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Functional Studies on the MRP1 Multidrug Transporter: Characterization of ABC-signature Mutant Variants

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Abstract. *Background: MRP1 is a key multidrug resistance ATP-binding Cassette (ABC) transporter in tumor cells. A functionally important signature motif is conserved within all ABC domains. Our current studies aimed to elucidate the role of these motifs in the cooperation of MRP1 ABC domains. Materials and Methods: We designed human MRP1 mutants based on a bacterial ABC structure. Conserved leucines (Leu) were replaced by arginines (Arg), while glycines (Gly) were substituted for aspartic acids (Asp). The activity of these mutants was assayed by measuring ATPase activity and vesicular transport. ATP-binding and transition-state formation were studied by a photoreactive ATP analog. Results: The Leu to Arg mutants retained both ATPase and transport activity, while the Gly to Asp mutants were inactive in all functional assays, while showing normal ATP-binding. Conclusion: Our results reinforce the notion that a single mutation in one of the ABC-signature regions affects the function of the whole protein. The relative role of the conservative leucines and glycines in MRP1 indicates a similar three-dimensional structure within the catalytic center of various ABC proteins.*

Multidrug resistance (MDR) is a major obstacle in the efficient chemotherapy of human malignancies. The emergence of this phenomenon is frequently associated with the overexpression of special membrane transporter proteins. Three members (MDR1, MRP1 and ABCG2) of the ATP-binding Cassette (ABC) transporter family have been suggested to play key roles in cancer multidrug resistance. It is well established that they carry out an ATP-dependent extrusion of the applied cytotoxic drugs, keeping the level of these agents below the cell-killing threshold, thus conferring resistance to many structurally dissimilar anticancer drugs.

One of the proteins causing this phenotype is the human Multidrug Resistance Protein (MRP1), which confers resistance to a wide variety of anticancer drugs (1), but has also been shown to be a high affinity primary active transporter for glutathione (GS)-conjugates (*e.g.* LTC_4 – see 2, 3). MRP1 transports large hydrophobic drugs, playing an important role in the chemotherapy resistance of several types of cancer cells, and cellular GS seem to be an important modulator in these transport functions (2, 4-9). The physiological functions of MRP1 range from the mediation of an inflammatory response to the elimination of certain xenobiotics (10,11).

All proteins in the ABC family consist of two major types of polypeptide regions; transmembrane domains (TMD), usually with six membrane-spanning helices, and conservative ABC units. The ABC units harbour two consensus polypeptide sequences, the so-called Walker A and Walker B motifs (12), which are present in many ATP-binding and/or utilizing proteins. In addition, in all ABC transporters a short, highly conserved polypeptide motif of about 13 amino acids (ABC-signature region) is found between the two Walker motifs. This ABC-signature motif is diagnostic for the whole ABC superfamily (13,14).

The membrane topology of MRP1 differs from most other members of the ABC superfamily. In addition to an MDR1-like core region, containing a tandem duplication of two TMDs (TMD1 and TMD2) and two ATP-binding (ABC) domains, MRP1 has a third TMD (TMD0) that is formed by the first 200 amino acids of the protein. This domain consists of five putative TM helices with an extracellular NH_2 terminus and is linked to the core region by a cytoplasmatic loop (L0) (15,16). Although the TMD0 region is characteristic of several members of the MRP1 family, it was found that it is not required for the MRP1 transport function nor for the proper routing of this protein to the basolateral plasma membrane (17). The MDR1-like core region was found to be inactive, while

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Key Words: MRP1, multidrug resistance, ATP-binding Cassette, ABC-signature, nucleotide trapping, ABC-ABC structure.

