Replacement of the positively charged Walker A lysine residue with a hydrophobic leucine residue and conformational alterations caused by this mutation in MRP1 impair ATP binding and hydrolysis

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MRP1 (multidrug resistance protein 1) couples ATP binding/hydrolysis at its two non-equivalent NBDs (nucleotide-binding domains) with solute transport. Some of the NBD1 mutants, such as W653C, decreased affinity for ATP at the mutated site, but increased the rate of ATP-dependent solute transport. In contrast, other NBD1 mutants, such as K684L, had decreased ATP binding and rate of solute transport. We now report that mutations of the Walker A lysine residue, K684L and K1333L, significantly alter the tertiary structure of the protein. Due to elimination of the positively charged group and conformational alterations, the K684L mutation greatly decreases the affinity for ATP at the mutated NBD1 and affects ATP binding at the unmutated NBD2. Although K684L-mutated NBD1 can bind ATP at higher concentrations, the bound nucleotide at that site is not efficiently hydrolysed. All these alterations result in decreased ATP-dependent solute transport to approx. 40 % of the wild-type. In contrast,

the K1333L mutation affects ATP binding and hydrolysis at the mutated NBD2 only, leading to decreased ATP-dependent solute transport to approx. 11 % of the wild-type. Consistent with their relative transport activities, the amount of vincristine accumulated in cells is in the order of $K1333L \geq CFTR$ (cystic fibrosis transmembrane conductance regulator) $> K684L \gg$ wild-type MRP1. Although these mutants retain partial solute transport activities, the cells expressing them are not multidrug-resistant owing to inefficient export of the anticancer drugs by these mutants. This indicates that even partial inhibition of transport activity of MRP1 can reverse the multidrug resistance caused by this drug transporter.

Key words: ATP binding/hydrolysis, ATP-dependent transport, conformation, multidrug resistance, multidrug resistance protein 1 (MRP1), nucleotide-binding domain.

INTRODUCTION

Cells overexpressing MRP1 (multidrug resistance protein 1) become multidrug-resistant [1] due to the action of the protein as an ATP-dependent export pump for glutathione (GSH)-conjugated compounds [2], including anticancer drugs. Since there are two NBDs (nucleotide-binding domains) in this protein [1], an important question to be asked is why does this transporter require two NBDs. Structural analyses of some ABC transporter (ATP-binding cassette transporter) NBDs clearly answered this question. The two NBDs form a dimer in which the two ATP molecules are each sandwiched between the Walker A motif from one NBD and the LSGGQ ABC transporter signature motif of the other [3–6]. The next question to be asked is whether these two NBDs play equal or unequal roles during solute transport. The two NBDs of P-gp (P-glycoprotein) have been shown to be functionally equivalent with identical ATP hydrolysis steps occurring alternately at each NBD [7]. Covalent modification of either site or mutation of an essential amino acid in one NBD completely abolished the function of P-gp [7,8]. Based on these results, Senior et al. [9] proposed a working model of coupling ATP hydrolysis alternately at each NBD and solute transport in each cycle, implying one solute transported per ATP hydrolysis. However, a different model, of two independent ATP hydrolysis events in a single drug transport cycle, has been proposed

[10]. The first ATP hydrolysis is associated with efflux of drug and the second ATP hydrolysis brings the P-gp back to its original conformational state so that the molecule can start a new cycle of ATP-dependent solute transport [11]. In this model, the ATP binding/hydrolysis sites of P-gp are recruited in a random fashion and only one is utilized at any given time because of the conformational changes in the catalytic site that drastically reduce the affinity of the second site for nucleotide [11], implying that the functions of the two NBDs are equal. Although some reports indicated that the two NBDs of P-gp were found to be essential for function but not entirely symmetrical [12,13], most NBD1 segments could be replaced by counterpart regions of NBD2 without loss of P-gp function [14]. Thus how the two events at NBD1 and NBD2 of P-gp co-operate during drug transport still remains unclear.

It is clear that the two NBDs of MRP1 have distinct properties and different functions. Modifications of the consensus Walker motifs in the two NBDs of MRP1 do not inactivate the protein completely and have different effects on solute transport [15–17]. Photoaffinity labelling experiments with 8-N₃ATP (8-azido-ATP) also revealed an asymmetry between NBD1 and NBD2, with [γ -³²P]8-N₃ATP preferentially labelling NBD1 [15,16], whereas trapping of the ATP hydrolysis product, ADP, occurred primarily at NBD2 [15,16,18]. ADP trapping at NBD2 stabilized ATP binding at NBD1 [16] and ATP binding at NBD1 allosterically

Abbreviations used: ABC transporter, AMP-PNP, adenosine 5'-[β , γ -imido]triphosphate; ATP-binding cassette transporter; ATR, attenuated total reflection; BHK, baby hamster kidney-21; CFTR, cystic fibrosis transmembrane conductance regulator; FTIR, Fourier-transform infrared; H/D, hydrogen/deuterium; LTC₄, leukotriene C₄; MRP1, multidrug resistance protein 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; 8-N₃ATP, 8-azido-ATP; NBD, nucleotide-binding domain; P-gp, P-glycoprotein; 8-N₃-AMP-PNP, 8-azido-AMP-PNP; Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane; V_i, vanadate.

