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Glutamine residues in Q-loops of multidrug resistance protein MRP1 contribute to ATP binding via interaction with metal cofactor☆

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ABSTRACT

Structural analyses of bacterial ATP-binding-cassette transporters revealed that the glutamine residue in Q-loop plays roles in interacting with: 1) a metal cofactor to participate in ATP binding; 2) a putative catalytic water molecule to participate in ATP hydrolysis; 3) other residues to transmit the conformational changes between nucleotide-binding-domains and transmembrane-domains, in ATP-dependent solute transport. We have mutated the glutamines at 713 and 1375 to asparagine, methionine or leucine to determine the functional roles of these residues in Q-loops of MRP1. All these single mutants significantly decreased Mg · ATP binding and increased the K_m (Mg · ATP) and V_{max} values in Mg · ATP-dependent leukotriene-C4 transport. However, the V_{max} values of the double mutants Q713N/Q1375N, Q713M/Q1375M and Q713L/Q1375L were lower than that of wtMRP1, implying that the double mutants cannot efficiently bind Mg · ATP. Interestingly, MRP1 has higher affinity for Mn · ATP than for Mg · ATP and the Mn · ATP-dependent leukotriene-C4 transport activities of Q713N/Q1375N and Q713M/Q1375M are significantly higher than that of wtMRP1. All these results suggest that: 1) the glutamine residues in Q-loops contribute to ATP-binding via interaction with a metal cofactor; 2) it is most unlikely that these glutamine residues would play crucial roles in ATP hydrolysis and in transmitting the conformational changes between nucleotide-binding-domains and transmembrane-domains.

1. Introduction

Over-expression of ATP-binding cassette (ABC) transporters, such as P-glycoprotein (P-gp or ABCB1) [1–3], breast cancer resistance protein (BCRP or ABCG2) [4,5] and/or multidrug resistance protein (MRP1 or ABCC1) [6,7], confers acquired multidrug resistance (MDR) owing to the fact that these ABC transporters catalyze ATP-dependent anticancer drug transport. Although some of the ABC transporters, such as ABCG2, contain one nucleotide binding domain (NBD) and one transmembrane domain (TMD), these transporters are thought to form either a homo- or a hetero-dimer in the plasma membrane [8–11]. Thus, all the functional ABC transporters contain two NBDs and at least two TMDs [3,7–12]. Upon Mg · ATP binding, the two NBDs form a dimer in which the two Mg · ATP molecules are each sandwiched between the Walker A motif from one NBD and the LSGGQ ABC signature motif from another [13–19]. In addition, the residues from Walker B motif, A-loop, D-loop, H-loop and Q-loop [13,14,16,17,19–36] also contribute to Mg · ATP binding and hydrolysis. Detailed structural analyses of several NBDs of ABC transporters revealed that the amide ε-oxygen of glutamine residue in Q-loop, as shown in Fig. S1, participates in Mg · ATP binding by interacting, together with other five oxygen atoms from the Walker A serine residue, the β- and γ-phosphate of the bound ATP and two water molecules, with a Mg^{++} cofactor to form the octahedral coordination geometry [13,16,21,25,27,29]. The amide ε-nitrogen of the glutamine residue in Q-loop may interact with the putative hydrolytic water molecule in the active center [13], implying that this residue may play a role in ATP hydrolysis. In addition, the amide ε-nitrogen of the glutamine residue in Q-loop may also interact with an amino acid from another NBD [13] to stabilize the NBD · ATP · ATP · NBD sandwich structure. Furthermore,the residues around the glutamine residue (in the linear sequence of an

Abbreviations: Sf21, *Spodoptera frugiperda* 21; ABC, ATP binding cassette; P-gp, P-glycoprotein; BCRP, breast cancer resistance protein; MRP1, multidrug resistance protein 1; NBD, nucleotide binding domain; TMD, transmembrane domain; LTC4, leukotriene C4; 8-N_3ATP, 8-azidoadenosine 5′-triphosphate; PBS, phosphate-buffered saline; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N,N,N,N*-tetraacetic acid; SDS, sodium dodecyl sulfate