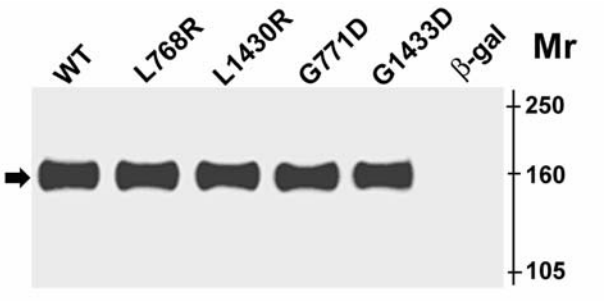

Figure 1. *Expression of human MRP1 and its signature mutant variants in the baculovirus-Sf9 expression system. Isolated membrane preparations of baculovirus-infected Sf9 cells, dissolved in disaggregating buffer, were subjected to electrophoresis on 7.5% Laemmli-type gels. Immunoblotting was performed using the anti-MRP1 monoclonal m6 antibody. Sf9 cells infected with a baculovirus containing the β-galactosidase cDNA were also examined. Each lane contains 3 μg of membrane protein. The arrow indicates the position of human MRP1 variants on the blot. The positions of molecular-mass markers are indicated (kDa) at the right.*

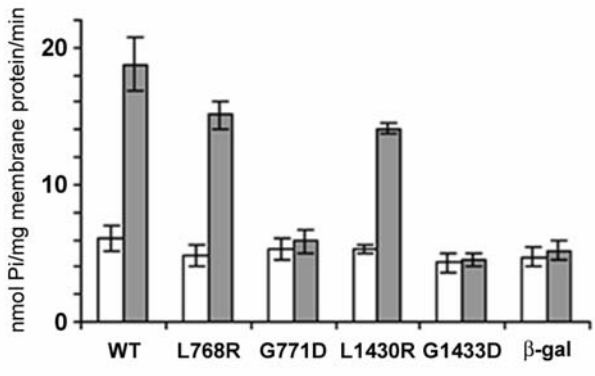

Figure 2. *Drug-stimulated, vanadate-sensitive ATPase activity in isolated membranes containing human MRP1 and its signature mutant variants. ATPase activity of the isolated Sf9 cell membranes was estimated by measuring inorganic phosphate liberation. Membrane ATPase activity was measured for 60 min at 37°C in the presence of 4 mM ATP. NEM-GS, when indicated (darker columns), was applied at a concentration of 5 mM. The differences between the ATPase activities were measured in the presence and absence of vanadate (800 μM), respectively.*

the L0 region was sufficient and essential for restoring MRP1-mediated transport activity and routing for this core region (18).

In MRP1 the sites interacting with the drug substrates are most probably harboured within the TMDs. These sites were identified by photoreactive probes in transmembrane helices 10, 11, 16 and 17 (19). In another study (20), the importance of Lys332 and His335 in determining substrate specificity and the role of Asp336 (all within TM6) in overall transport activity was suggested. Substitution of a highly conserved tryptophan at position 1246 with cysteine in TM17 was also shown to modulate the substrate specificity of MRP1 (21).

In ABC transporters ATP hydrolysis takes place within the ABC domains. According to our present knowledge, in all ABC transporters a close collaboration of two ABC domains is required for transport and ATPase function. The sequences of the two ABC domains in the multidrug transporter MDR1 protein are very similar, and MDR1 hydrolyses ATP in both ABC domains. In contrast, the sequences of the two ABC domains in MRP1 are less similar; the N-ABC of MRP1 contains a deletion of 13 amino acids between the Walker A and Walker B motifs, which is not present in the C-ABC.

Recently three research groups have provided data, independently, showing that the two ABCs of MRP1 are functionally non-equivalent (22-26). In photoaffinity labeling experiments with 8-azido-ATP, ATP-binding was found to occur preferentially in the N-ABC, while vanadate-induced nucleotide trapping of 8-azido-[^{32}P]-ADP was predominantly found in the C-ABC. When a highly conserved lysine residue within the Walker A motif in MRP1 was substituted for methionine in either the N-ABC or in the C-ABC unit, the K1333M mutation in the C-ABC nearly abolished ATP-dependent LTC_4 uptake, whereas the K684M substitution in the N-ABC had a less pronounced effect (22,23). The two ABC-signature regions in MRP1 have not been investigated in detail as yet, thus currently little is known about their function or dissimilarity.