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enhanced ADP trapping or AMP-PNP (adenosine 5'-[β , γ -imido]triphosphate) binding at NBD2 [19]. The ATP-binding sites of MRP1 seem not to be recruited in a random fashion. For example, photolabelling by the non-hydrolysable [α - ^{32} P]8-N₃-AMP-PNP (8-azido-AMP-PNP), which in the absence of other nucleotides occurred predominately at NBD1, was enhanced at NBD2 by a low concentration of 8-N₃ATP, implying that NBD1 has higher affinity for nucleotide than NBD2 [19]. Further studies confirmed that the NBD1 of MRP1 has higher affinity for nucleotide than NBD2 [20]. Replacement of the putative catalytic residue Glu¹⁴⁵⁵, directly adjacent to the Walker B motif in NBD2, with a non-acidic amino acid almost completely abolished ATP-dependent LTC₄ (leukotriene C₄) transport [21,22]. Conversely, replacement of the putative catalytic residue Asp⁷⁹³, directly adjacent to the Walker B motif in NBD1, with a non-acidic amino acid increased the rate of ATP-dependent LTC₄ transport [17,22]. Replacement of the aromatic residue Trp⁶⁵³, which interacts with the adenine ring of the bound ATP [3,6], with a polar cysteine residue decreased affinity for ATP, but dramatically increased the rate of ATP-dependent LTC₄ transport [23]. We speculated that ATP hydrolysis at NBD2 and dissociation of the bound ATP from NBD1, regardless of whether it is hydrolysed or not, brought MRP1 back to its original conformation so that the molecule can start a new cycle of ATP-dependent solute transport. However, this speculation cannot explain why the K684L mutation decreases affinity for ATP at the mutated NBD1, in other words, increases the release rate from the mutated NBD1, but does not increase the rate of ATP-dependent solute transport. We have now found that replacement of the Walker A lysine residue with a leucine residue in either NBD1 (K684L) or NBD2 (K1333L) significantly alters the tertiary structure of the protein and affects ATP binding/hydrolysis and ATP-dependent solute transport.

MATERIALS AND METHODS

Materials

Verapamil, daunomycin, colchicine, vincristine, sodium orthovanadate, EGTA, EDTA, ATP and Tos-Phe-CH₂Cl (tosylphenylalanylchloromethane; 'TPCK')-treated trypsin were purchased from Sigma. [α - ^{32} P]8-N₃ATP, [γ - ^{32} P]8-N₃ATP and [α - ^{32} P]8-N₃-AMP-PNP were purchased from Affinity Labeling Technologies. [^{14} C, ^{15} C, ^{19} C, ^{20} - ^{3} H]LTC₄ was from NEN Life Science Products. [^{3} H]Vincristine was from Amersham Biosciences. Protein G beads were purchased from Invitrogen. Stratalinker UV Crosslinker 2400 model (wavelength 254 nm) was from Stratagene.

Cell culture and cell lines expressing MRP1

Cell lines expressing wild-type, K684L- and K1333L-mutated MRP1s and CFTR (cystic fibrosis transmembrane conductance regulator) were established previously [16,24,25] (but see [25a]). These BHK (baby hamster kidney-21) cell lines were cultured at 37°C in 5% CO₂. Cells for membrane vesicle preparation and purification were grown in roller bottles (Bellco).

Membrane vesicle preparations

Membrane vesicles were prepared according to the procedure described previously [16]. The membrane vesicle pellet was resuspended in a solution containing 10 mM Tris/HCl (pH 7.5), 250 mM sucrose and 1× protease inhibitors {2 µg/ml aprotinin, 121 µg/ml benzamidin, 3.5 µg/ml E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane], 1 µg/ml leupeptin and 50 µg/ml Pefabloc}. After passage through a Liposofast™ vesicle

extruder (Avestin, Ottawa, ON, Canada), they were divided into 200 µl aliquots and stored at -80°C.

Identification of NBD1- and NBD2-containing fragments

Western blotting was performed according to the method described previously [16]. 42.4 monoclonal antibody was used to identify the NBD1-containing fragments of MRP1, whereas 897.2 monoclonal antibody was used to detect the NBD2-containing fragments [16].

Membrane vesicle transport

ATP-dependent LTC₄ transport was assayed by a rapid filtration technique [26,27]. A solution (15 µl) containing 3 µg of membrane proteins, 50 mM Tris/HCl (pH 7.5), 250 mM sucrose, 10 mM MgCl₂, 200 nM LTC₄ (17.54 nCi of ^{3} H-labelled LTC₄) and 15 µl of a solution containing 8 mM ATP (8 mM AMP was used as control), 50 mM Tris/HCl (pH 7.5), 250 mM sucrose, 10 mM MgCl₂ and 200 nM LTC₄ were pre-warmed to 37°C, mixed (so that the final ATP concentration should be 4 mM) and incubated at 37°C for 30 s. The samples were chilled on ice, diluted with 1 ml of ice-cold 1× transport buffer (50 mM Tris/HCl, pH 7.5, 250 mM sucrose and 10 mM MgCl₂) immediately and trapped on nitrocellulose membranes (0.2 µm) that had been equilibrated with 1× transport buffer. The filter was then washed with 10 ml of ice-cold 1× transport buffer, air-dried and placed in 10 ml of biodegradable counting scintillant (Amersham Biosciences). The amount of radioactivity bound to the nitrocellulose membrane was determined by liquid-scintillation counting (Beckman LS 6000SC).

Photoaffinity labelling of MRP1 protein

V_i (vanadate) preparation and photoaffinity labelling of MRP1 were performed according to procedures described previously [16]. Briefly, photolabelling experiments were carried out in 10 µl of a solution containing membrane proteins, 40 mM Tris/HCl (pH 7.5), 2 mM ouabain, 10 mM MgCl₂ and 0.1 mM EGTA. The amount of membrane proteins, concentration of ^{32} P-labelled nucleotide, concentration of V_i, incubation time and temperature are specifically indicated in Figures 2 and 3. The labelled proteins were separated by 7% PAGE and electroblotted on to a nitrocellulose membrane.

In order to determine which NBD was labelled, samples, after UV irradiation, were resuspended in 20 µl of a solution containing 40 mM Tris/HCl (pH 7.4) and 1 mM EDTA, digested with Tos-Phe-CH₂Cl-treated trypsin (trypsin/protein ratio = 1:16) at 37°C for 15 min, chilled on ice and loaded on to a gel directly, or immunoprecipitated in 0.5 ml of RIPA buffer containing 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1× protease inhibitors and antibodies against NBD1 (5 µl of 42.4 antibody) and NBD2 (5 µl of 897.2 antibody). Protein G-agarose beads, after overnight incubation in a cold room (4°C), were added to the above mixture and gently shaken for 60 min in a cold room. The complex formed with the Protein G-agarose beads was washed four times with RIPA buffer and eluted with 1× electrophoresis sample buffer [50 mM Tris/HCl, pH 6.8, 100 mM dithiothreitol, 2% (w/v) SDS, 0.1% (w/v) Bromophenol Blue and 10% (v/v) glycerol] for 60 min at room temperature (23°C).

