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Non-equivalent cooperation between the two nucleotide-binding folds of P-glycoprotein

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Abstract

To identify the roles of the two nucleotide-binding folds (NBFs) in the function of human P-glycoprotein, a multidrug transporter, we mutated the key lysine residues to methionines and the cysteine residues to alanines in the Walker A (W_A) motifs (the core consensus sequence) in the NBFs. We examined the effects of these mutations on *N*-ethylmaleimide (NEM) and ATP binding, as well as on the vanadate-induced nucleotide trapping with 8-azido-[α-^{32}P]ATP. Mutation of the W_A lysine or NEM binding cysteine in either of the NBFs blocked vanadate-induced nucleotide trapping of P-glycoprotein. These results suggest that if one NBF is non-functional, there is no ATP hydrolysis even if the other functional NBF contains a bound nucleotide, further indicating the strong cooperation between the two NBFs of P-glycoprotein. However, we found that the effect of NEM modification at one NBF on ATP binding at the other NBF was not equivalent, suggesting a non-equivalency of the role of the two NBFs in P-glycoprotein function.

Keywords: ATP hydrolysis; MDR1; Multidrug resistance; Transporter

1. Introduction

P-Glycoprotein functions as an ATP-dependent efflux pump that extrudes cytotoxic drugs from cells before the drugs reach their intracellular targets, and in this way confers multidrug resistance on the cancer cells [1–3]. P-Glycoprotein is a member of the ABC transporter superfamily, characterized by two multiple transmembrane domains and two nucleotide-binding folds (NBFs) [4].

Both NBFs of P-glycoprotein can hydrolyze nucleotides, and their ATPase activity is necessary for drug transport [5–9]. Mutation of the lysine residue in the Walker A (W_A) motif of either NBF abolishes the ATPase activity of P-glycoprotein and its ability to confer multidrug resistance [10–12]. This lysine residue is predicted to interact with the phosphoryl moiety of the bound nucleotide [13,14]. The W_A motifs of P-glycoprotein also contain cysteine residues and covalent modification of either of these cysteine

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residues has been shown to be sufficient to inactivate the ATPase activity of P-glycoprotein [15–17]. Furthermore, vanadate-induced trapping of a nucleotide at just one NBF is sufficient to inhibit this ATPase activity [7]. These results suggest that the NBFs alternate in steady-state catalysis [8], and mutations and modifications in the W_A motif of either of the NBFs inhibit a catalytic cycle. Alternatively, mutations or modifications in one NBF might prevent the hydrolysis of even a single ATP at the other (still intact) NBF.

Vanadate-induced nucleotide trapping is useful in the analysis of ATP hydrolysis by ABC transporter proteins [9,18,19]. Hydrolysis of a single ATP may be enough to trap the nucleotide in the catalytic site in the presence of vanadate. Using this technique, it may be possible to determine a single ATP hydrolytic event during the catalytic cycle. We examined the effect of mutating the lysine and cysteine residues within the W_A motifs of human P-glycoprotein and the interaction of NEM with both ATP binding and vanadate-induced nucleotide trapping. We demonstrate that NEM modification or mutation of the W_A lysine of a single NBF is sufficient to prevent vanadate-induced nucleotide trapping presumably at the other (intact) NBF. However, we find that the cooperative effect of NEM modification at one NBF on ATP binding at the other NBF is not equivalent.

2. Materials and methods

2.1. Materials

8-Azido-[α-^{32}P]ATP was purchased from ICN Biomedicals. Monoclonal antibody C219 was obtained from Centocor.

2.2. Construction of mutants and expression vectors

Sculptor in vitro mutagenesis system (Amersham) was used to introduce amino acid substitutions. P-Glycoprotein-S, in which a 15 amino acid S-tag peptide derived from pancreatic ribonuclease A is fused to the C terminus of P-glycoprotein, was constructed by fusing of a DNA fragment containing the S-tag with a His-tag from pET-29b plasmid (Novagen). This fusion did not affect the function of P-glycoprotein, because the pattern and degree of multidrug resistance conferred by P-glycoprotein-S were similar to those conferred by P-glycoprotein (data not shown).

