Characterization of the human multidrug resistance protein containing mutations in the ATP-binding cassette signature region

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A number of mutants with single amino acid replacements were generated in the highly conserved ATP-binding cassette (ABC)-signature region (amino acids 531–543) of the N-terminal half of the human multidrug resistance (MDR1) protein. The cDNA variants were inserted into recombinant baculoviruses and the MDR1 proteins were expressed in *Spodoptera frugiperda* (Sf9) insect cells. The level of expression and membrane insertion of the MDR1 variants was examined by immunostaining, and MDR1 function was followed by measuring drug-stimulated ATPase activity. We found that two mutations, L531R and G534V, practically eliminated MDR1 expression; thus these amino acid replacements seem to inhibit the formation of a stable MDR1 protein structure. The MDR1 variants G534D and I541R were expressed at normal levels with normal membrane insertion, but showed a complete loss of drug-stimulated ATPase activity, while mutant R538M yielded full protein expression but with greatly decreased ATPase activity. Increasing the ATP concentration did not restore MDR1 ATPase activity in these variants. Some amino acid replacements in the ABC-signature region (K536I, K536R, I541T and R543S) affected neither the expression and membrane insertion nor the ATPase function of MDR1. We found no alteration in the drug-sensitivity of ATP cleavage in any of the MDR1 variants that had measurable ATPase activity. These observations suggest that the ABC-signature region is essential for MDR1 protein stability and function, but alterations in this region do not seem to modulate MDR1–drug interactions directly.

INTRODUCTION

The multidrug resistance phenotype in cancer cells is often caused by the overexpression of special membrane transport proteins, the P-glycoprotein [also known as the multidrug resistance protein (MDR1)] and/or the multidrug resistance-associated protein. It is well established that these proteins carry out the ATP-dependent extrusion of the relevant cytotoxic drugs and that the hydrolysis of ATP provides the energetic basis for these transport processes. Both MDR1 and the multidrug resistance-associated protein are members of the ABC (ATP-binding cassette) transporter family. All proteins in this family consist of two major types of polypeptide region: transmembrane domains, usually with six membrane-spanning helices, and conservative ABC units. The human MDR1 protein is built from a tandem repeat of transmembrane regions followed by ABC units (for reviews, see [1,2]).

The ABC units harbour two short consensus polypeptide sequences, first described by Walker et al. [3] (the so called Walker A and Walker B motifs), which are present in many ATP-binding and/or ATP-utilizing proteins. In addition, in all ABC transporters a short, highly conserved peptide motif of 13 amino acids (ABC-signature region) is found between the two Walker motifs [4,5] (Figure 1). The three-dimensional structure of the ABC unit is not known, but models constructed on the basis of the three-dimensional structure of two Walker A plus Walker B-type ATP-binding proteins are available [4,6]. These models suggest that the ABC-signature motif has a special, surface-exposed localization in the ABC unit chain, and the authors speculate that this part of the molecule may be responsible for the coupling of ATP hydrolysis to the transport function.

Several mutations in the ABC-signature region that seriously affect biological function have been described for various ABC transporters, e.g. for FstE, a cell division protein of *Escherichia coli* [7], for the yeast a-mating factor transporter STE6 [8,9] and for the human cystic fibrosis transmembrane conductance regulator (CFTR) [10–12]. In the case of the human P-glycoprotein, Hoof et al. [13] studied the effects of replacement of three amino acids (Ser^{532}, Gly^{534} and Lys^{536}) in the ABC-signature region of the N-terminal ABC unit. They found that the MDR1 mutants containing a Ser → Arg (S532R) or a Gly → Asp (G534D) substitution were present at very low levels in the plasma membrane, and thus did not confer drug resistance. The mutant K536Q (Lys to Gln) was expressed in comparable amounts to the wild-type protein, but conferred decreased drug resistance, while the K536R (Lys to Arg) replacement increased multidrug resistance, with a preferential resistance to colchicine. These findings further supported the possible functional importance of this region.

In the experiments reported below we have examined the effects of a range of amino acid substitutions in the ABC-signature region of the N-terminal half of human MDR1. By using an *in vitro* (baculovirus-infected insect cell) expression system, which has been found to be suitable for studying structure–function relationships of MDR1 in several reports [14–16], we have examined the expression and function of the mutant MDR1 proteins. The level of expression of these MDR1 variants was measured by quantitative immunoblotting, while their membrane insertion was assessed by immunoflow cytometry. For a functional characterization of the mutant proteins, drug-stimulated MDR1 ATPase activity was studied in isolated membrane preparations. The results suggest a profound effect of changes in this ABC-signature motif on both the expression and function of human MDR1.

