300 µl lysis buffer [50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NP-40 (Pierce), 100 µM leupeptin, 1 mM benzamidine, 4 µg/ml pepstatin and 1 mM ethylenediaminetetra-acetic acid (EDTA)]. Cell lysate (75 µg unless otherwise stated) was separated by SDS–PAGE and the proteins transferred electrophoretically to a nitrocellulose membrane (HiBond, Amersham). The membrane was probed with the anti-P-gp monoclonal antibody, C219 (Cis-Bio International) and developed using horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody and enhanced chemiluminescence (Amersham).

Biotinylation of P-gp

Transfected cells were incubated with 5 ml reducing buffer [50 mM dithiothreitol (DTT), 1 mM EDTA in PBS] for 5 min at room temperature. The cells were washed twice with PBS and then incubated with 2 µg/ml R123 (Sigma), with or without verapamil (50 µM) as indicated, and incubated for a further 30 min at 37°C. The cells were washed three times with versene, then incubated with 20 µg/ml anti-P-gp antibody (UC2–PE; Immunotech) diluted in FACS buffer (1% FCS in PBS) for 15 min at 4°C. The cells were washed three times in FACS buffer before resuspension in 300 µl FACS buffer. Samples were stored in the dark at 4°C until analysis. Flow cytometric analysis of the cells was carried out using a FACSScan Calibur flow cytometer (Becton Dickinson). PE fluorescence was measured at 606 nm and R123 fluorescence at 515 nm. Fluorescence data were collected from 50 000 events on a four-decade log scale and analysed using CELLQuest (Becton Dickinson) software.

Materials and methods

Mammalian expression plasmids pMDR-wt and pMDR-cys

The wild-type human MDR1 gene and the mutant gene MDR1-cys, encoding a cysteine-less P-gp in which the seven endogenous cysteine residues of the wild-type protein have been replaced with serines, have been generated, flanked by a 5’ SalI site and Kozak sequence and a 3’ NcoI site (K.J.Linton and L.Soceneantu, unpublished data). These genes were subcloned into the mammalian expression vector pCI-neo (Promega) to yield the plasmids pMDR-wt and pMDR-cys, respectively. Both plasmids encode functional P-gp protein with a His₆ tag at the C-terminus.

Assay for P-gp function and membrane localization

HEK 293T cells (3 × 10⁶) were transfected using lipofectamine (Gibco-BRL) in 80 mm cell culture dishes. Forty-eight hours post-transfection, the cells were harvested by incubation with versene [2 mM EDTA in phosphate buffered saline (PBS)] for 10 min at 37°C. The cells were washed twice with PBS and then incubated with 2 µg/ml R123 (Sigma), with or without verapamil (50 µM) as indicated, and incubated for a further 30 min at 37°C. The cells were washed three times with versene, then incubated with 20 µg/ml anti-P-gp antibody (UC2–PE; Immunotech) diluted in FACS buffer (1% FCS in PBS) for 15 min at 4°C. The cells were washed three times in FACS buffer before resuspension in 300 µl FACS buffer. Samples were stored in the dark at 4°C until analysis. Flow cytometric analysis of the cells was carried out using a FACSScan Calibur flow cytometer (Becton Dickinson). PE fluorescence was measured at 606 nm and R123 fluorescence at 515 nm. Fluorescence was measured at 606 nm and R123 fluorescence at 515 nm. Fluorescence data were collected from 50 000 events on a four-decade log scale and analysed using CELLQuest (Becton Dickinson) software.
Table I. Oligonucleotides used for site-directed mutagenesis

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<th>pSC-name</th>
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<td>CGCAAACCCAAATTATGCCGTGCGATGAGGCCGCC</td>
</tr>
<tr>
<td>S565C</td>
<td>+BsmI</td>
<td>GACACAGAATCCGGAAGGACG</td>
</tr>
<tr>
<td>V569C</td>
<td>+BsmI</td>
<td>GACACAGAATCCGGAAGGACG</td>
</tr>
<tr>
<td>K578C</td>
<td>+BglII</td>
<td>GAGAACGAGGCGCCAGAGTGGTGAGATGGAGAGA</td>
</tr>
<tr>
<td>R580C</td>
<td>−MolI</td>
<td>GCCCAAAAAGGTGTCGACAGACCAGTTTGTA</td>
</tr>
<tr>
<td>T581C</td>
<td>+ApaI</td>
<td>GAAAGAGTGCGTGCCACCATTGTTG</td>
</tr>
<tr>
<td>E638C</td>
<td>+EcoXI</td>
<td>GAAGTTGAAATATGCGATGCGGCAATGAGATC</td>
</tr>
</tbody>
</table>

‘+’ represents the introduction of a new restriction endonuclease site; ‘−’ represents the removal of an existing site. Nucleotide changes are underlined.

Protease assay
NIH 3T3–MDR cells are NIH 3T3 murine fibroblasts permanently transfected with human MDR1, and selected for high-level resistance to colchicine (Shen et al., 1986). The cells (4 × 10⁶) were incubated with various concentrations of protease (trypsin or proteinase K; Sigma), with or without 0.1% saponin as indicated, in a final volume of 100 μl in PBS. The reaction was incubated at 37°C for 30 min with occasional gentle mixing. Leupeptin (100 μM final concentration) or phenylmethylsulfonyl fluoride (PMSF, 1 mM final concentration) was added to the samples to inhibit trypsin or proteinase K activity, respectively, and the incubation continued for 10 min at room temperature. The cells were lysed by addition of 100 μl lysis buffer and the lysates analysed by SDS–PAGE and Western blotting. The membranes were first probed with C219 antibody to detect P-gp, and then re-probed with anti-β-tubulin antibody (15 μg/ml; Amersham).

Deglycosylation of P-gp
NIH 3T3–MDR cells (4 × 10⁶) were resuspended in 100 μl deglycosylation buffer (G7 buffer; NEB) with 0–50 U/μl PNGase F (NEB) and incubated at 37°C for 30 min, with occasional gentle mixing. The cells were lyzed by addition of 100 μl lysis buffer and the lysates analysed by SDS–PAGE and Western blotting. The membranes were first probed with C219 antibody to detect P-gp, and then re-probed with anti-β-tubulin antibody (15 μg/ml; Amersham).

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References


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