The aim of the present study was to characterise the highly conserved ABC-signature regions in the two non-equivalent ABC units of MRP1. In the experiments reported below, we have examined the effects of two conservative amino acid substitutions in the ABC-signature regions of both the N-terminal and the C-terminal half of MRP1. The conserved leucines of the LSGGQ motifs were replaced by arginines (L768R, L1430R) and the conserved glycines in the fourth position of the signature motifs were substituted for aspartic acids (G771D, G1433D). We expressed the wild-type and mutant MRP1 proteins in the baculovirus-Sf9 insect cell expression system and examined the expression and function of the mutant variants. For the functional characterization of the mutant variants, vanadate-sensitive, drug-stimulated ATPase- and vesicular transport activities were measured in isolated membrane preparations. The individual steps of the ATPase cycle, that is ATP-binding and nucleotide trapping, were studied by using a labeled photoreactive ATP analog.

Our data suggest a major functional role of the conserved Gly residues in both ABC units of MRP1 and the formation of composite catalytically active sites, involving both signature regions within the ABC domains.

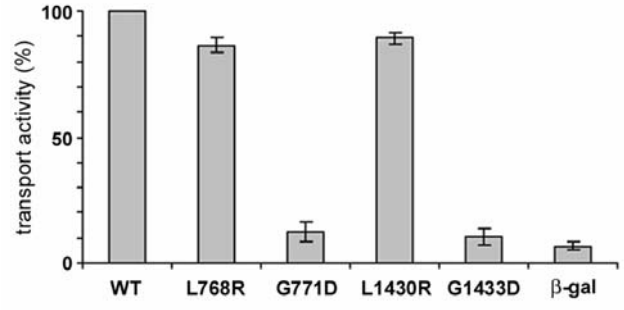

Figure 3. *ATP-dependent LTC_4 uptake by human MRP1 and its signature mutant variants in isolated Sf9 membrane vesicles. Membrane preparations (6 mg/ml) were incubated with 50 nM LTC_4 at 23˚C for 30 sec in the presence or absence of 4 mM ATP. LTC_4 uptake was followed by rapid filtration assay. The relative transport rates were corrected for the amount of membrane vesicles (see Methods).*

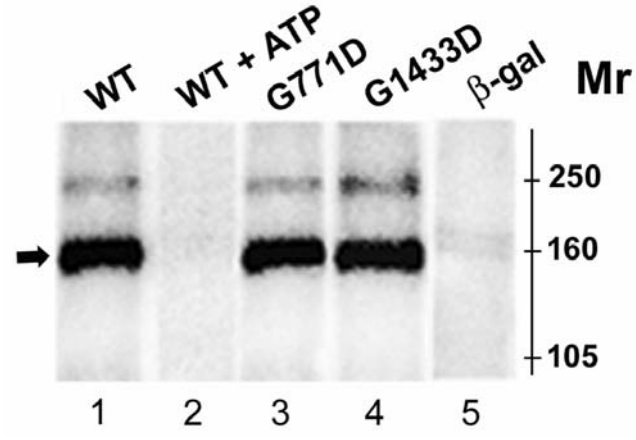

Figure 4. *Binding of [α-^{32}P]8-azido-ATP to human MRP1 and its signature mutant variants in isolated Sf9 membrane vesicles. Isolated Sf9 cell membranes (10 mg/ml) were incubated at 0˚C for 5 min in the presence of 5 μM final concentration of 8-azido-ATP, containing [α-^{32}P]8-azido-ATP, and irradiated with an UV lamp. The membranes were then washed and dissolved in disaggregating buffer and run on 7.5% Laemmli-type gels. The proteins were electroblotted, the blots were dried and subjected to autoradiography. As a control, Sf9 cell membrane containing β- galactosidase was also examined. Lane 2 shows the effect of pretreatment with 4 mM unlabeled ATP on the wild-type MRP1. The arrow indicates the position of human MRP1 variants on the blot. The positions of molecular-mass markers are indicated (kDa) at the right.*

Materials and Methods

Construction of the recombinant transfer vectors. For cloning various cDNA constructs, the modified baculovirus transfer vector pAcUW21-L was used (30). Mutagenesis was performed by overlap-extension PCR (38) with pAcUW21-L-MRP1 as template. Sequencing was performed to confirm the mutations and to ensure that no additional mutations occurred.