Purification and reconstitution of MRP1

MRP1 was purified as described previously ([25] but see [25a]) in the absence of phospholipids. Reconstitution of wild-type, K684L- and K1333L-mutated MRP1 was achieved by employing

SM-2 Bio-Beads to remove detergent from the protein/detergent/lipid mixture as described previously [28].

ATR (attenuated total reflection)–FTIR (Fourier-transform infrared) spectroscopy

ATR–FTIR spectra were recorded at room temperature on a Bruker IFS55 FTIR spectrophotometer equipped with a liquid nitrogen-cooled MCT (mercury-cadmium-telluride) detector at a resolution of 2 cm^{-1} and encoded every 1 cm^{-1} . The spectrophotometer was continuously purged with an FTIR purge gas generator 75-62 Balston (Maidstone, Kent, U.K.) at a flow rate of 5.8 l/min. The internal ATR element is a germanium plate ($50\text{ mm} \times 20\text{ mm} \times 2\text{ mm}$) with an aperture angle of 45° , yielding 25 internal reflections [29].

Sample preparation

Reconstituted wild-type, K684L- or K1333L-mutated MRP1s ($20\text{ }\mu\text{g}$) was mixed with either ATP + V_i or AMP-PNP (the molar ratio of protein to ATP is 1:6). Thin films of orientated multilayers were obtained by slowly evaporating the sample on one side of the ATR plate under a stream of nitrogen [29]. The ATR plate was then sealed in a universal sample holder (Harrick Scientific).

Secondary structure analysis

The sample on the ATR plate was equilibrated with $^2\text{H}_2\text{O}$ -saturated N_2 for 2 h at room temperature. Scans (256) were averaged for each measurement. The determination of the secondary structure was based on the shape of the amide I band ($1600\text{--}1700\text{ cm}^{-1}$), which is sensitive to the secondary structure [30]. The analysis was performed on the amide I region of deuterated samples because the $\text{H}/^2\text{H}$ (hydrogen/deuterium) exchange allows differentiation of the α -helical secondary structure from the random secondary structure for which the absorption band shifts from 1655 to 1642 cm^{-1} [31]. A combination of Fourier self-deconvolution and a least squares iterative curve fitting was applied to narrow and to quantify the area of the different components of the amide I band between 1700 and 1600 cm^{-1} [32]. To avoid the introduction of artefacts due to the self-deconvolution procedure, the fitting was performed on the non-deconvoluted spectrum. The proportion of a particular structure was computed as the sum of the area of all the fitted Lorentzian bands having their maximum in the frequency region where that structure occurs divided by the total area of the amide I. The frequency limits for each structure were assigned according to theoretical [33] or experimental [34] data: $1662\text{--}1645\text{ cm}^{-1}$, α -helix; $1689\text{--}1682$ and $1637\text{--}1613\text{ cm}^{-1}$, β -sheet; $1644.5\text{--}1637\text{ cm}^{-1}$, random; $1682\text{--}1662.5\text{ cm}^{-1}$, β -turns.

$\text{H}/^2\text{H}$ exchange kinetics

Films containing $20\text{ }\mu\text{g}$ of reconstituted protein were formed on a germanium plate as described above. Nitrogen was saturated with $^2\text{H}_2\text{O}$ by bubbling in a series of three vials containing $^2\text{H}_2\text{O}$. Before starting the deuteration, ten spectra of the sample were recorded to test the stability of the measurements. At zero time, the $^2\text{H}_2\text{O}$ -saturated N_2 flux, at a flow rate of 100 ml/min , was connected to the sample. For measurement of $\text{H}/^2\text{H}$ exchange kinetics at each time point, 24 scans were recorded and averaged at a resolution of 2 cm^{-1} . The signal from the atmospheric water was subtracted as described by Goormaghtigh and Ruyschaert [35]. As previously described [33], deuteration of protein side chains induces modifications in the amide I ($1700\text{--}1600\text{ cm}^{-1}$) and amide II ($1600\text{--}1500\text{ cm}^{-1}$) regions. Several parameters, including the ionization state of the carboxylic amino acids and the fraction of

deuterated and undeuterated amino acid side chains, affect the modification for each spectrum of the kinetics in the amide I and II. We used homemade software, which can compute the contribution of the amino acid side chains as a function of the extent of deuteration [36]. The area of amide II, characteristic of the $\delta(\text{N-H})$ vibration, was obtained by integration between 1596 and 1502 cm^{-1} . For each spectrum, the area of amide II was divided by the corresponding lipidic $\nu(\text{C=O})$ area to take into account small but significant variations in the total intensity due to the presence of $^2\text{H}_2\text{O}$, which induces swelling of the sample layer at the beginning of the kinetics [31]. This ratio expressed as a percentage was plotted against deuteration time. The value corresponding to 0% deuteration is defined by the amide II/lipid ratio before deuteration. The 100% value corresponds to a zero absorption in the amide II region, observed for full deuteration of the protein.

Chemosensitivity assay

The resistance of BHK cells to anticancer drugs was determined by employing the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay [37]. Briefly, cells were plated in a volume of $200\text{ }\mu\text{l}$ at 2500 cells/well in 96-well plates. After incubation at 37°C overnight, the media were replaced with fresh media containing various concentrations of anticancer drugs. The cells were incubated for a further 4 days. At the end of drug exposure, medium containing 0.5 mg/ml MTT was added to the wells and the plates were incubated for an additional 4 h. After removing the medium containing MTT, the cells were dried completely and dissolved in $200\text{ }\mu\text{l}$ of a solution containing propan-2-ol and 1 M HCl (ratio of 24:1). Cell survival was determined by measuring the absorbance (A) at 570 nm .

