The “LSGGQ” Motif in Each Nucleotide-binding Domain of Human P-glycoprotein Is Adjacent to the Opposing Walker A Sequence*

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The human multidrug resistance P-glycoprotein (P-gp, ABCB1), a member of the ATP-binding cassette (ABC) family of transport proteins, actively transports many cytotoxic compounds out of the cell. ABC transporters have two nucleotide-binding domains (NBD) and two transmembrane domains. The presence of the conserved “signature” sequence (LSGGQ) in each NBD is a unique feature in these transporters. The function of the signature sequences is unknown. In this study, we tested whether the signature sequences (531LSGGQ535 in NBD1; 1176LSGGQ1180 in NBD2) in P-gp are in close proximity to the opposing Walker A consensus nucleotide-binding sequences (1070GSSGCGKS1077 in NBD2, 427GNSGCGKS434 in NBD1). Pairs of cysteines were cross-linked into a Cys-less P-gp at the signature and “Walker A” sites and the mutant P-gps were subjected to oxidative cross-linking. At 4 °C, when thermal motion is low, P-gp mutants (L531C(Signature)/C1074(Walker A) and C431(Walker A)/L1176C(Signature) were cross-linked. Cross-linking inhibited the drug-stimulated ATPase activities of these two mutants. Their activities were restored, however, after addition of the reducing agent, dithiothreitol. Vanadate trapping experiments, the membranes were incubated with an equal volume of TBS containing 1 mM CuSO4(phenanthroline). The samples were incubated for 30 min at 4 °C, 15 min at 21 °C, or 5 min at 37 °C. The reactions were stopped by addition of SDS sample buffer (125 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, and 4% (w/v) SDS) containing 50 mM EDTA and no reducing agent. The reaction mixtures were subjected to SDS-PAGE (7.5% polyacrylamide gels) and immunoblot analysis with a rabbit polyclonal antibody against P-gp (5).

For vanadate trapping experiments, the membranes were incubated with an equal volume of TBS containing 12 mM ATP, 24 mM MgCl2, and 0.6 mM sodium vanadate for 10 min at 37 °C before cross-linking at 4, 21, or 37 °C. Purification of histidine-tagged P-gp mutants and assay of verapamil-stimulated ATPase activities were done as described previously (15).

RESULTS

The contact sites between the NBDs of P-gp are not known. It is possible that the conserved signature sequence in each NBD (531LSGGQ535 in NBD1 and 1176LSGGQ1180 in NBD2) may interact with the Walker A site (427GNSGCGKS434 in NBD1 and 1070GSSGCGKS1077 in NBD2) in the opposing NBD. Accordingly, we used disulfide cross-linking analysis to determine whether a cysteine introduced into the signature sequence in one NBD could be cross-linked to another cysteine introduced into the Walker A site in the opposing NBD. P-gp is an ideal membrane protein for cross-linking analysis because

The human multidrug resistance P-glycoprotein (P-gp)1 is a member of the ATP-binding cassette (ABC) family of transporters. It transports a wide variety of structurally diverse compounds of different sizes (recently reviewed in Ref. 2). The 1280 amino acids of P-gp are organized in two repeating units of 610 amino acids that are joined by a linker region of about 60 amino acids (3). Each repeat has six transmembrane (TM) segments and a hydrophilic domain containing an ATP-binding site (3, 4). The minimum functional unit is a monomer (5), but the two halves of the molecule do not have to be covalently linked for function (6, 7).

A potentially important region of P-gp is the “signature” sequences (LSGGQ) in each NBD. The signature sequences are present in all ABC transporters, but not in any other transporter (1). The function of the signature sequences is unknown. Both NBDs must interact with each other, since both halves of P-gp are required for drug-stimulated ATPase activity (8, 9). Therefore, it is possible that the conserved signature sequence in each NBD may interact with Walker A consensus nucleotide-binding sequence (Walker A site) (10) in the opposing NBD. In this study, we used cysteine-scanning mutagenesis and cross-linking analysis to test whether the signature sequence in each NBD is close to the site in the opposing NBD.