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NBD) in Q-loop form a Q-loop cleft that interacts with the residues in L-loop of the transmembrane subunits [14,17], suggesting that these interactions may facilitate inter-domain communications between the transport-substrate-binding sites, such as TMDs, and ATP binding/hydrolysis sites, such as NBDs [13,14,22]. Thus, the interactions of the glutamine residue in Q-loop with the residues mentioned above may play very important roles in ATP binding, hydrolysis and ATP-dependent solute transport. However, whether the glutamine residue in Q-loop of human MRP1 will play so many important roles has not been experimentally proved yet. In order to determine the functional roles of the glutamine residue in the Q-loop of human MRP1, we have substituted the glutamine residue in Q-loop of MRP1 with: 1) an asparagine (Q713N and Q1375N) that remains the amide group but with one methylene shorter in asparagine than in glutamine; 2) a methionine (Q713M and Q1375M) that eliminates the amide group but contains paired electrons in the sulfur atom of the methionine residue that might potentially interact with the Mg^{++} cofactor; 3) a leucine (Q713L and Q1375L) that eliminates the amide group and abolishes the interactions with Mg^{++} cofactor and the putative hydrolytic water molecule, and used them to determine the consequence of these mutations in ATP-dependent leukotriene C4 (LTC4) transport. The data presented in this manuscript indicate that the glutamine residues in the Q-loops of MRP1 mainly play a role in Mg · ATP binding, but not in ATP hydrolysis and in inter-domain communications between the NBDs and TMDs.

2. Materials and methods

2.1. Materials

Sodium orthovanadate, EGTA and ATP were purchased from Sigma. [14,15,19,20-^{3}H]-leukotriene C4 was from PerkinElmer Life Sciences. [α-^{32}P]-8-N_3ATP and [γ-^{32}P]-8-N_3ATP were from Affinity Labeling Technologies. Fetal bovine serum was from Gemini Bio-Products. QuikChange site-directed mutagenesis kit was from Stratagene. Anti-mouse Ig conjugated with horseradish peroxidase was from Amersham Biosciences. Chemiluminescent substrates for western blotting were from Pierce.

2.2. Site-directed mutagenesis of human MRP1 cDNA

Wt N-half (NH, 1–932) and C-half (CH, 933–1531) of human MRP1 cDNA cloned into pDual expression vector [37,38], named as pDual/NH/CH, was used as a template for the in vitro mutagenesis. The glutamine residue at position of 713 in Q-loop of NBD1 or 1375 in Q-loop of NBD2 was mutated to either asparagine (N), methionine (M) or leucine (L) by using the forward and reverse primers and the QuikChange site-directed mutagenesis kit [39]. The forward and reverse primers used to introduce these mutations are: Q713/forward, 5′-TCC GTG GCC TAT GTG CCA XXX CAG GCC TGG ATT CAG AAT-3′; Q713/reverse, 5′-ATT CTG AAT CCA GGC CTG XXX TGG CAC ATA GGC CAC GGA-3′; Q1375/forward, 5′-AAG ATC ACC ATC ATC CCC XXX GAC CCT GTT TTG TTT TCG GG-3′; Q1375/reverse, 5′-CC CGA AAA CAA AAC AGG GTC XXX GGG GAT GAT GGT GAT CTT-3′. The underlined XXX sequences are codons for mutated residues as shown in Table S1. Regions containing these mutations were sequenced to confirm that the correct clones were obtained. Recombinant viral DNA preparation and baculovirus viral particle production were carried out according to the procedures described [38].