Generation of recombinant baculoviruses. Recombinant baculoviruses were generated and Sf9 (*Spodoptera frugiperda* ovarian) cells were infected and cultured as described by Bakos *et al.* (29). Individual clones expressing a high level of the MRP1 variants were obtained by endpoint dilution and subsequent amplification.

Membrane preparation and immunoblotting. The virus infected Sf9 cells were harvested and the membrane fractions and the membrane protein concentrations were determined as described earlier (27, 29). Membranes were suspended in a disaggregation buffer and samples were run on 7.5% Laemmli-type SDS-containing gels and electroblotted to PVDF membranes (27). MRP1 was detected with the monoclonal antibody m6 (39).

Membrane ATPase activity measurements. Drug-stimulated ATPase activity was measured in isolated membranes as described earlier (27, 29), with an incubation time of 60 min at 37˚C. The figures represent mean values from three independent experiments.

Transport measurements. [^{3}H]LTC_4 (obtained from Perkin Elmer Life Sciences, 182.2 Ci/mmol) transport measurements in isolated Sf9 cell membrane vesicles were performed as described by Bakos *et al.* (17). In brief, vesicles were incubated in the presence of 4 mM ATP in a transport buffer at 23˚C for 30 sec, then aliquots were rapidly filtered through nitrocellulose membranes. Radioactivity associated with the filters was measured by liquid scintillation counting. The relative amount of transport-competent inside-out vesicles in Sf9 membrane preparations was estimated by measuring the rate of endogenous ATP-dependent calcium uptake in each preparation (28).

Photoaffinity labeling by [α-^{32}P]8-azido-ATP. This method has been described in detail (36). In brief, for measurement of ATP-binding, isolated Sf9 cell membranes were incubated with 8-azido-ATP (Sigma), containing 0.1 MBq of [α-^{32}P]8-azido-ATP (Affinity Labeling Technologies, Lexington, KY, USA 229.4 GBq/mmol). The samples were kept on ice and irradiated for 10 min with a UV lamp at 4˚C, then washed twice in the presence of 10 mM ATP.

In the nucleotide trapping experiments, the Sf9 cell membranes were incubated for 5 min at 37˚C in the presence of 5 μM final concentration of 8-azido-ATP containing [α-^{32}P]8-azido-ATP, with or without a trapping agent (Na_3VO_4). The reaction was stopped by the addition of an ice-cold buffer containing 10 mM MgATP. Membranes were washed twice in the same buffer and then irradiated with UV (λmax about 250 nm) at a distance of 3 cm at 4˚C.

The labeled samples were run on 7.5% Laemmli-type SDS-containing gels, the proteins were electroblotted to PVDF membranes and the blots were dried and subjected to quantitative autoradiography in a PhosphorImager (BioRad). The identity of the labeled bands was confirmed by immunostaining of the same blot with anti-MRP1 mAb, m6.

Results

In the present study we used the heterologous Sf9 expression system for the production of human MRP1 protein at a high level, to allow its functional characterization. It has been previously documented that the basic structural and