MATERIALS AND METHODS

Construction of Mutants—A histidine-tagged Cys-less P-gp was constructed (4, 11). Cysteines were re-introduced into the Cys-less P-gp in the signature sequences (531LSGGQ535 in NBD1 and 1176LSGGQ1180 in NBD2) in P-gp and in the Walker A sites (427GNSGCGKS434 in NBD1 and 1070GSSGCGKS1077 in NBD2) (12). Expression, Disulfide Cross-linking Analysis, and Purification—The mutant cDNAs were expressed in HEK 293 cells in the presence of cyclosporin A (13) and membranes prepared as described previously (11, 14). For cross-linking, aliquots of membranes were added to equal volumes of TBS containing 1 mM CuSO4(phenanthroline). The samples were incubated for 30 min at 4 °C, 15 min at 21 °C, or 5 min at 37 °C. The reactions were stopped by addition of SDS sample buffer (125 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, and 4% (w/v) SDS) containing 50 mM EDTA and no reducing agent. The reaction mixtures were subjected to SDS-PAGE (7.5% polyacrylamide gels) and immunoblot analysis with a rabbit polyclonal antibody against P-gp (5).

1 The abbreviations used are: P-gp, P-glycoprotein; ABC, ATP-binding cassette; DTT, dithiothreitol; NBD, nucleotide-binding domain; NBD1, NH2-terminal NBD; NBD2, COOH-terminal NBD; TM, transmembrane; HEK, human embryonic kidney.
Cross-linking of P-glycoprotein Signature and Walker A Sites

The cross-linking results of three mutants, L531C/G1070C, L531C/S1072C, and L531C/C1074, are shown in Fig. 1. No cross-linked product was detected in mutant L531C/G1070C at any temperature. In mutant L531C/S1072C, no cross-linked product was detected when treated with oxidant at 4 °C. By contrast, there was partial cross-linking at 21 °C and almost complete cross-linking at 37 °C. The slow migrating cross-linked product was not detected when the reducing agent di-thiothreitol (DTT) was added after cross-linking. This indicates that the disulfide bond was cleaved by DTT. In mutant L531C/C1074, cross-linked product was detected at all three temperatures. Again, addition of DTT resulted in the disappearance of the cross-linked product and the appearance of the 170-kDa P-gp. The complete cross-linking results between the cysteines in the NBD1 terminal signature sequence (531LSGGQ535) and the cysteines in the NBD2 Walker A site (1070GSSGCGKS1077) are shown in Table I. Residues G1073C and C1074 in the NBD2 Walker A site and L531C and S532C in the NBD1 signature sequence appear to be closest, since mutants L531C/G1073C, L531C/C1074, and S532C/G1073C were cross-linked when treated with oxidant at 4 °C. The cysteines in other mutants (e.g., L531C/S1072C, S532/S1072C, and G533C/G1073C) must be further apart, since cross-linked product was only observed at either 21 or 37 °C. In all cases, there was no evidence of intermolecular cross-linking, since a product with a molecular mass greater than that of the cross-linked P-gp was not detected (data not shown). Also, no cross-linked product was observed in P-gp containing one cysteine (data not shown).

Structural changes that occur in P-gp immediately after ATP hydrolysis can be studied by vanadate trapping of the molecule in a transition state (21). Vanadate traps ADP at one of the two NBDs by occupying the position of the γ-phosphate adjacent to ADP. Vanadate trapping at one site inhibits ATP hydrolysis at the second site. Accordingly, we examined the effect of vanadate trapping on cross-linking of all the mutants. Representative cross-linking results of two (NBD1 signature/NBD2 Walker A) mutants (L531C/G1073C and L531C/C1074) are shown in Fig. 2. For both mutants, cross-linking was done at 21 °C, after preincubation of the membranes with ATP plus vanadate for 10 min at 37 °C (22). Similar results were obtained when cross-linking was done at 4 or 37 °C after vanadate trapping (Table I). Cross-linking of mutant L531C/C1074 was almost completely inhibited by vanadate trapping of nucleotide. Similar results were observed in mutants L531C/S1071C, S532C/C1074, G533C/C1074, and L531C/K1076C (Table I). Mutants L531C/C1074, L531C/S1071C, S532C/C1074, G533C/C1074, and L531C/K1076C had verapamil-stimulated ATPase activities of 22, 22, 30, 90, and 11% respectively, relative to Cys-less P-gp.

The other mutants (Table I) such as L531C/G1073C (Fig. 2) showed no detectable inhibition of cross-linking when preincubated with ATP plus vanadate. A prerequisite step in vanadate trapping is hydrolysis of ATP, and an inactive mutant would not be expected to show evidence of vanadate trapping. This appears to be the case for mutants such as L531C/G1073C that showed no inhibition of cross-linking after treatment with ATP plus vanadate. All of these mutants had little or no ATPase activity (<5%).