Vincristine accumulation

Cells (10^5 per well) were seeded (in triplicate) in a 24-well plate and incubated overnight in the absence of vincristine. Fresh medium containing $1\text{ }\mu\text{M}$ ^3H -labelled vincristine ($0.1\text{ }\mu\text{Ci/well}$) was used to replace the old medium and incubated at 37°C for various times. Cells were washed three times with ice-cold PBS, lysed with 2% SDS and used to determine the amount of radioactivity.

RESULTS

Mutation of NBD2 has a greater effect on the ATP-dependent LTC4 transport than the corresponding mutation of NBD1

We have found that the K1333L-mutated MRP1 almost completely abolished ATP-dependent solute uptake, whereas the corresponding mutation in NBD1, K684L, reduced it by approximately half [16]. In order to further characterize the properties of these mutants, the amount of MRP1 in membrane vesicles was quantified by scanning the densities of the MRP1 bands, yielding a ratio of approx. 0.564 (K684L) or approx. 1.608 (K1333L) compared with wild-type MRP1 (Figure 1A). The ATP-dependent LTC4 transport activities of K684L- and K1333L-mutated MRP1, after adjusting the amount of MRP1 in the membrane vesicles to a similar amount with membrane vesicles containing CFTR, are approx. 40% (K684L) and 11% (K1333L) of the wild-type MRP1 (Figure 1B), indicating that mutation of NBD2 has a larger effect on the ATP-dependent LTC4 transport than the corresponding mutation of NBD1. In addition, the ATP-dependent LTC4 transport activity of parental BHK cell membrane vesicles (results not shown) and similar vesicles from cells expressing CFTR is approx. 8% of the wild-type MRP1, implying that

Figure 1 Expression and functional analysis of wild-type and Walker A lysine mutants

(A) Expression of wild-type (WT), K684L- and K1333L-mutated MRP1 in BHK cells. Membrane vesicles were prepared from BHK cells expressing wild-type, K684L- or K1333L-mutated MRP1 and used in Western-blot analyses. The amounts of membrane proteins loaded are indicated at the top of the gel, proteins were resolved by SDS/PAGE (7% gel), electroblotted on to a nitrocellulose membrane and probed with MRP1-specific antibodies 42.4 and 897.2 [16]. Molecular mass markers (kDa) are indicated on the left. The intensities of the MRP1 bands were measured by a scanning densitometer. The ratios of the band intensities are: 1.000 (wild-type MRP1), 0.564 ± 0.093 (K684L, $n = 3$) and 1.608 ± 0.175 (K1333L, $n = 3$). (B) Relative LTC4 transport activity by membrane vesicles containing wild-type, K684L- and K1333L-mutated MRP1. ATP-dependent LTC4 transport assay was performed according to the method described in the Materials and methods section. Since the amounts of MRP1 proteins in the membrane vesicles containing wild-type, K684L- and K1333L-mutated MRP1 were different, they were adjusted to a similar amount of MRP1 with membrane vesicles containing CFTR (1.692 μg of wild-type MRP1 + 1.308 μg of CFTR; 3 μg of K684L; 1.05 μg of K1333L + 1.95 μg of CFTR; 3 μg of CFTR) to determine the ATP-dependent LTC4 transport activity.

there are some endogenous ATP-dependent solute transporters that contribute to approx. 8% transport activity. Thus the ATP-dependent LTC4 transport activity of K684L- or K1333L-mutated MRP1 should be less than 40% or 11% of the wild-type MRP1.

Mutation of NBD1 impairs ATP binding at NBD1 and NBD2, whereas mutation of NBD2 does not affect ATP binding at NBD1

Since the Walker A lysine residue is involved in ATP binding [3], we speculated that replacement of the positively charged lysine residue with a hydrophobic leucine residue should decrease the affinity for ATP at the mutated NBD. To test this hypothesis, membrane vesicles containing wild-type, K684L- and K1333L-mutated MRP1s were labelled with various concentrations of [α - ^{32}P]8-N₃ATP on ice (Figures 2A–2C). Since ATP hydrolysis is greatly decreased on ice, the labelling should mainly reflect the equilibrium between binding and release of ATP. Figures 2(A) and 2(C) show that wild-type and K1333L-mutated MRP1 are heavily labelled by 16 μM [α - ^{32}P]8-N₃ATP, whereas K684L is not heavily labelled even at 256 μM (Figure 2B), implying that ATP binding to K684L-mutated NBD1 and unmutated NBD2 is significantly decreased. Unexpectedly, the K_d value of K1333L is only approximately one-third of the wild-type MRP1 (Table 1), similar to the K_d value of wild-type NBD1 determined by digesting the labelled wild-type MRP1 with trypsin [20], implying that the labelling of K1333L mainly occurs at the unmutated NBD1 and mutation of the NBD2 does not have a significant effect on the unmutated NBD1. The results in Figures 2(G) and 2(H) strongly support this hypothesis. Using the same rationale, the labelling of K684L-mutated MRP1 should mainly occur at the unmutated NBD2. This conclusion is supported by the results in Figures 2(G) and 2(H). However, the K_d value of K684L is increased to 56 μM , which is much higher than that of wild-type MRP1 (Table 1) or the K_d value of wild-type NBD2 determined by digesting the labelled wild-type MRP1 with trypsin [20], implying that K684L mutation impairs the regulatory effect of ATP binding to NBD1 [19].

Since ATP-dependent anticancer drug transport experiments were performed in cell culture at 37°C, the K_d values determined on ice may not reflect the real situation at 37°C. However, K_d (ATP) values cannot be determined at 37°C due to ATP hydrolysis. We then decided to use the non-hydrolysable ATP analogue, [α - ^{32}P]8-N₃-AMP-PNP, to label the MRP1 protein at 37°C. Figures 2(D–F) (wild-type MRP1, K684L and K1333L respectively) show that the labelling patterns of these MRP1 proteins with [α - ^{32}P]8-N₃-AMP-PNP at 37°C are similar to those performed on ice with [α - ^{32}P]8-N₃ATP (Figures 2A–2C). For example, the labelling intensity of K684L is much weaker than either wild-type or K1333L-mutated MRP1. Due to very low levels of labelling, the K_d (AMP-PNP) of K684L-mutated MRP1 cannot be accurately determined. In addition, the labelling of K1333L-mutated MRP1 at a higher AMP-PNP concentration is greatly increased (Figure 2F), implying that the K1333L-mutated NBD2 could also be labelled.