Abbreviations used: ABC, ATP-binding cassette; CFTR, cystic fibrosis transmembrane conductance regulator; MDR1, multidrug resistance protein; Sf9 cells *Spodoptera frugiperda* cells.

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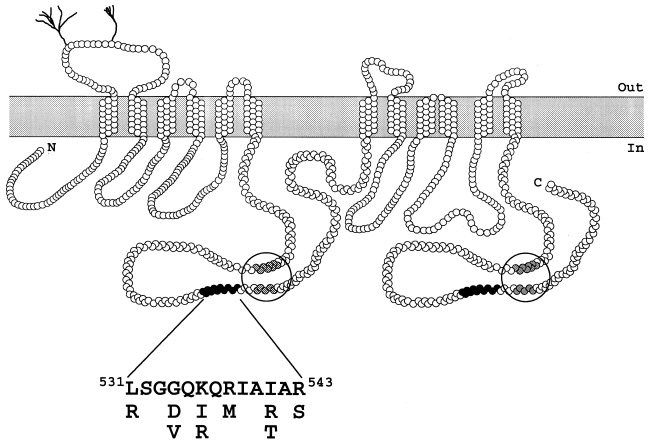

Figure 1 Membrane topology model of the human MDR1 protein: localization, composition and point mutations in the ABC-signature regions

N-linked glycosylation sites are shown by solid lines; predicted ATP-binding sites, containing the Walker A and Walker B motifs, are circled. The ABC-signature regions are represented by filled symbols, and the N-terminal ABC-signature region (residues 531–543) and the amino acid replacements carried out in this work are represented by single-letter codes of the relevant amino acids.

EXPERIMENTAL

Construction of recombinant transfer vectors

Recombinant baculovirus transfer vectors carrying the MDR1 ABC-signature mutants were constructed as follows. The full-length human MDR1 cDNA was cloned into the transfer vector ΔpAcUW21, as described previously [17]. A 2.2 kb *Eco*RI–*Pst*I fragment (corresponding to nucleotides 1177–3372) was removed from the MDR1 cDNA (ΔpAcUW21MDR1) and inserted into M13mp18. Mutations were engineered by the site-directed mutagenesis technique of Kunkel [18] utilizing the following mutagenic oligonucleotides: L531R, 5′ CCACCACTCCGCTGGGCCCCT; G534D, 5′ TGCTTCTGAACACCACTCAAT; G534V, 5′ TGCTTCTGATCACCACTCAAT; K536R, 5′ GATCCTCTGTCTCTGCCCACCAC; K536I, 5′ GATCCTCTGTATCTGCCCACCAC; R538M, 5′ GCACGTGCAATGGCGATCATCTGCTTG; I541R, 5′ GCACGTGCTCTGGCGATCCTCTGCTTG; I541T, 5′ GCACGTGCTGTGGCGATCCTCTGCTTG; R543S, 5′ AACCAGGGCACTTGCAATGGCGAT. All mutations were confirmed by DNA sequencing. The fragments containing the mutants from two parallel clones of M13mp18 were inserted back into the original position in ΔpAcUW21MDR1.

Generation of recombinant baculoviruses

Recombinant baculoviruses, carrying the different mutants of the human MDR1 cDNA, were generated by using the BaculoGold Transfection Kit (PharMingene), according to the manufacturer's suggestions. Sf9 (*Spodoptera frugiperda*) cells were infected and cultured according to the procedures described previously [16,19]. Two independent clones of viruses for each MDR1 variant were used for protein expression.

Membrane preparation and ATPase measurements

The virus-infected Sf9 cells were harvested and their membranes were isolated and stored, and the membrane protein concentrations determined, as described by Sarkadi et al. [14] and Müller et al. [16]. The ATPase activity of the isolated Sf9 cell membranes was estimated by measuring the liberation of P_i, as described in [14,16]. The data points in the Figures show the means of triplicate determinations in representative experiments. The differences between ATPase activities measured in the absence and the presence of vanadate (100 μM) are plotted.