2.3. Cell culture and cells expressing N-half and C-half of MRP1

Spodoptera frugiperda 21 (Sf21) cells were cultured in Grace's insect cell medium supplemented with 5% heat-inactivated fetal bovine serum at 27 °C. Viral infection was performed according to Invitrogen's recommendations, i.e., a multiplicity of infection (MOI) of 5 to 10 was used to infect the insect cells. The expression levels of the dually expressed N-half and C-half with varying MOI were determined by western blot analysis. The MOI producing similar amount of N-half (by comparing the wt N-half with the mutated N-half) and C-half (by comparing the wt C-half with the mutated C-half) was used to infect Sf21 cells for membrane vesicle preparations.

2.4. Identification of N-half and C-half of MRP1 protein

Western blot was performed according to the method described previously [39]. A 42.4 mAb was used to identify the NBD1-containing N-half fragment of MRP1, whereas 897.2 mAb was used to detect the NBD2-containing C-half fragment [37,39]. The secondary antibody used was anti-mouse Ig conjugated with horseradish peroxidase.

2.5. Membrane vesicle preparation

Membrane vesicles were prepared according to the procedure described previously [39]. The membrane vesicle pellet was re-suspended in a solution containing 10-mM Tris–HCl (pH 7.5), 250-mM sucrose and 1× protease inhibitor cocktail (2 μg/ml aprotinine, 121 μg/ml benzamidine, 3.5 μg/ml E64, 1 μg/ml leupeptin and 50 μg/ml Pefabloc). Aliquots of the membrane vesicles, after passage through a Liposofast™ vesicle extruder (Avestin, Ottawa, Canada), were stored in −80 °C.

2.6. Membrane vesicle transport

ATP-dependent LTC4 transport was assayed by a rapid filtration technique [39–41]. The assays were carried out in triplicate determinations in a 30-μl solution containing 3 μg of membrane proteins, 50-mM Tris–HCl (pH 7.5), 250-mM sucrose, 10-mM $MgCl_2$ (or 10-mM $MnCl_2$), 200-nM LTC4 (17.54 nCi of ^{3}H-labeled LTC4) and 4-mM AMP (used as negative control) or 4-mM ATP (with an ATP regenerating system consisting of 120 mU of creatine kinase and 10 mM of creatine phosphate). For percentage determination, above components were mixed on ice, transferred to 37 °C water bath, incubated at 37 °C for 4 min, brought back to ice and diluted with 1 ml of ice-cold 1× transport buffer (50-mM Tris–HCl, pH 7.5, 250-mM sucrose and 10-mM $MgCl_2$). For kinetic analysis, the ATP solutions and the membrane vesicles were prepared in two different eppendorf tubes on ice, transferred to 37 °C heating block (filled with water), warmed up to 37 °C, mixed the two solutions, incubated at 37 °C for 1 min, brought back to ice and diluted with 1 ml of ice-cold 1× transport buffer.

2.7. Photoaffinity labeling of N-half and C-half of MRP1 protein

Vanadate preparation (from sodium orthovanadate) and photoaffinity labeling of MRP1 protein were performed according to procedures described previously [39,42,43]. Briefly, photolabeling experiments were carried out in a 10-μl solution containing 10 μg of membrane proteins, 40-mM Tris–HCl (pH7.5), 2-mM ouabain, 10-mM $MgCl_2$, 0.1-mM EGTA and either 10-μM [α-^{32}P]-8-N_3ATP or 10-μM [γ-^{32}P]-8-N_3ATP. The other conditions, such as incubation time, temperature and vanadate, are indicated in figure legend. After UV-irradiation ($\lambda = 254$ nm) on ice for 2 min, the labeled proteins were separated by polyacrylamide gel (7%) electrophoresis and electro-blotted to a nitrocellulose membrane.

2.8. Statistical analysis

The results in Figs. 1C and 4 and Tables 1 and S3 were presented as means ± SD from three independent experiments. The two-tailed *P* values were calculated based on the unpaired *t*-test from GraphPad Software Quick Calcs. By conventional criteria, if *P* value is less than