ATP bound to the mutated NBDs is not efficiently hydrolysed

Since the ATP-dependent LTC4 transport activity of K1333L is extremely low (Figure 1B), we expected that even if nucleotide can bind to the K1333L-mutated NBD2 at higher concentrations, the bound nucleotide would not be efficiently hydrolysed by this mutated NBD2. To test this hypothesis, wild-type, K684L- and K1333L-mutated MRP1s were labelled with 8 μM of either [α - ^{32}P]8-N₃ATP or [γ - ^{32}P]8-N₃ATP at 37°C in the presence of V_i (Figure 3A). The ratio of [γ - ^{32}P]8-N₃ATP labelling to [α - ^{32}P]8-N₃ATP labelling is approx. 60.4% (wild-type MRP1), approx. 83.9% (K684L) and approx. 109.3% (K1333L), implying that the ATP bound to K684L is more efficiently hydrolysed than the ATP bound to K1333L and less efficiently hydrolysed than the ATP bound to wild-type MRP1. These conclusions are supported by the results in Figure 3(B), i.e. (i) both [α - ^{32}P]8-N₃ATP and [γ - ^{32}P]8-N₃ATP labelled the unmutated NBD1 of K1333L-mutated MRP1 with similar intensity; (ii) [α - ^{32}P]8-N₃ATP mainly labelled the unmutated

Figure 2 Nucleotide binding to wild-type and mutant MRPs

Samples were mixed in 10 μ l of a solution containing 10 μ g of wild-type MRP1 (**A**), K684L (**B**) or K1333L (**C**) membrane proteins and various concentrations of [α - ^{32} P]8-N $_3$ ATP, incubated on ice for 1 min and UV-irradiated on ice for 2 min. Due to high background at a higher concentration of [α - ^{32} P]8-N $_3$ ATP, the labelled K684L and K1333L proteins were immunoprecipitated with MRP1-specific antibodies 42.4 and 897.2 [16]. The labelled samples were subjected to SDS/PAGE (7% gel) and electroblotted on to a nitrocellulose membrane. MRP1 indicates the monomer 190 kDa MRP1 protein and higher molecular mass aggregation products (verified by Western blotting using antibodies against MRP1) caused by immunoprecipitation. In order to check nucleotide binding (without hydrolysis) at 37 $^{\circ}$ C, the non-hydrolysable ATP analogue AMP-PNP was used for labelling at 37 $^{\circ}$ C. Samples were mixed in 10 μ l of a solution containing 10 μ g of wild-type MRP1 (**D**), K684L (**E**) or K1333L (**F**) and various concentrations of [α - ^{32} P]8-N $_3$ AMP-PNP, incubated at 37 $^{\circ}$ C for 10 min and UV-irradiated after washing with 500 μ l of ice-cold Tris/EGTA buffer (0.1 mM EGTA and 40 mM Tris/HCl, pH 7.5) [16]. In order to prove that the labelling at K684L- or K1333L-mutated MRP1 mainly occurs at the unmutated NBD, the labelled wild-type (WT), K684L- and K1333L-mutated MRP1 were digested with trypsin (**G**). The same nitrocellulose membrane was probed with either the NBD1-specific antibody 42.4 or the NBD2-specific antibody 897.2 (**H**).

NBD2 of K684L, whereas [γ - ^{32} P]8-N $_3$ ATP barely labelled NBD1 or NBD2 of K684L; (iii) [α - ^{32} P]8-N $_3$ ATP labelled both NBD1 and NBD2 of wild-type MRP1, whereas [γ - ^{32} P]8-N $_3$ ATP mainly labelled NBD1 of wild-type MRP1. At a higher concentration of ATP (128 μ M in Figure 3C), K684L-mutated NBD1 and K1333L-mutated NBD2 are clearly labelled by either [α - ^{32} P]8-N $_3$ ATP or [γ - ^{32} P]8-N $_3$ ATP. Interestingly, the [γ - ^{32} P]8-N $_3$ ATP labelling intensity of the unmutated NBD2 of K684L-mutated MRP1 is much weaker than the [α - ^{32} P]8-N $_3$ ATP labelling, whereas

the [γ - ^{32} P]8-N $_3$ ATP labelling intensity of the K684L-mutated NBD1 is similar to that of [α - ^{32} P]8-N $_3$ ATP labelling, implying that ATP bound to the K684L-mutated NBD1 is not efficiently hydrolysed. In contrast, the [γ - ^{32} P]8-N $_3$ ATP labelling intensity of the unmutated NBD1 of K1333L-mutated MRP1 is much weaker than the [α - ^{32} P]8-N $_3$ ATP labelling, whereas the [γ - ^{32} P]8-N $_3$ ATP labelling intensity of the K1333L-mutated NBD2 is similar to that of the [α - ^{32} P]8-N $_3$ ATP labelling, implying that ATP bound to the K1333L-mutated NBD2 is not efficiently hydrolysed.

Table 1 Mean K_d (ATP) of wild-type and mutant MRP1

Protein	K_d (μ M ATP)*
Wild-type MRP1	32.3 ± 10.9
K684L	55.9 ± 16.5
K1333L	10.8 ± 5.3

* The K_d values of wild-type MRP1 ($n = 6$), K684L ($n = 6$) and K1333L ($n = 6$) were derived from Figures 2(A)–2(C).

Walker A lysine mutations of MRP1 and nucleotide binding/hydrolysis do not alter the secondary structure of the protein

Since mutation of these two lysine residues in Walker A motifs have drastic effect on the ATP-dependent solute transport, we expected that these point mutations might alter the secondary structure of the protein. The secondary structures of wild-type and mutated MRP1s were determined by using purified and reconstituted proteins. The IR spectra (Figure 4) of wild-type and mutated MRP1s were recorded in the absence or presence of AMP-PNP that mimics ATP binding or ATP + V_i that maintains the protein in a conformation in which the ATP hydrolysis product ADP remains trapped. The secondary structure of the protein was determined by Fourier deconvolution and curve-fitting analysis of the amide I band of a deuterated sample as described previously [32]. The results in Table 2 show that these Walker A lysine mutations as well as the binding of AMP-PNP or ATP + V_i did not significantly alter the secondary structure of the protein.