Quantification of the MDR1 protein by immunoblotting

Electrophoresis and quantitative immunoblotting with the 4077 polyclonal antibody, which recognizes human MDR1 [20], were carried out as described by Sarkadi et al. [14] and Müller et al. [16]. The amounts of expressed MDR1 were calculated from the luminescence values, based on calibration using a dilution series of standard Sf9 MDR1 membrane preparations.

Flow cytometry

For immunofluorescence staining, 10^6 virus-infected Sf9 cells were permeabilized by 0.5% Triton X-100 in PBS containing 1% BSA, and incubated with monoclonal antibodies UIC2 [21] and MRK16 [22] (4 μg/ml) for 40 min at 4 °C. Isotype-matched non-specific antibodies were used as controls. The cells were washed three times and an FITC-conjugated second antibody (10 μg/ml; DAKO, Glostrup, Denmark) was applied similarly to the first antibody. Finally the cells were resuspended in PBS and cellular fluorescence was measured using a Becton Dickinson FACSCalibur flow cytometer. Data were analysed using Winlist software (Verity Software House, Inc.).

RESULTS

Determination of the expression levels of the mutant MDR1 proteins

Several single amino acid replacements were introduced into the ABC-signature region located in the N-terminal half of human MDR1 (amino acids 531–543; see Figure 1), and the baculovirus/Sf9 insect cell expression system was applied to produce the mutant MDR1 protein variants. The expression levels of the MDR1 variants were measured by quantitative immunoblotting. As demonstrated in Figure 2 and Table 1, for

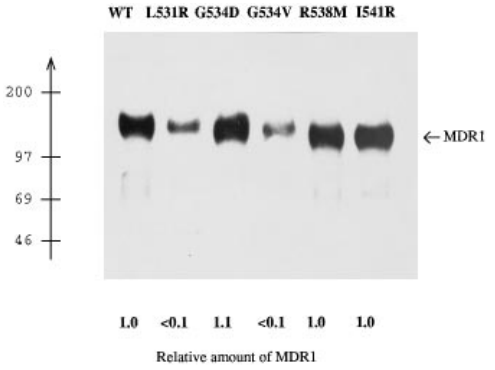

Figure 2 Immunoblot detection of human MDR1 expressed in the baculovirus/Sf9 cell system

Isolated membranes of baculovirus-infected Sf9 cells were subjected to electrophoresis and to immunoblotting with the anti-MDR1 polyclonal antibody 4077, as described in the Experimental section. Peroxidase-dependent luminescence on the immunoblots was quantified by liquid scintillation counting. The relative amounts of the expressed MDR1 protein variants are indicated below the lanes. Values on the left are molecular mass (kDa). WT, wild type.

Table 1 Characterization of the ABC-signature mutants

Expression is given relative to that of the wild-type MDR1 protein. ATPase activity is also given relative to that of the wild-type protein, and was measured in the presence of 30 μM verapamil and 3.5 mM ATP. The values are means of three independent experiments.

Mutant	Relative expression level	Relative ATPase activity
L531R	< 0.1	< 0.05
G534V	< 0.1	< 0.05
G534D	1.0	< 0.05
K536I	0.9	1.0
K536R	1.1	0.9
R538M	1.1	0.4
I541R	1.2	< 0.05
I541T	1.0	1.1
R543S	1.1	1.1

the mutants G534D, K536I, K536R, R538M, I541R, I541T and R543S the MDR1-immunoreactive proteins appeared with the expected size of underglycosylated wild-type MDR1 (about 130 kDa), characteristic of MDR1 expression in Sf9 cells [14,19]. These mutants in the insect cells gave approximately the same yield of protein expression as the wild-type MDR1, with an average expression level of about 30 μg of MDR1/mg of membrane protein (see also [14]). In contrast, two of the mutants, L531R and G534V, gave very low yields of expression ($< 10\%$ of the wild-type). Since plasma membrane targetting is not a limiting factor for MDR1 expression in the insect cells [14,19], the most plausible explanation for the low level of production of these two MDR1 variants is that these amino acid replacements are not fully compatible with the formation of a stable protein structure.

Immunoreactivity of the ABC-signature mutants in flow cytometry

In order to investigate the correct membrane insertion of the mutant proteins, we examined their interactions with two monoclonal antibodies having specific recognition properties. The UIC2 antibody has been shown to interact with extracellular epitope(s) of the human MDR1 [21], and this interaction is destroyed if a segment in the first extracellular loop (amino acids 78–97) is removed [17,23]. The localization of the complex binding sites of monoclonal antibody MRK16 on the extracellular domains of human MDR1 have been determined [24], and were found to be different from those reacting with UIC2 [17,23].