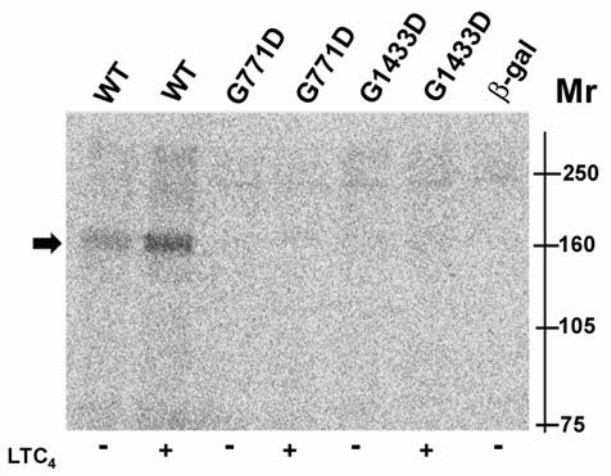

Figure 5. *Nucleotide trapping of wild-type and G771D and G1433D signature mutant MRP1 variants. Sf9 cell membranes (10 mg/ml) expressing the wild-type MRP1 and its mutant variants or membranes from cells infected with β-galactosidase were incubated at 37˚C for 5 min in the presence of 5 μM Mg-[α-^{32}P]8-azido-ATP and 1 mM sodium orthovanadate. The reaction buffer contained 800 nM LTC4. The arrow indicates the position of human MRP1 variants on the blot. The positions of molecular-mass markers are indicated (kDa) at the right.*

transport characteristics of human MRP1 expressed in Sf9 cells are identical to those seen in mammalian cells (34).

In the first set of experiments, we compared the expression levels of the wild-type and mutant MRP1 variants in the baculovirus-infected Sf9 cells by quantitative immunoblotting. As shown in Figure 1, all mutant variants were efficiently produced in Sf9 cells and gave approximately the same yield of expression as the wild-type MRP1. The expressed proteins had an apparent molecular weight of 160 kDa, which represents the underglycosylated form of MRP1 and its variants. As a control, Sf9 cells were infected with a baculovirus construct containing the β-galactosidase cDNA and these cells had no detectable amount of MRP1 (see Figure 1).

MRP1 utilises the energy of ATP hydrolysis to exclude drugs from the cells. Drug-stimulated, vanadate-sensitive ATPase activity in multidrug transporters reflects the whole catalytic cycle, which is tightly coupled to the transport of the drug substrates (31). In the following experiments we examined whether the signature mutant variants are able to perform drug-stimulated, vanadate-sensitive ATPase activity.

Figure 2 shows the vanadate-sensitive ATPase activity measured in the Sf9 cell membrane preparations, containing the wild-type and signature mutant variants of MRP1. We performed these measurements both in the absence and presence of 5 mM NEM-GS, a known model-substrate of human MRP1 (3). It has been shown previously that this concentration of NEM-GS ensures maximum stimulation of the ATPase activity of wild-type MRP1 (31).

As documented in Figure 2, the vanadate-sensitive ATPase activity of wild-type MRP1 was relatively low in the absence of NEM-GS (about 6 nmol Pi/mg membrane protein/min). When NEM-GS was added, the vanadate-sensitive ATPase activity was significantly stimulated, to about 19 nmol Pi/mg membrane protein/min in the wild-type MRP1- containing cell membranes. Membranes from cells expressing β-galactosidase had a low basal ATPase activity (4-5 nmol Pi/mg membrane protein/min) and no measurable stimulation was detected in the presence of NEM-GS.

In Figure 2 we document that, when measuring the ATPase activity of the L768R and L1430R signature mutants, we found that they possessed a significant level of vanadate-sensitive, drug-stimulated ATPase activity. These values were about 15 and 14 nmol Pi/mg membrane protein/min, respectively, which is about 80 and 75% of that of the wild-type activity. On the other hand, in the G771D and G1433D mutants the NEM-GS-stimulated ATPase activities were not significantly higher than those in the negative control β-galactosidase-infected cell membranes (5.8 and 4.5 nmol Pi/mg membrane protein/min, respectively).

In order to compare the transport characteristics of the wild-type and mutant variants of MRP1, we studied the uptake of the radiolabeled LTC_4, a natural substrate of human MRP1, in isolated Sf9 cell membrane vesicles. The applied final concentration of LTC_4 was 50 nM, an optimum concentration for measuring the transport activity of the wild-type MRP1 (2). In these transport studies for the various MRP1 variants, a similar amount of transport-competent inside-out vesicles in the Sf9 membrane preparations was assured by measuring the rate of endogenous ATP-dependent calcium uptake in each preparation (see Materials and Methods).