2.3. Cell culture, transfection, and drug resistance assay

An expression vector, pCAGGS [20], was used for transient expression. Human cultured cells HEK293 were transfected by human MDR1 expression vectors with LipofectAMINE (Gibco). Membrane proteins were prepared 3 days after transfection as described previously [19].

2.4. Vanadate-induced trapping of 8-azido-[α-^{32}P]ATP and photoaffinity labeling of P-glycoprotein

In a study of the effect of NEM on vanadate-induced nucleotide trapping, membrane proteins (20 μg) were incubated with or without NEM for 10 min at 20°C. Dithiothreitol was added to a final concentration of 400 mM, and the membrane proteins were precipitated by centrifugation. Membrane proteins were suspended in 400 μl of 40 mM Tris-HCl buffer (pH 7.5) containing 100 μM EGTA and precipitated again by centrifugation. Membrane proteins were then reacted with 5 μM 8-azido-[α-^{32}P]ATP in the presence of 20 μM verapamil, 200 μM vanadate, 3 mM $MgSO_4$, 2 mM ouabain, 0.1 mM EGTA, and 40 mM Tris-Cl (pH 7.5) in a total volume of 10 μl for 10 min at 37°C. The reactions were stopped by addition of 400 μl of ice-cold Tris-EGTA buffer (0.1 mM EGTA, 40 mM Tris-Cl (pH 7.5)), and free ATP, Mg^{2+}, and vanadate were removed after centrifugation (15 000×g, 10 min, 4°C). The pellet was washed in the same buffer and resuspended in 8 μl of Tris-EGTA buffer, placed on ice, and irradiated for 5 min (λ = 254 nm, 5.5 mW/cm^2). Samples were analyzed as described previously [18]. Experiments were done in triplicate.

2.5. ATP binding of P-glycoprotein

Membrane proteins (about 20 μg) prepared from cells expressing similar amounts of P-glycoprotein-S

or its mutant proteins were incubated with 5 μM 8-azido-[α-^{32}P]ATP in the presence of 3 mM $MgSO_4$, 2 mM ouabain, 0.1 mM EGTA, and 40 mM Tris-Cl (pH 7.5) in a total volume of 10 μl at 0°C for 5 min. The mixture was then irradiated with a UV lamp for 5 min without removing free 8-azido-[α-^{32}P]ATP. The labeled membranes were then made soluble with 10 mM Tris-HCl containing 1% Nonidet-P40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl, and 0.5 mM EDTA, and P-glycoprotein-S was precipitated with S-protein agarose (Novagen). The S-tag peptide and the 104 amino acid S-protein derived from pancreatic ribonuclease A form a specific and strong complex. Experiments were done in triplicate.

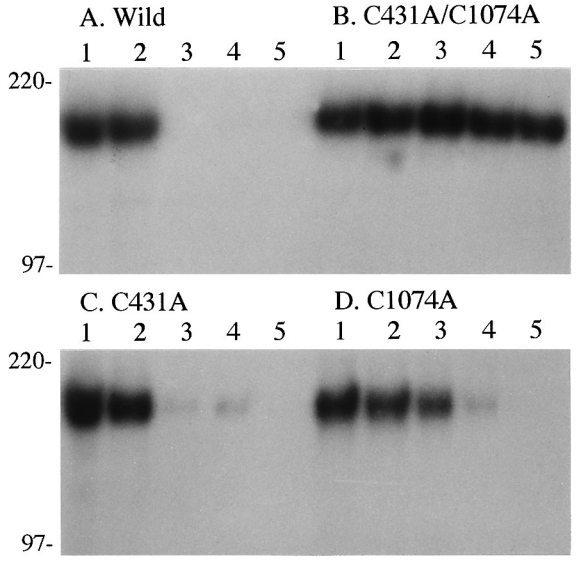

Fig. 1. Effects of NEM on vanadate-induced nucleotide trapping in P-glycoprotein. Plasma membrane proteins (about 20 μg) from stable KB-3-1 transfectants expressing equivalent amounts of the wild-type human P-glycoprotein (A), the C431A/C1074A double-mutant form, in which the cysteine residues of Walker A in both NBFs were replaced by alanine (B), or the C431A (C) or C1074A (D) single-mutant form were treated with NEM at 1 μM (lane 2), 10 μM (lane 3), 50 μM (lane 4), or 100 μM (lane 5), or with NEM (lane 1). Then membrane proteins were reacted with 5 μM 8-azido-[α-^{32}P]ATP in the presence of 20 μM verapamil, 200 μM vanadate, 3 mM $MgSO_4$, 2 mM ouabain, and 0.1 mM EGTA, and analyzed as described in Section 2. Experiments were done in triplicate.