Walker A lysine mutations of MRP1 alter the tertiary structure of the protein

Since mutations of these two lysine residues in the Walker A motifs did not alter the secondary structure of the protein, we expected that these point mutations might alter the tertiary structure of the protein. Conformational alterations were monitored by H^2H exchange. The decrease in the amide II band area (located between 1600 and 1500 cm^{-1}) as a function of deuteration time is a measure of the rate of amide hydrogen (N–H) exchanged with deuterium (N– ^2H) (Figure 4). This kinetic analysis provides information on the protein's accessibility to the aqueous phase and/or on the stability of the secondary structure of the protein. The kinetics of deuteration were followed by monitoring the decrease in the amide II absorption peak of the protein exposed to nitrogen-saturated ^2H_2O as a function of time (Figure 5A). The kinetics of H^2H exchange of wild-type, K684L- and K1333L-mutated MRP1s in the absence of nucleotide were different from each other, with 37, 21 and 43 % of amide hydrogen remaining unexchanged after a 2 h exposure to ^2H_2O (Figure 5A), implying that replacement of the lysine residue with leucine altered the tertiary structure of the protein. The amide hydrogens that are poorly accessible to the aqueous phase may be involved in stable secondary structures. Thus the protein with the K684L mutation becomes more 'relaxed' (greater extent of water accessibility) than that of the wild-type MRP1, whereas the protein with the K1333L mutation becomes slightly more 'compact' (lesser extent of water accessibility) than that of the wild-type MRP1.

Conformational changes induced by nucleotide binding/hydrolysis at NBD1 are different from that at NBD2

Since binding of ATP to NBD1 induced conformational changes of the protein and enhanced ATP binding to NBD2 [19], we expected that conformational changes induced by ATP binding/

Figure 3 Walker A lysine mutations affect ATP binding and hydrolysis

Samples were mixed in 10 μ l of a solution containing wild-type (10 μ g), K684L- (15 μ g) or K1333L-mutated MRP1 (10 μ g), 800 μ M V_i and 8 μ M of either [α - ^{32}P]8-N $_3$ ATP or [γ - ^{32}P]8-N $_3$ ATP, incubated at 37 °C for 4 min and UV-irradiated on ice for 2 min (A). The labelled samples were subjected to SDS/PAGE (7% gel) and electroblotted on to a nitrocellulose membrane. The amounts of radioactivity incorporated into MRP1 were determined by using a Packard instant imager, yielding a ratio ([γ - ^{32}P]8-N $_3$ ATP labelling versus [α - ^{32}P]8-N $_3$ ATP labelling) of 60.4 ± 16.6 % (wild-type, $n = 7$); 83.9 ± 17.5 % (K684L, $n = 4$); and 109.3 ± 20.6 % (K1333L, $n = 5$). Samples were mixed in 10 μ l of a solution containing wild-type (10 μ g), K684L- (15 μ g) or K1333L-mutated MRP1 (10 μ g), 800 μ M V_i and 8 μ M (B) or 128 μ M (C) of either [α - ^{32}P]8-N $_3$ ATP or [γ - ^{32}P]8-N $_3$ ATP, incubated at 37 °C for 10 min, UV-irradiated on ice for 2 min, washed with 500 μ l of ice-cold Tris-EGTA buffer and digested with trypsin (trypsin/protein ratio = 1:16). The digested samples were immunoprecipitated with antibodies 42.4 against NBD1 and 897.2 against NBD2 [16]. NBD1 and NBD2 indicate the labelled fragments containing NBD1 and NBD2.

hydrolysis at NBD1 should be different from those at NBD2. Binding of the non-hydrolysable ATP analogue AMP-PNP to wild-type MRP1 increased water accessibility, with 8 % (from 37 to 29 %) extra amide hydrogen exchanged (Figure 5B), implying that binding of the nucleotide, not hydrolysis, to NBD1 and NBD2 induced conformational changes of the protein. Incubation of the same protein with ATP in the presence of V_i resulted in 11 % (from 37 to 26 %) extra amide hydrogen exchanged

Figure 4 IR spectra of MRP1 reconstituted into lipids

Thin films were obtained by slowly evaporating a sample containing 20 μg of the reconstituted MRP1 on an ATR element. Spectra were recorded as a function of time exposed to $^2\text{H}_2\text{O}$ -saturated N_2 . Negative values refer to the spectra recorded before starting the deuteration procedure. The inset shows the decrease in intensity of the amide II band during the $\text{H}/^2\text{H}$ exchange kinetics of MRP1.

Table 2 Secondary structure analysis of wild-type, K684L- and K1333L-mutated MRP1 in the presence or in the absence of nucleotide

Protein and substrate	Proportion (%)			
	α -Helix	β -Sheet	β -Turn	Random coil
MRP1	42 $\pm$ 3	29 $\pm$ 3	12 $\pm$ 4	17 $\pm$ 6
MRP1 + AMP-PNP	40 $\pm$ 2	28 $\pm$ 2	11 $\pm$ 3	21 $\pm$ 4
MRP1 + ATP + V_i	43 $\pm$ 2	27 $\pm$ 3	12 $\pm$ 3	18 $\pm$ 3
K684L	41 $\pm$ 2	31 $\pm$ 4	9 $\pm$ 4	19 $\pm$ 3
K684L + AMP-PNP	39 $\pm$ 3	30 $\pm$ 4	10 $\pm$ 3	21 $\pm$ 5
K684L + ATP + V_i	40 $\pm$ 2	32 $\pm$ 3	11 $\pm$ 3	17 $\pm$ 4
K1333L	42 $\pm$ 2	28 $\pm$ 4	11 $\pm$ 3	19 $\pm$ 3
K1333L + AMP-PNP	41 $\pm$ 3	29 $\pm$ 2	12 $\pm$ 2	18 $\pm$ 4
K1333L + ATP + V_i	39 $\pm$ 3	30 $\pm$ 3	13 $\pm$ 4	18 $\pm$ 4

(Figure 5B), indicating that ATP binding, hydrolysis and trapping of the hydrolysis product ADP by V_i induced conformational changes slightly different from those observed after AMP-PNP binding.