In immunoflow cytometry experiments, the mutant MDR1 proteins G534D, R538M and I541R were recognized by both monoclonal antibodies, in a manner indistinguishable from that of the wild-type protein (Figure 3). We found similar full recognition for the mutants K536I, K536R, I541T and R543S, whereas the mutant proteins showing low expression levels on the immunoblots (L531R and G534V) were not detectable by immunoflow cytometry either (results not shown). These findings strongly suggest that all of the mutants that have expression levels similar to that of the wild-type protein are correctly inserted into the membrane plane, i.e. the proper combinations of all relevant epitopes appear on the relevant membrane surface.

Drug-stimulated ATPase activity

In the isolated membrane preparations of Sf9 cells expressing the MDR1 protein, a high-capacity, drug-stimulated ATPase activity can be detected [14]. It has been demonstrated previously that the stimulatory effects of drugs on this ATPase activity are closely related to the drug-transporting activities measured in the wild-type or mutant forms of MDR1 [15,16]. In order to study the functional consequences of the mutations introduced into the

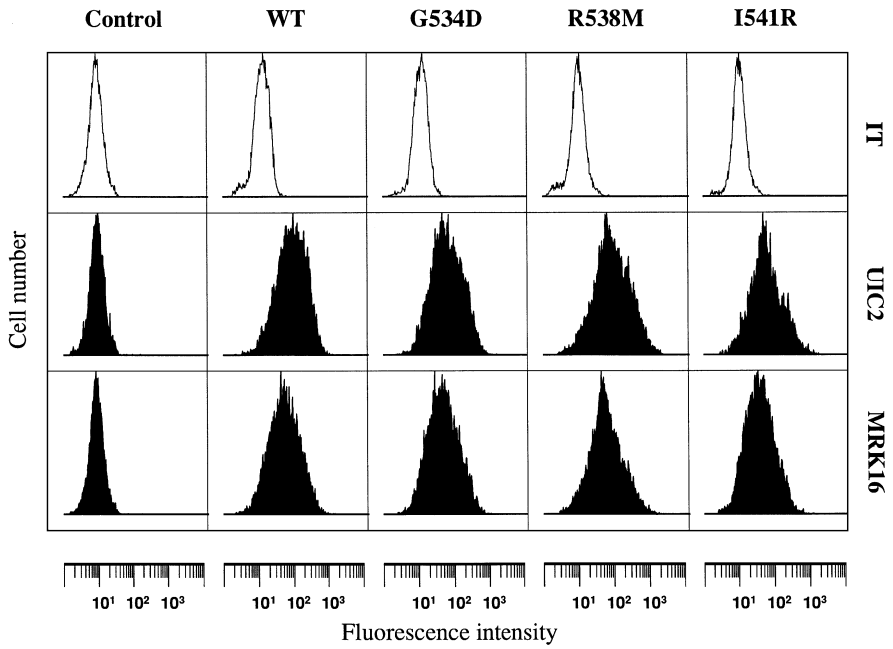

Figure 3 Immunochemical analysis by flow cytometry of Sf9 cells expressing wild-type and mutant MDR1 protein variants

Baculovirus-infected Sf9 cells were labelled with isotype control (IT; top panels), UIC2 (middle panels) and MRK16 (bottom panels) monoclonal antibodies, and then with FITC-conjugated anti-mouse second antibody, as described in the Experimental section. Data are shown as cell numbers plotted against green fluorescence intensity (log scale).