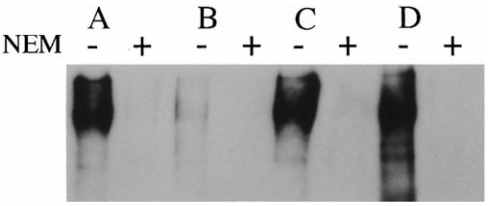

Fig. 2. Effects of NEM on labeling with biotin maleimide. Plasma membrane proteins (about 20 μg) from HEK293 cells transiently expressing equivalent amounts of P-glycoprotein-S (in which an S-tag is fused to the C terminus of P-glycoprotein) and its cysteine-to-alanine mutants were incubated with 100 μM NEM and then reacted with 5 μM biotin maleimide at 20°C. P-Glycoprotein-S was precipitated with S-protein agarose and analyzed as described in Section 2. A, P-glycoprotein-S; B, C431A/C1074A; C, C431A; D, C1074A. Experiments were done in duplicate.

3. Results

3.1. Inhibition of vanadate-induced nucleotide trapping in P-glycoprotein by NEM

When membrane proteins containing human P-glycoprotein were incubated with vanadate, Mg^{2+}, verapamil, and 5 μM 8-azido-[α-^{32}P]ATP, a 170 kDa protein was specifically photoaffinity-labeled (Fig. 1). For such labeling of P-glycoprotein, both vanadate and Mg^{2+} are needed, and verapamil is stimulatory [19]. Nucleotide trapping in P-glycoprotein was inhibited by NEM in a concentration-dependent way, and was inhibited completely by 10 μM NEM (Fig. 1A). The C431A/C1074A double-mutant form of P-glycoprotein, in which the cysteine residues of the W_A motif in both NBFs were replaced by alanine, trapped nucleotides even after treatment with 100 μM NEM (Fig. 1B). Also, these cysteine-to-alanine mutations did not affect the function of P-glycoprotein, because the pattern and degree of multidrug resistance conferred by the C431A/C1074A double-mutant form were similar to those conferred by the wild-type protein (data not shown). By contrast, vanadate-induced nucleotide trapping of the C431A and C1074A mutant forms, in which the cysteine residue of the W_A motif in only one of the NBFs was replaced by alanine, was affected by NEM (Fig. 1C,D). Nucleotide trapping with the C431A mutant form was inhibited by 10 μM NEM (Fig. 1C), and nucleotide trapping with the C1074A mutant form was inhibited by 50 μM NEM (Fig. 1D).

3.2. Modification of the Walker A cysteines by NEM

To confirm the NEM modification of the cysteine residues of the W_A motifs, the effects of NEM on labeling of P-glycoprotein with biotin maleimide were examined. P-Glycoprotein-S was labeled by 5 μM biotin maleimide and specifically inhibited by the presence of 100 μM NEM (Fig. 2A), but the C431A/C1074A mutant form was labeled little if at all (Fig. 2B). The C431A and C1074A mutant forms were also both labeled in an NEM-dependent manner by biotin maleimide (Fig. 2C,D), suggesting that biotin maleimide at a concentration of 5 μM specifically and uniformly labels the W_A cysteines in both NBFs, as previously reported [16]. These results strongly indicate that the cysteines of the W_A motifs in both NBFs are responsible for the effects of NEM on vanadate-induced ATP trapping shown in Fig. 1.