For the K684L mutant, non-hydrolysable ATP analogue AMP-PNP binding increased the amount of unexchanged amide hydrogen from 21 to 25 % (Figure 5C), whereas binding of hydrolysable ATP in the presence of V_i increased the amount of unexchanged amide hydrogen from 21 to 31 % (Figure 5C). These results indicate that although nucleotide binding to the K684L-mutated NBD1 is significantly reduced (Figure 2), AMP-PNP binding made the protein more 'compact' than the original state, and ATP binding, hydrolysis and trapping of ADP (probably at

the unmutated NBD2) led the protein to an even more 'compact' structure (Figure 5C).

Interestingly, non-hydrolysable ATP analogue AMP-PNP binding to the K1333L-mutated MRP1 induced the same (from 43 to 26 % with 17 % extra amide hydrogen exchanged) extent of conformational changes as did ATP + V_i (Figure 5D). The fact that the $\text{H}/^2\text{H}$ exchange patterns in the presence of either AMP-PNP or ATP + V_i are almost identical indicates that the bound ATP at either unmutated NBD1 or K1333L-mutated NBD2 is not efficiently hydrolysed. This conclusion is consistent with the results shown in Figure 3(A).

Cells expressing K684L- or K1333L-mutated MRP1s are not multidrug-resistant

Considering that the transport activities of K684L, K1333L and CFTR (or parental BHK) are approx. 40, 11 and 8 % of the wild-type MRP1, we expected that cells expressing K684L-mutated MRP1 should be partially resistant to anticancer drugs, whereas the cells expressing K1333L-mutated MRP1 should not. Indeed, the cells expressing K1333L-mutated MRP1 are not resistant to daunomycin (Figure 6A), colchicine (Figure 6B) or vincristine (Figure 6C). However, the cells expressing K684L-mutated MRP1 are also not resistant to daunomycin (Figure 6A), colchicine (Figure 6B) or vincristine (Figure 6C). The amount of vincristine that accumulated in cells expressing K1333L-mutated MRP1 is similar to that in cells expressing CFTR (Figure 7), whereas the accumulation of vincristine in cells expressing either wild-type or K684L-mutated MRP1 is slightly less than in cells expressing K1333L-mutated MRP1 or CFTR within 15 min incubation at 37 °C (Figure 7), implying that either wild-type or K684L-mutated MRP1 has greater ability to transport vincristine out of the cells than the K1333L-mutated MRP1. The amount of vincristine that accumulated in cells expressing K684L-mutated MRP1 is more than in cells expressing wild-type MRP1, but less than in cells expressing either K1333L-mutated MRP1 or CFTR after 30, 60 or 120 min incubation at 37 °C (Figure 7), implying that K684L-mutated MRP1 is more active than K1333L-mutated MRP1, but less active than wild-type MRP1. This lower transport activity resulted in the cells expressing K684L-mutated MRP1 not being resistant to vincristine (Figure 6C).

Cells expressing either K684L- or K1333L-mutated MRP1s are not hypersensitive to verapamil

We have found that cells expressing wild-type MRP1 are hypersensitive to verapamil [38]. Since K684L- or K1333L-mutated MRP1 has approx. 40 or 11 % of the wild-type MRP1 transport activity (Figure 1B), we expected that cells expressing K1333L-mutated MRP1 should have similar sensitivity to verapamil as the cells without MRP1 expression, whereas the cells expressing K684L-mutated MRP1s might be more sensitive to verapamil than the cells without MRP1 expression. Indeed, the cells expressing K1333L-mutated MRP1 have similar sensitivity to verapamil as the parental BHK cells (Figure 6D). However, the cells expressing K684L-mutated MRP1s are not more sensitive to verapamil than its parental BHK cells (Figure 6D), implying that the intracellular glutathione is not efficiently co-transported out with verapamil by the K684L-mutated MRP1.

DISCUSSION

MRP1 transports its substrate in an ATP-dependent manner by utilizing its two NBDs and transmembrane domains. Both NBDs of MRP1 can bind nucleotide and contribute to solute transport

Figure 5 Evolution of the proportion of unexchanged amide hydrogen in wild-type and mutant MRP1 as a function of the deuteration time

The data were derived from Figure 4 by using wild-type (A, B), K684L- (A, C) or K1333L-mutated (A, D) MRP1s in the absence or presence of AMP-PNP or ATP + V_i . The molar ratio of MRP1 protein to nucleotide (AMP-PNP or ATP + V_i) is 1:6. The peak value before the samples were exposed to $^2\text{H}_2\text{O}$ -saturated N_2 is considered as 100% of unexchanged amide hydrogen, whereas the disappearance of the absorption in that region is considered as 0% of unexchanged amide hydrogen. The curves are the means for at least three experiments.

[15,16,18]. However, the functions of the two NBDs do not seem to be identical. Sequence dissimilarities [1] between the two NBDs suggest that their tertiary structures might be different. This asymmetry is visualized by IR spectroscopy that shows distinct conformational alterations for the Walker A lysine mutant K684L in NBD1, and the corresponding NBD2 mutant K1333L (Figure 5A), suggesting that the original tertiary structures of the two NBDs are different. It is therefore reasonable that mutations of the consensus Walker A lysine residues in the two NBDs have different effects (Figure 1B) on solute transport [15,16].