As shown in Figure 3, in harmony with the ATPase activity measurements, the L768R and L1430R signature mutants presented 87-89 % of the transport activity of the wild-type MRP1, while the G771D and G1433D signature mutants had only a negligible level of ATP-dependent LTC_4 uptake, similar to that found in the β-galactosidase-expressing control membranes.

All these functional studies indicated that the catalytic cycles of the L768R and L1430R mutants were similar to the wild-type, while that of the G771D and G1433D mutants were seriously diminished. In the following experiment, we studied if this loss of function was due to the loss of ATP-binding and/or the formation of a catalytic intermediate in these MRP1 variants.

In order to examine whether the loss of ATPase and transport activity in the G771D and G1433D signature mutants was due to an impaired binding of ATP, we performed photoaffinity-labeling experiments using [α-^{32}P]8-azido-ATP under nonhydrolytic conditions. Isolated

Sf9 membranes containing the wild-type and the G771D and G1433D signature MRP1 variants were incubated on ice in the presence of 5 μM [α-^{32}P]8-azido- ATP.

As shown in Figure 4, after cross-linking with UV, followed by gel electrophoresis and immunoblotting, radioactive bands corresponding to the MRP1 variants were observed in the wild-type MRP1 and in both mutants. As shown in lane 2, the addition of 4 mM unlabeled MgATP to the reaction medium abolished 8-azido-ATP labeling of the wild-type MRP1 protein, indicating the specificity of the ATP-binding. Quantification of the radioactive signal associated with the individual bands showed that the amount of radioactivity incorporated was similar in the mutants and in the wild-type MRP1 protein. The radioactive label was absent from control membranes prepared from cells expressing β-galactosidase.

These data indicate that, under the applied conditions, even the ATPase- and transport inactive MRP1 mutant variants are capable of binding the ATP analog, [α-^{32}P]8-azido- ATP, similarly to that found in the wild-type MRP1.

In the following experiments we studied the effects of these mutations on the partial reactions of the ATPase cycle of human MRP1. Vanadate-dependent nucleotide trapping in ABC transporters reflects a posthydrolytic intermediate-like state of the ATPase cycle, and in drug transporters this step is stimulated by the transported substrates (34-36).

Vanadate arrests the catalytic cycle of ABC proteins, most probably by replacing the inorganic phosphate in the transition-state intermediate and stabilizes a protein-trapped form of ADP. In the presence of vanadate and MgATP, MRP1 has already been shown to form a stable complex (37). Experimentally this reaction can be followed by using [α-^{32}P]8-azido-ATP as an energy donor substrate.

In experiments documented in Figure 5, isolated Sf9 membranes containing the wild-type MRP1, or the G771D and G1433D signature mutants, were incubated under hydrolytic conditions for 5 min, in the presence of vanadate as an inhibitory anion, and 5 μM [α-^{32}P]8- azido-ATP. When LTC_4, a substrate and stimulator of the MRP1 ATPase activity (see above), was also included in the reaction mixture, it had a stimulatory effect on the vanadate-induced nucleotide trapping of the wild-type MRP1 (see lane 2).

As shown, the G771D and G1433D mutants, although displaying significant 8-azido-ATP-binding (see above), did not perform any nucleotide trapping. This was true even if higher 8-azido-ATP concentration (20 μM) was used (data not shown). The corresponding immunoblots ensured that equal amounts of the MRP1 variants were applied in all experiments (see methods). These mutants did not present labeling, even when LTC_4 was also applied.

Collectively these data indicate that the G771D and G1433D signature mutants are capable of proper ATP-binding, but a later step of the catalytic cycle, namely the transition state formation, cannot be detected.

Discussion

MRP1 plays an important role in the multidrug resistance phenotype, which is a major obstacle in cancer therapy. For this reason a better understanding of the structure and function of MRP1 is of great importance. However, no high-resolution three-dimensional structure is available as yet for any of the mammalian ABC transporters. The presence of the ABC-signature region is characteristic for all ABC proteins, but the exact function of this region is also still largely unknown.