3.3. Effects of NEM on ATP binding

The effects of NEM on 8-azido-ATP binding were examined. 8-Azido-ATP binding with the wild type P-glycoprotein was inhibited by 100 μM NEM (Fig. 3A). 8-Azido-ATP binding with the C431A/C1074A mutant form was not inhibited by 100 μM NEM, but possibly increased (Fig. 3B), whereas 8-azido-ATP binding of the C431A mutant form appeared not to be affected (Fig. 3C). However, ATP binding to the C1074A mutant form was significantly reduced

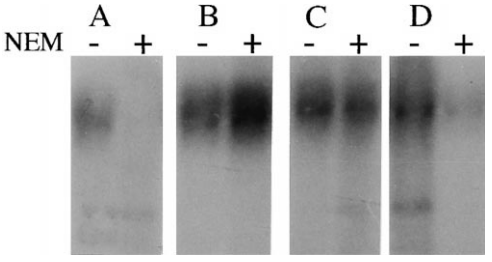

Fig. 3. Effects of NEM on ATP binding with P-glycoprotein. Plasma membrane proteins (about 20 μg) from HEK293 cells transiently expressing equivalent amounts of P-glycoprotein-S and its cysteine-to-alanine mutant forms were incubated in the presence or absence of 100 μM NEM. Membrane proteins were then reacted with 5 μM 8-azido-[α-^{32}P]ATP at 0°C for 5 min, for photoaffinity labeling. The labeled membranes were then made soluble with detergents. P-Glycoprotein-S was precipitated with S-protein agarose and analyzed as described in Section 2. A, P-glycoprotein-S; B, C431A/C1074A; C, C431A; D, C1074A. Experiments were done in triplicate.

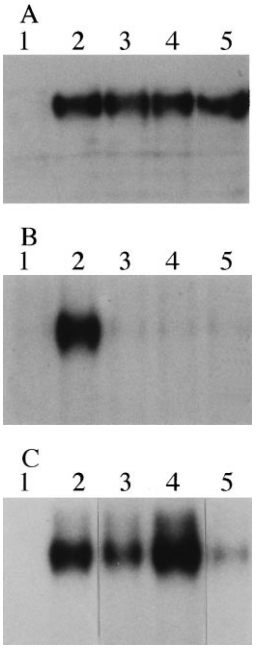

Fig. 4. Immunoblot analysis (A), vanadate-induced nucleotide trapping (B), and 8-azido-ATP binding (C) of wild-type protein and lysine-to-methionine mutants of P-glycoprotein-S. Lanes: 1, cells not transfected; 2, P-glycoprotein-S; 3, K433M; 4, K1076M; 5, K433M/K1076M. (A) Membrane proteins (about 20 μg) from HEK293 cells expressing equivalent amounts of different forms of P-glycoprotein-S were separated on 7% SDS-PAGE, and P-glycoprotein-S and mutants detected by immunoblotting with monoclonal antibody C219. (B) Membrane proteins were reacted with 5 μM 8-azido-[α-^{32}P]ATP in the presence of 20 μM verapamil, 200 μM vanadate, 3 mM $MgSO_4$, 2 mM ouabain, and 0.1 mM EGTA for 10 min at 37°C, and analyzed as described in Section 2. (C) Membrane proteins were reacted with 5 μM 8-azido-[α-^{32}P]ATP at 0°C, for photoaffinity labeling. The labeled membranes were then made soluble with detergents. P-Glycoprotein-S was precipitated and analyzed as described in Section 2.

by treatment with 100 μM NEM (Fig. 3D), similar to the wild-type P-glycoprotein.

3.4. Vanadate-induced nucleotide trapping and ATP binding in K433M and K1076M mutant P-glycoproteins

To further examine the roles of the two NBFs in the function of P-glycoprotein, we constructed three other mutants, K433M, K1076M, and K433M/K1076M, in which the lysine residue in the W_A motif of either or both NBFs was replaced by methionine. Membranes prepared from cells expressing similar

amounts of the wild-type and mutant forms of P-glycoprotein-S (Fig. 4A) were used for studying the interaction with ATP. The three mutant proteins did not trap nucleotide in the presence of vanadate (Fig. 4B). This is consistent with previous reports suggesting that the lysine residues in the W_A motifs in both NBFs are important in the function of P-glycoprotein [10–12]. The effect of these mutations on 8-azido-ATP binding is also shown in Fig. 4C. As previously reported all these mutants bound ATP, although the binding to the K433M/K1076M double-mutant form appeared substantially reduced (lane 5).