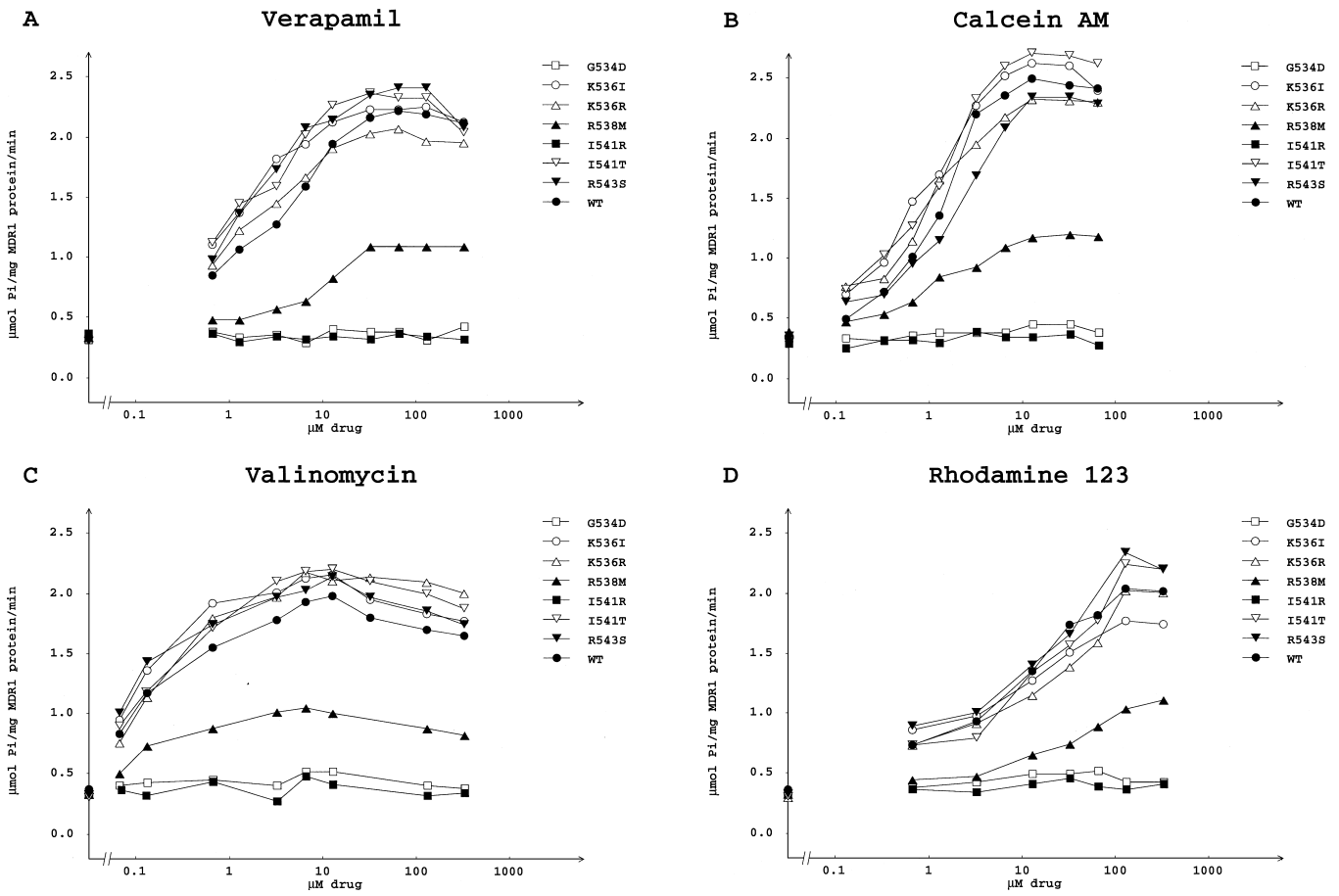

Figure 4 Drug-stimulation of vanadate-sensitive MDR1 ATPase activity in Sf9 cell membranes expressing wild-type or mutant MDR1 proteins

The ATPase activity of the isolated Sf9 cell membranes was estimated by measuring vanadate-sensitive P_i liberation, as described in the Experimental section. MDR1 ATPase activity was calculated by using quantitative immunoblot data as shown in Figure 2. The data points are means of triplicate determinations in representative experiments. The respective drug concentrations were varied as shown on the abscissa. WT, wild type.

ABC-signature region, we have studied the effects of various concentrations of four different compounds (verapamil, calcein AM, valinomycin and Rhodamine 123) on MDR1 ATPase activity. Since all these agents were shown to be transported substrates of MDR1 (see [1]), and to result in significant stimulation of the ATPase activity of wild-type MDR1 with greatly variable concentrations required for half-maximal activation, they should represent a useful screening panel for any major changes in the substrate activation of the multidrug transporter.

For the MDR1 variants K536I, K536R, I541T and R543S, all four drugs concentration-dependently induced ATPase activities that were not significantly different from those seen in the wild-type MDR1 (Figure 4). The K536R mutant MDR1 had been described previously to result in altered cross-resistance towards different drugs, i.e. it caused a preferential resistance to colchicine [13]. However, in our hands the drug-stimulated ATPase activity of this mutant was similar to that of the wild-type MDR1. We also found that the stimulation of ATPase activity by various concentrations of colchicine (33–1300 μM) was similar for this mutant to that seen for the wild-type MDR1 (results not shown).