Results suggesting a profound effect of changes in the ABC signature motif on the expression and function of the human MDR1 protein were published (29), even before the first bacterial three-dimensional ABC structure appeared. A number of mutants with single amino acid replacement were generated in the highly conserved ABC-signature region of the N-terminal half of the protein and the observations suggested that this region is essential for MDR1 protein stability and function (29,30).

Recently the structure of Rad50, a bacterial ABC-ABC dimer, was solved (32). It represents two functionally interacting ABC subunits, dimerizing in a "head-to-tail" orientation. In this dimer the two ABC domains complement each other's active site. This structure provided detailed information about the position of the signature region and its relation to the bound ATP and the Walker A region in a functional ABC-ABC dimer. According to the structure, the Walker A sequence of one Rad50cd subunit and the ABC-signature motif of the opposite one are involved in the formation of a single ATP-binding site, and the two ATP molecules are completely buried in the dimer interface. A similar arrangement of the ABC ATPase catalytic sites has been found recently in the BtuCD ABC-transporter of *E.coli* (33), indicating that the composite catalytic sites found in the Rad50cd dimer is a general feature of the ABC-ATPases.

Single amino acid residues can be studied on the basis of the structure. The conserved leucines in the first position of the LSGGQ motifs are not oriented towards the ATP in the Rad50 (neither in the BtuCD structure). The highly conserved serine residue in the second position and the glycine residue in the fourth position of the signature motifs interact with the oxygen of the γ-phosphate of the ATP bound in the active site(s). The conservation of the glycine residue in the fourth position of the ABC-signature region is almost universal in the family.

In order to study the possible position and orientation of the signature region of MRP1, we prepared mutants designed on the basis of the Rad50 bacterial ABC ATPase structure. We chose leucine, a residue not oriented towards the ATP, which we substituted for arginine (L768R, L1430R). The glycines of the fourth position of the signature motif, laying on the ATP-binding surface, were replaced by aspartic acids (G771D, G1433D). Based on the

crystal structure, the Leu-Arg replacement was not expected to seriously affect the catalytic function, while the Gly-Asp replacement causes such a distortion that it most probably should prevent any significant ATP hydrolysis.

As documented above, the wild-type and mutant MRP1 proteins were expressed in Sf9 insect cells and their catalytic activities were assayed by ATPase and vesicular transport experiments. The individual steps of the catalytic cycle – ATP-binding and transition state formation – were studied by using [α-^{32}P]8-azido-ATP, a labeled photoreactive ATP analog. All mutant variants could be expressed in Sf9 insect cells in similar quantities to the wild-type MRP1. We found that the ATPase and transport activity of the L768R and L1430R signature mutants, reflecting the whole catalytic cycle of MRP1, were not significantly different from that of the wild-type MRP1. However, the G771D and G1433D signature mutants were practically inactive, both in the ATPase and in the vesicular transport assays.

In order to examine whether the loss of ATPase activity in the G771D and G1433D signature mutants was due to an impaired binding of ATP, we performed photoaffinity-labeling experiments. These data indicated that, under the applied conditions, the inactive MRP1 mutant variants are capable of binding the ATP analog, [α-^{32}P]8-azido-ATP, similarly to that seen in the wild-type MRP1. Thus this step of the catalytic cycle is still preserved in these mutants.

When studying the formation of a transition state of the ATPase cycle, reflected by vanadate-dependent nucleotide trapping, we found that in the G771D and G1433D mutants this partial reaction could not be detected. These results suggest that either they are unable to perform the steps following ATP-binding, or that their transition state can not be detected as a result of a steric effect of the mutations. An important result in these studies was that the GD mutation in any of the two ABC units eliminated both the full catalytic cycle and the transition state formation in both ABC units.

Collectively these results reinforce the notion that mutation of an important amino acid residue within the ABC-signature regions in either ABC domains can drastically affect the function of the whole protein. This implies that MRP1 also contains composite-type active sites, in which the two ABC domains complement each other. These observations also suggest that the two conservative leucines and glycines may occupy similar positions and orientations in MRP1 as in the Rad50 dimer and in the BtuCD transporter, indicating a similar three-dimensional structure within the catalytic center of the ABC proteins.

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