4. Discussion

NEM modification of either of the NBFs blocks vanadate-induced nucleotide trapping in P-glycoprotein. A similar effect is also observed by mutation of either of the W_A lysines suggesting an equivalent and cooperative effect of the two domains and that abolishing function at either NBF is sufficient to prevent the hydrolysis of a single bound nucleotide at the other NBF. However, although NEM modification of NBF2 did not impair 8-azido-ATP binding of P-glycoprotein (presumably at NBF1), we find that NEM modification of NBF1 is sufficient to prevent 8-azido-ATP binding at NBF2. Taken together, these results further confirm the strong cooperation between the two NBFs of P-glycoprotein. However, they also indicate a possible non-equivalency of the two NBFs in the function of P-glycoprotein.

Wild-type human P-glycoprotein contains seven cysteines, and two of them are located in the W_A motifs of the NBFs. We replaced only these two cysteine residues with alanine to avoid the allosteric effects which might be caused by replacing other cysteines. The C431A/C1074A mutant form of P-glycoprotein was indistinguishable from the wild-type in its function. Also the C431A/C1074A mutant P-glycoprotein trapped nucleotides even after treatment with 100 μM NEM, indicating that the other five cysteines were probably not accessible to NEM. Indeed, none of the other cysteines were accessible to biotin maleimide at 5 μM (Fig. 2). However, the C431A/C1074A mutant P-glycoprotein showed an increase in ATP binding after NEM treatment (Fig. 3B), indicating that NEM modification of other cysteine residues outside NBFs may allosterically affect ATP binding in a positive manner.

Liu and Sharom [17] have reported that Chinese hamster P-glycoprotein is still able to bind ATP after modification of two cysteine residues by 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid (MIANS). However, NEM binding of both NBFs of human P-glycoprotein impaired binding of 5 μM 8-azido-ATP (Fig. 3A). We used 8-azido-[^{32}P]ATP at 5 μM to minimize non-specific photolabeling. Higher concentrations of 8-azido-ATP might be able to bind the NEM modified NBFs. Alternatively, the 8-azido moiety may interact with the modified cysteine to hinder ATP binding.

Muller et al. have reported a concentration dependence of 8-azido-ATP binding to the W_A lysine mutant forms of P-glycoprotein [12]. They showed that the mutation in a single NBF did not affect 8-azido-ATP binding but the double mutation reduced 8-azido-ATP binding. Fig. 4C shows that the W_A lysine mutation in NBF1 slightly reduced 8-azido-ATP binding while the mutation in NBF2 slightly increased it. In the experiments of Muller et al. [12], P-glycoprotein was expressed in Sf9 insect cells. Isolated cell membranes were used for the photoaffinity labeling. The membrane proteins were separated on electrophoresis gels, and the photoaffinity labeled band of P-glycoprotein was analyzed. We expressed P-glycoprotein-S and its mutant proteins in HEK293 cells, and precipitated the photoaffinity labeled P-glycoprotein with S-protein agarose to eliminate other specifically and non-specifically photoaffinity labeled proteins. The slight differences in these results could be due to the different experimental conditions.

The C431A mutant P-glycoprotein showed no change in 8-azido-ATP binding after NEM treatment. However, NEM treatment significantly reduced 8-azido-ATP binding in the C1074A mutant P-glycoprotein. These results suggest that NEM modification of NBF1 has an allosteric effect on ATP binding at NBF2 and is responsible for the NEM inhibition of ATP binding in the wild-type protein. However, NEM modification of NBF2 does not affect further ATP binding presumably at NBF1. This suggests that while the effects of NEM modification of the NBFs on vanadate-induced nu-

cleotide trapping appear equal and cooperative, the effect on ATP binding is not.

Because nucleotide trapping of P-glycoprotein was abolished when one NBF was modified or mutated, the cooperative role of the two NBFs in the function of P-glycoprotein may be equivalent as proposed by Urbatsch et al. [9]. However, the roles of the two NBFs in the function of SUR1 and CFTR appear to be different [19,21,22], providing evidence for the non-equivalency of the roles of the two NBFs in the function of the ABC superfamily proteins. Our results, for the first time, also suggest that the NBFs of P-glycoprotein may show some non-equivalency in their function.

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