We then performed cross-linking analysis between cysteines in the NBD2-signature sequence (1176LSGGQ1180) and cysteines in the NBD1 Walker A site (427GNSGCGKS434). Cross-linking involving residues G427C, N428C, and S434C was not done because the single cysteine mutants, G427C and S434C, showed little or no activity (<5%), while N428C was defectively processed and rapidly degraded. The cross-linking results from the other 25 double cysteine mutants are shown in Table II. One mutant, L1176C/C431, was cross-linked at 4, 21, and 37 °C. Mutants L1176C/S429C and L1176C/G432C were cross-linked only at 21 and 37 °C, while cross-linking of mutants L1176C/G430C, S1177C/S429C, S1177C/G430C, S1177C/C431, S1177C/G432C, and G1179C/S429C was only observed at 37 °C.

Inhibition of cross-linking by vanadate trapping was tested with all the mutants. Cross-linking of four mutants, L1176C/

![Cross-linking of P-gp mutants.](image)

**Table I.**

| Cysteine Mutants | Cross-linking at 4 °C | Cross-linking at 21 °C | Cross-linking at 37 °C |
|------------------|----------------------|-----------------------|-----------------------|
|                  | L531C (21%)<sup>a</sup> | SS32C (38%)<sup>a</sup> | G533C (91%)<sup>a</sup> | G534C (4%)<sup>a</sup> | Q535C (0%)<sup>a</sup> |
| G1070C (0%)<sup>b</sup> | + | +<sup>c</sup> | +<sup>c</sup> | +<sup>c</sup> | +<sup>c</sup> |
| S1072C (23%)<sup>b</sup> | +<sup>c</sup> | ++<sup>c</sup> | +<sup>c</sup> | +<sup>c</sup> | +<sup>c</sup> |
| G1073C (4%)<sup>b</sup> | ++<sup>c</sup> | ++<sup>c</sup> | ++<sup>c</sup> | ++<sup>c</sup> | ++<sup>c</sup> |
| C1074 (103%)<sup>b</sup> | ++<sup>c</sup> | ++<sup>c</sup> | ++<sup>c</sup> | ++<sup>c</sup> | ++<sup>c</sup> |
| G1075C (0%)<sup>b</sup> | +<sup>c</sup> | +<sup>c</sup> | +<sup>c</sup> | +<sup>c</sup> | +<sup>c</sup> |
| L1076C (12%)<sup>b</sup> | ++<sup>c</sup> | ++<sup>c</sup> | ++<sup>c</sup> | ++<sup>c</sup> | ++<sup>c</sup> |
| S1077C (0%)<sup>b</sup> | +<sup>c</sup> | +<sup>c</sup> | +<sup>c</sup> | +<sup>c</sup> | +<sup>c</sup> |

<sup>a</sup> Activity of the single cysteine mutant relative to Cys-less P-gp.
<sup>b</sup> No cross-linked product detected in SDS-PAGE.
<sup>c</sup> Relatively weak cross-linking (<50% of P-gp cross-linked).
<sup>d</sup> Relatively strong cross-linking (>50% of P-gp cross-linked).
<sup>e</sup> Asterisk sign in bold indicates cross-linking inhibited when preincubated with ATP plus vanadate.
S429C, S1177C/S429C, L1176C/C431, and S1177C/C431, was inhibited by preincubation with ATP plus vanadate (Table II). A representative blot of mutant L1176C/C431 is shown in Fig. 2. No inhibition of cross-linking by vanadate trapping was observed in mutants such as L1176C/G430C, L1176C/G432C, S1177C/G430C, or S1177C/G432C. Inhibition of cross-linking by vanadate plus ATP again correlated with the ATPase activity of the mutants. All of the mutants that had cross-linking inhibited by vanadate trapping had verapamil-stimulated ATPase activity (>23% of Cys-less P-gp), while those that did not show inhibition had little or no verapamil-stimulated ATPase activity (<5%).

Is the cross-linking method specific? To address this question we also constructed 120 other double cysteine mutants. Each mutant had a cysteine in the NBD1-signature sequence and another in a segment of the second cytoplasmic loop (residues Asn280 to Ile289) connecting TMs 4 and 5, in the NBD2-signature sequence, in the conserved Q-loop (residues 1114Q to 1121I) in NBD2, or in the D-loop (residues Ser1204 to Ser1211). The p- and Q-loops are highly conserved regions in ABC proteins (23). When these mutant P-gps were expressed in HEK 293 cell and subjected to oxidative cross-linking with copper phenanthroline at 37 °C, there was no cross-linked product detected in SDS-PAGE (data not shown). These results indicate that cross-linking between residues in the signature sequences and in the Walker A sites was specific.