On the other hand, when the isolated membranes contained similar amounts of the MDR1 proteins harbouring the mutation G534D or I541R (Table 1), none of the drugs examined was able to stimulate ATPase activity. This was true even if the drugs were applied at significantly higher concentrations than those producing maximal stimulation of the ATPase activity of the wild-type MDR1 (Figure 4). An interesting finding was that the MDR1 mutant R538M showed a reduced maximal level of drug-stimulated ATPase activity in the presence of each of the four drugs studied. However, the drug concentrations producing half-maximal stimulation were the same as for the wild-type MDR1 (Figure 4).

Determination of K^{ATP}_m values

In order to examine the possible causes of the loss of MDR1 ATPase activity in the low-activity mutants, their ATPase activities were determined in the presence of various concentrations of ATP (Figure 5). It has been demonstrated previously that the K^{ATP}_m value of the drug-stimulated ATP hydrolysis reaction catalysed by the human MDR1 is between 0.3 and 0.5 mM [14]. We found similar K^{ATP}_m values for the ABC-signature mutants showing normal drug-stimulated ATPase activity (K536I, K536R, I541T and R543S; results not shown). As demonstrated in Figure 5, the ATPase reaction catalysed by the R538M mutant MDR1 also had a similar K^{ATP}_m value as the

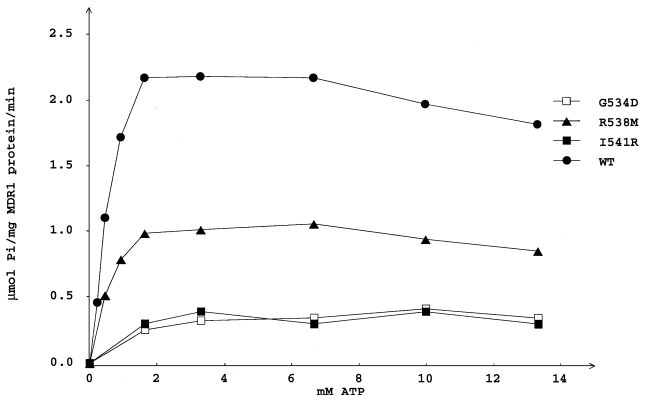

Figure 5 MgATP-concentration-dependence of vanadate-sensitive ATPase activity in isolated Sf9 cell membranes

The ATPase activity of the isolated Sf9 cell membranes was estimated by measuring P_i liberation in the presence of 30 μM verapamil, as described in the Experimental section. The data points are means of triplicate determinations in representative experiments. The differences between the ATPase activities measured in the absence and presence of vanadate (100 μM) are plotted. MgATP concentrations were varied as shown on the abscissa. WT, wild type.

wild-type MDR1, although the maximal level of ATP hydrolysis (V_{max}) by the mutant was only about 40 % of that of the wild-type. Mutants G534D and I541R were unable to catalyse ATP hydrolysis, even when high concentrations of ATP were present in the incubation medium (Figure 5).

DISCUSSION

The ABC transporter family is one of the largest protein families known; a recent release of the protein sequence database (Swissprot rel. 32.0) stores 238 entries with protein sequences falling into the category of 'ABC transporters', containing a total of 282 ABC units (44 proteins possess two ABC units). A short peptide region of 13 amino acids between the two Walker motifs, in close N-terminal proximity to the Walker B motif, is conserved exclusively in the ABC transporters. This region has no counterpart in other 'Walker A/Walker B-type' ATP-binding proteins, and its presence is diagnostic for an ABC unit (ABC signature). The ABC-signature regions of the N- and C-terminal ABC units of the human MDR1 are identical (Figure 1).

There is as yet no experimental evidence to assign a characteristic role to the ABC-signature region, but several mutations in this region have been described to seriously affect function, e.g. in the bacterial histidine permease HisP [5], in FstE, a cell division protein of *E. coli* [7], in the yeast pheromone transporter STE6 [8,9] and in the human CFTR [10–12]. Hyde et al. [4] speculated that the remarkable conservation of the ABC-signature region (called 'loop 3' by these authors) suggests that this region has some transport-related function, rather than participating only in ATP binding and/or hydrolysis by these proteins.