ATP binding at NBD1 mainly plays a regulatory role during ATP-dependent solute transport [20]. ATP binding to NBD1, but not hydrolysis, increases binding and hydrolysis of nucleotide at NBD2 [19,39]. Replacement of the putative catalytic base Asp⁷⁹³ in NBD1 of MRP1 with a non-acidic amino acid, such as leucine or asparagine, decreased affinity for ATP at the mutated NBD1, but did not have a significant effect on ATP binding at the unmutated NBD2 [22]. In addition, these mutations did not abolish the enhancement effect of ATP on the $[\alpha\text{-}^{32}\text{P}]\text{8-N}_3\text{ADP}$ trapping (R. Yang and X.-b. Chang, unpublished work) and the ATP-dependent LTC4 transport [21,22]; instead, it enhanced the transport [22]. In contrast, replacement of the positively charged Walker A Lys⁶⁸⁴ in NBD1 with a hydrophobic leucine greatly decreased the ATP binding at the mutated NBD1 (Figure 2) and significantly diminished the enhancing effect of ATP on the $[\alpha\text{-}^{32}\text{P}]\text{8-N}_3\text{ADP}$ trapping [19] and the ATP-dependent LTC4 transport [15,16], probably due to the elimination of a positively charged group and the conformational alterations caused by the K684L mutation (Figure 5A). Replacement of the positively charged Walker A Lys¹³³³ in NBD2 with a hydrophobic leucine

greatly reduced the ATP binding at the mutated NBD2, but did not have a significant effect on ATP binding at the unmutated NBD1 (Figure 2 and Table 1). However, ATP can still bind to the K1333L-mutated NBD2 at a higher concentration (Figure 3C). Interestingly, ATP can efficiently bind to a recombinant MRP1-NBD1 fragment, but cannot bind to a recombinant MRP1-NBD2 fragment at all [15]. We interpret these results as indicating that the NBD1–NBD2 heterodimer formation may not be crucial for ATP binding at NBD1, but may play a vital role in ATP binding at NBD2.

The results in Figure 3(C) indicate that the ATP bound to either K684L-mutated NBD1 or K1333L-mutated NBD2 is not efficiently hydrolysed. Since the Walker A lysine residue is neither a putative catalytic base [3] nor a key histidine residue involved in substrate-assisted catalysis [40], there must be other reasons preventing efficient ATP hydrolysis at the mutated site. One possible reason could be that the tertiary structure changes caused by these mutations (Figure 5A) might alter the stereo-geometry of the putative catalytic base or the key histidine residue involved in substrate-assisted catalysis and the bound ATP in the mutated NBDs. Considering the data accumulated from other NBD2 mutants, such as Y1302C, E1455Q, H1486F and H1486L, the conformational alterations caused by the K1333L mutation may not be the only reason preventing ATP hydrolysis at the mutated site. Replacement of the aromatic Tyr¹³⁰², which interacts with the adenine ring of the bound ATP at NBD2, with a polar cysteine residue greatly increased the K_d of ATP binding, in other words, it greatly decreased affinity for ATP, but significantly increased the rate of ATP-dependent LTC4 transport [23]. In contrast, replacement of the putative catalytic base Glu¹⁴⁵⁵,

Figure 6 BHK cells expressing K684L- or K1333L-mutated MRP1 are neither multidrug-resistant nor hypersensitive to verapamil

Cell survival experiments were performed according to the chemosensitivity assay as described in the Materials and methods section. Various concentrations of daunomycin (**A**), colchicine (**B**), vincristine (**C**) and verapamil (**D**) were applied to 96-well plates containing parental, wild-type MRP1-, K684L- or K1333L-transfected BHK cells. Cell survival was determined by measuring the absorbance at 570 nm. Results shown are the means $\pm$ S.D. for six repeat determinations.

Figure 7 Vincristine accumulation in cells expressing wild-type, K684L- and K1333L-mutated MRP1

Intracellular accumulation of ^3H -labelled vincristine was carried out according to the vincristine accumulation method as described in the Materials and methods section. ^3H -labelled vincristine ($1\ \mu\text{M}$) was applied to the 24-well plate containing CFTR-, MRP1-, K684L- or K1333L-transfected BHK cells. The cells (in triplicate) were harvested at various times and the radioactivity trapped in each sample was determined by liquid-scintillation counting.

directly adjacent to Walker B motif in NBD2, with a non-acidic amino acid glutamine also increased the K_d value by approx. 4–5-fold, but almost completely abolished the ATP-dependent LTC₄ transport [21,22]. Interestingly, although replacement of His¹⁴⁸⁶ (H-loop in NBD2), which forms a hydrogen bond with the γ -phosphate of the bound ATP in NBD2, with an aromatic phenylalanine residue or a hydrophobic leucine residue

has different effects on ATP binding, i.e. the H1486F mutation had no effect on ATP binding at the unmutated NBD1 and the H1486F-mutated NBD2, whereas the H1486L mutation increased the K_d value by approx. 2-fold, the mutation of either H1486F or H1486L abolished the ATP-dependent LTC₄ transport activity (R. Yang and X.-b. Chang, unpublished work). In addition, replacement of Gly⁷⁷¹, which should form a hydrogen bond with the γ -phosphate of the bound ATP in NBD2 [3,4,6], with an alanine residue did not affect ATP binding at NBD1 or NBD2 of MRP1, but abolished the ATP-dependent LTC₄ transport completely [41]. These results suggest that: (i) the aromatic stacking interaction between Trp⁶⁵³ in NBD1 or Tyr¹³⁰² in NBD2 and the adenine ring of the bound ATP may play a role in nucleotide binding, but may not play a very important role in holding ATP during the hydrolysis; (ii) the hydrogen bonds formed between the β - and γ -phosphates of the bound ATP and the residues in the Walker A motif, H-loop and LSGGQ ABC transporter signature sequences from the opposite NBD, such as the residues responsible for ATP binding in NBD2 of MRP1, including Thr¹³²⁹, Lys¹³³³, Ser¹³³⁴, His¹⁴⁸⁶, Ser⁷⁶⁹ and Gly⁷⁷¹, may play a vital role in holding the bound ATP so that the catalytic base-activated water molecule can efficiently attack the γ -phosphate of the bound ATP; (iii) ATP hydrolysis at NBD2 of MRP1 reverts the heterodimer into the resting-state conformation [42] and release of the ATP hydrolysis products ADP and phosphate from NBD2 and ATP from NBD1 brings the protein back to its original conformation so that the molecule can start a new cycle of ATP-dependent solute transport [22].

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