The cross-linking results indicate that the signature sequence in each NBD is very close to the Walker A site in the opposing NBD. To confirm that this structural arrangement is present in the active molecule, we tested the effect of cross-linking on drug (verapamil)-stimulated ATPase activity. Two mutants, L531C/C1074 and L1176C/C431, were selected for analysis; because these residues are found at identical positions when the two halves of P-gp are aligned, both mutants can be cross-linked with oxidant at 4 °C and both retained ATPase activity (22 and 41%, respectively, relative to Cys-less P-gp).

Fig. 3 shows that the activities of mutants L531C/C1074 and L1176C/C431 after treatment with oxidant were inhibited 82 and 72%, respectively. The activities of both mutants were almost completely recovered after treatment with DTT. These results are consistent with the idea that disulfide bond formation is occurring in functional molecules and that cross-linking between the signature sequence in each NBD with the opposing Walker A site inhibits activity.

**DISCUSSION**

This study shows that the NBD1 Walker A site is close to the NBD2 signature sequence, while the NBD2 Walker A site is close to the NBD1 signature sequence. The residues closest to each other are Cys431(NBD1)/Cys1176(NBD2) at one ATP-bind-

![Image 1](https://via.placeholder.com/150)

![Image 2](https://via.placeholder.com/150)

**FIG. 2.** *Effect of ATP plus vanadate on cross-linking.* Membranes prepared from HEK 293 cells expressing mutants L531C/G1073C, L531C/C1074, or L1176C/C431 were preincubated for 10 min at 37 °C in the presence (+) or absence (−) of ATP plus vanadate. The membranes were then treated with oxidant for 15 min at 21 °C and the reactions stopped by addition of sample buffer containing EDTA and no reducing agent. The mixtures and untreated membranes (control) were subjected to immunoblot analysis. The positions of the cross-linked (X-link) product and mature (170 kDa) P-gp are indicated.

**FIG. 3.** *Effect of oxidative cross-linking on ATPase activity.* Mutants L531C/C1074 and L1176C/C431 were isolated by nickel-chelate chromatography and treated with (+ CP) or without (− CP) oxidant, copper phenanthroline (CP), for 15 min at 21 °C. The reaction was stopped by addition of EDTA and the mixture passed through a gel filtration column. The eluted samples were reconstituted with lipid and assayed for verapamil-stimulated ATPase activity in the presence (+) or absence (−) of 10 min DTT. The activities are expressed relative to that of a sample that was mock-treated with oxidant and is the average of two different experiments.

**FIG. 4.** *Summary of cross-linking.* Lines indicate residues cross-linked at 4 °C.

**Table II**

| Cross-linking between residues in the NBD2 signature sequence and in the NBD1 Walker A site |
|-----------------------------------------------|
| L1176C (35%)                  | S1177C (35%)                  | G1178C (78%)                  | G1179C (14%)                  | Q1180C (23%)                  |
| 4 °C  | 21 °C  | 37 °C  | 4 °C  | 21 °C  | 37 °C  | 4 °C  | 21 °C  | 37 °C  | 4 °C  | 21 °C  | 37 °C  | 4 °C  | 21 °C  | 37 °C  |
| S429C (38%)                   | − b | − e | ++ | − b | − e | ++ | − b | − e | ++ | − b | − e | ++ | − b | − e | ++ |
| G430C (12%)                   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   |
| G431 (100%)                   | −   | ++ | + d | −   | ++ | + d | −   | ++ | + d | −   | ++ | + d | −   | ++ | + d |
| G432C (0%)                    | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   |
| K433C (12%)                   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   |

a Activity of the single cysteine mutant relative to Cys-less P-gp.

b No cross-linked product detected in SDS-PAGE.

c Relatively weak cross-linking (<50% of P-gp cross-linked).

d Relatively strong cross-linking (>50% of P-gp cross-linked).

 Asterisk sign in bold indicates cross-linking inhibited when preincubated with ATP plus vanadate.
Cross-linking P-glycoprotein Signature and Walker A Sites

The crystal structures of three other bacterial proteins support the biochemical data from P-gp. In the DNA repair enzyme Rad50 (32), bacterial vitamin B₁₂ transporter BtuCD (33), and the inactive NBD from Methanococcus jannaschii MJ0796 (34), the signature sequences were reported to be close to the Walker A site and contributed to ATP binding. A recent biochemical study of the MalK NBDs also suggested potential interaction between the signature sequences and the Walker A site (35). They reported vanadate-catalyzed photo-cleavage of the signature sequence. Therefore, the NBDs of some bacterial and eukaryotic ABC transporters may have some similar features.

Major structural changes occur in the NBDs (this study) and in the TM domains (19, 22) of P-gp immediately after ATP hydrolysis. Further studies will be required to determine how conformational changes in the NBDs are transmitted to the TM domains.

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