No experimental data are currently available on the three-dimensional structure of any of the ABC units. However, based on the three-dimensional structures of two Walker A/Walker B-type ATP-binding (but not ABC transporter) proteins, i.e. adenylate kinase and $p21^{ras}$, models for this domain have been proposed [4,6]. Although the two published models propose somewhat different three-dimensional structures for the ABC unit, they agree that the ABC-signature region must loop out from the core structure of these proteins. According to Hyde et al. [4], the ABC signature is inserted in a region which may undergo the most extensive conformational changes upon ATP binding in adenylate kinase; thus in ABC transporters this motif may be responsible for coupling of the ATP-dependent conformational changes to a transport process.

In the case of the human MDR1 protein expressed in mammalian cells, replacement of the lysine residue in the N-terminal ABC signature with arginine (K536R) resulted in a P-glycoprotein variant that was found to be more effective in conferring multidrug resistance than the wild-type protein, with a preferential resistance to colchicine [13]. Based on these results, the authors proposed that modification of this region modifies the drug-selectivity of MDR1.

In the present work we have studied the role of the ABC-signature region by engineering several single amino acid substitutions within the N-terminal ABC signature of the human MDR1 (Figure 1). The functional consequences of these amino acid substitutions were detected by expressing the mutant proteins in insect (Sf9) cells, and by measuring drug-stimulated ATPase activity in isolated membrane preparations. When expressed in mammalian cells, several ABC-signature mutants of CFTR and MDR were found to be incorrectly glycosylated and/or delivered to the cell surface membrane [11,13], and thus their transport function could not be estimated reliably. It is important to note that results obtained by our experimental approach do not depend on MDR1 glycosylation or on the targetting of this protein to the cell surface membrane (see [14,19]). Also, the ATPase assay directly reflects the enzymic features of the transporter and allows detailed analysis of its substrate activation and ATP requirements. It has been demonstrated previously that the different stimulatory effects of drugs on the ATPase activity of the wild-type MDR1 and on its various mutants are closely related to differences in drug-transporting activities [15,16,25].

According to our experiments, two amino acid substitutions affecting the ABC-signature region, namely mutations L531R and G534V, led to the formation of unstable MDR1 proteins, which were probably mostly degraded before insertion into the membrane. The conservation of a leucine in a position equivalent to amino acid 531 in MDR1 was found in 224 of the 282 ABC units in the Swissprot database, and only hydrophobic side chains were found in this position. Thus the L531R mutation, which introduces an extra positive charge, apparently resulted in a major folding problem for the MDR1, and prevented the formation of a stable molecular architecture even in the Sf9 expression system.

A glycine residue in the fourth position of the ABC-signature region (Gly^{534} in MDR1) is almost fully conserved in the whole ABC-transporter family, i.e. 273 of the 282 known ABC units contain glycine, while none of them possesses valine in this position. Since the two glycine residues in positions 533 and 534 may allow greater flexibility, e.g. a tight turn in the protein structure, the bulkier valine residue may substantially modify this normal protein fold.

In the case of the mutants G534D and I541R, we found full MDR1 protein expression but a complete loss of drug-stimulated ATPase activity. None of the drugs examined was able to activate these two mutants, even at extremely high concentrations (Figure 4). We also demonstrated that these mutants were inactive even when high concentrations of MgATP (more than 20 times higher than the K^{ATP}_m of the wild-type MDR1) were present in the assay medium (Figure 5). The basic membrane topology of these two inactive mutants was found to be similar to that of the wild-type MDR1, since two monoclonal MDR1-specific antibodies, reacting with complex epitopes, were bound to the mutants in an identical fashion to that seen with the wild-

type MDR1 (Figure 3). Thus the loss of activity was not the result of major changes in the membrane topology of these mutant proteins.

Mutation G534D in MDR1 is equipositional with the G551D mutant of the human CFTR, which causes severe cystic fibrosis and is the second most frequently observed mutation in this disease [26]. Impaired nucleotide binding by the N-terminal ABC unit of the CFTR carrying the G551D mutation was observed when this portion of the CFTR was expressed in bacteria [27]. The G509D amino acid replacement, affecting the equipositional glycine residue of the N-terminal ABC signature in yeast STE6, also resulted in a complete loss of function [8,9]. In the experiments of Hoof et al. [13] the G534D MDR1 mutant was found to be expressed only in very low amounts at the surface of the recombinant mammalian host cells; thus this mutation may also affect the maturation of MDR1. Since in our studies the human MDR1 ATPase activity was measured in isolated insect cell membranes, the present experiments confirm that the G534D mutant, although expressed normally, is non-functional.

Mutation I541R introduces a positive charge into a highly conserved hydrophobic patch (Ile-Ala-Ile^{541}-Ala) of the ABC-signature region. In fact, 280 of the 282 known ABC units contain a hydrophobic amino acid in this position, whereas the other two contain threonine. No equipositional mutation of this amino acid replacement has been reported so far in the literature, but our current experiments strongly suggest that the function of ABC transporters could be abolished by the introduction of a charged residue in this position, probably by perturbing crucial intramolecular interactions. This view is supported by the finding that a mutation at the same position that did not introduce an extra charge (I541T) caused no apparent change in the activity of the MDR1 protein (Figure 4).

In the position equivalent to Lys^{536} of MDR1, in a central, more polar region of the ABC signature (Gln-Lys^{536}-Gln-Arg), there is a dominance of positively charged residues (arginine or lysine is present in 202 of the 282 ABC units), but uncharged and/or hydrophobic residues are found in some ABC transporters. In our experiments, full MDR1 protein expression and full MDR1 ATPase activity were observed when Lys^{536} was replaced either by arginine (K536R), causing no net charge difference, or even by isoleucine (K536I), which removes this positive charge. Hoof and co-workers [13] found that the K536R mutation in mammalian cells generated a transporter which was more active than the wild type, and this greater multidrug resistance was manifested in a preferential resistance to colchicine. In our experiments, when expressing the mutant K536R in insect cells, we found no major alteration in its drug-stimulated ATPase activity (Figure 4), including its stimulation by colchicine. We have demonstrated previously that for the G185V mutation, which conferred increased resistance to colchicine, the MDR1 ATPase activity measurements in Sf9 cell membranes correlated well with the drug resistance profile [16]. The discrepancy between the results of Hoof et al. [13] and those shown here may be due to the different expression and assay systems employed.

In the central polar region of the ABC signature, the replacement of arginine at position 538 with methionine (R538M) created an MDR1 protein with a lowered maximum level of drug-stimulated ATPase activity (approx. 40% of that of wild-type MDR1; Table 1). This difference was observed with each of the four drugs used in this study (Figure 4), suggesting that the R538M MDR1 shows lower maximum activity but not altered substrate specificity. As shown in Figure 5, the lower ATPase activity is indeed due to a lower maximum level of ATP hydrolysis, since the K^{ATP}_m value of the mutant is about the same as that of the wild-type MDR1 (about 0.5 mM). Since this amino acid replacement did not change either the value of K^{ATP}_m or the drug-interaction profile, we conclude that this residue may not be directly involved in the interactions of MDR1 with ATP or the transported drugs.

According to the database search, a positively charged residue is present in most ABC units in the position equivalent to amino acid 543 of MDR1. However, our present experiments indicate that the replacement of arginine with serine at this position (R543S) does not induce a major decrease in MDR1 expression or in MDR1 ATPase activity.

In summary, in the present study the most dramatic changes caused by single amino acid replacements within the ABC-signature motif were obtained when strongly conserved residues were affected. The L531R and G534V mutants were expressed in very low amounts in Sf9 cells, as they apparently could not form stable three-dimensional structures. The (fully expressed) R538M mutant had decreased maximum MDR1 ATPase activity, while the G534D and I541R mutants showed a complete loss of activity despite retaining a wild-type ABC signature region in the C-terminal half of the protein. As our previous study showed [16], discrete substitutions of the conserved Lys residue with Met within the Walker A motif in either the N- or C-terminal half of human MDR1 abolished drug-stimulated ATPase activity. The experiments of Azzaria et al. [28] gave similar results: mutations in either half of mouse MDR1 abrogated the multidrug resistance phenotype. These data are in accordance with our present findings and support the idea that interaction between the two ABC domains is necessary for P-glycoprotein function. It is important to note that, in the case of residue 541, an amino acid replacement causing no charge difference (I541T) was fully tolerated. These results suggest that the conservation of the ABC-signature region, and especially of its specific charged and hydrophobic amino acids, is essential for the stability and/or function of MDR1, and support the view that this motif is involved in functional intramolecular interactions. At the same time, our experiments do not indicate that major changes in the drug substrate specificity or ATP recognition characteristics of MDR1 are evoked by mutations in the ABC-signature region.

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