Drug-stimulated ATPase Activity of Human P-glycoprotein Requires Movement between Transmembrane Segments 6 and 12*

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Transmembrane segments (TM) 6 and 12 are directly connected to the ATP-binding domain in each homologous half of P-glycoprotein and are postulated to be important for drug-protein interactions. Cysteines introduced into TM6 (L332C, F343C, G346C, and P350C) were oxidatively cross-linked to cysteines introduced into TM12 (L975C, M986C, G989C, and S993C, respectively). The pattern of cross-linking was consistent with a left-handed coiled coil arrangement of the two helices. To detect conformational changes between the helices during drug-stimulated ATPase activity, we tested the effects of substrates and ATP on cross-linking. Cyclosporin A, verapamil, vinblastine, and colchicine inhibited cross-linking of mutants F343C/M986C, G346C/G989C, and P350C/S993C. By contrast, ATP promoted cross-linking between only L332C/L975C. Enhanced cross-linking between L332C/L975C was due to ATP hydrolysis, since cross-linked product was not observed in the presence of ATP and vanadate, ADP, ADP and vanadate, or AMP-PNP. Cross-linking between P350C/S993C inhibited verapamil-stimulated ATPase activity by about 75%. Drug-stimulated ATPase activity, however, was fully restored in the presence of dithiothreitol. These results show that TM6 and TM12 undergo different conformational changes during drug-binding or during ATP hydrolysis. To test this hypothesis, we replaced the residues in TM6 and TM12 with cysteine and tested for the effect of drug substrates and ATP on the oxidative cross-linking between TM6 and TM12. The pattern of cross-linking was consistent with the TM6 and TM12 helices arranged as a left-handed coiled coil, and cross-linked pairs were affected differently by substrates or ATP. We also show that TM6 and TM12 must undergo conformational changes during drug-stimulated ATPase activity, since formation of a cross-link between TM6 and TM12 inhibited drug-stimulated ATPase activity. Activity could be restored by breaking the disulfide bond with dithiothreitol.

EXPERIMENTAL PROCEDURES

Construction of Mutants—Cysteine residues were introduced into a Cys-less mutant of P-glycoprotein containing a histidine tag at the COOH terminus as described previously (4, 9, 14). The presence of a histidine tag facilitated purification of the mutant P-glycoprotein by nickel-chelate chromatography (9).

Sulfhydryl Cross-linking with Copper Phenanthroline—For each mutant, ten 10-cm-diameter culture plates of HEK 293 cells were transfected with mutant MDR1 cDNA. After 24 h, the media were replaced with fresh media containing 10 μM cyclosporin A. The cells were harvested after another 24 h, and membranes were prepared as described previously (9). The membranes were suspended in 200 μl of Tris-buffered saline. A sample of the membrane suspension (12 μl) was mixed with 15 μl of 2 × ATPase buffer (100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl2) containing the desired nucleotide or drug substrate for 5 min at room temperature. Cross-linking was initiated by addition of 3 μl of 2 mM or 20 mM Cu2+ (phenanthroline)3−, and the samples were incubated for 10 min at 37 °C (22). The reactions were stopped by addition of EDTA to a final concentration of 30 mM in SDS sample buffer containing no dithiothreitol reducing agent. The samples were subjected to SDS-PAGE, transferred onto a sheet of nitrocellulose, and probed with a rabbit polyclonal antibody against P-glycoprotein and enhanced chemiluminescence (22).

Purification of Cross-linked P-glycoprotein Mutant and Measurement of Verapamil-stimulated ATPase Activity—Purification was carried out as described previously (9). Briefly, forty 10-cm diameter culture plates of HEK 293 cells were transfected with the mutant MDR1 cDNA, followed by addition of cyclosporin A as described above. Membranes

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‡ The abbreviations used are: TM, transmembrane segment(s); PAGE, polyacrylamide gel electrophoresis; AMP-PNP, adenylyl-5′-ylimidophosphate; DTT, dithiothreitol.

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RESULTS

Mutagenesis and Oxidative Cross-linking—Predicted TM6 and TM12 directly connect the transmembrane domains to nucleotide-binding fold NBF1 and NBF2, respectively. A relatively high degree of homology exists between TM6 and TM12 since 11 of 21 residues are identical, when both transmembrane segments are aligned. In a previous study, we showed that residues 332 (TM6) and 975 (TM12) are close to each other in the tertiary structure of P-glycoprotein (22). Using this observation as a starting point, we modeled the two helices as left- or right-handed coiled coils (reviewed in Refs. 24–26) to predict other sites in TM6 and TM12 that may be close to each other. Fig. 1, A and B, shows the arrangement of the residues in TM6 and TM12 as α-helical nets, while C shows the imposition of TM6 on TM12 in a left-handed coiled coil. According to Chothia (26), the helices have ridges (consisting of the side chains of residues) and grooves between the side chains. The ridges are formed by the residues that are spaced 1, 3 (i, i + 3), or 4 (i, i + 4) apart. During packing in a right-handed coiled coil, the i + 4 axes in each helix are superimposed, whereas in a left-handed coiled coil, the i + 3 axis of one helix is superimposed on the i + 4 axis of the other. Both left- and right-handed coiled coils are equally common in globular proteins, but left-handed coiled coils appear to be more common in the membrane proteins whose crystal structures have been determined (27, 28). Modeling of TM6 and TM12 in a left-handed coiled coil showed the pairs of residues between TM6 and TM12 that are modeled in a left-handed coiled coil (27, 28). The membrane fractions containing each mutant P-glycoprotein (29) were then prepared and cross-linked with Cu2⁺ (phenanthroline), (2 mM final concentration) for 10 min at 37 °C. The cross-linked sample was then diluted 100-fold with Tris-buffered saline and centrifuged at 200,000 × g for 2 h at 4 °C. The membranes were then solubilized with 1% (w/v) n-dodecyl-β-D-maltoside (Sigma) and P-glycoprotein-(His)10 isolated by nickel-chelate chromatography using nickel-spin columns (Ni-NTA, Qiagen).

To measure ATPase activity, the purified P-glycoprotein was diluted with an equal volume of 100 mg/ml crude sheep brain phosphatidylethanolamine (Sigma, Type II), which had been previously washed with Tris-buffered saline to remove traces of phosphate and then sonicated. 100 μg of purified P-glycoprotein was incubated with 1 mM verapamil, and ATPase activity was initiated by addition of an equal volume of buffer containing 100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl2, 10 mM ATP, with or without 10 mM DTT. The samples were incubated at 37 °C, and the amount of inorganic phosphate liberated was determined by the method of Chifflet et al. (23).

Inhibition of ATPase Activity by Cross-linking—An interesting observation was that the amount of cross-linking seen in mutant L332C/L975C in whole cells (9) varied considerably if the cells were starved before cross-linking (data not shown). A possible explanation was that cross-linking was most efficient with freshly isolated cells and decreased with the metabolic state of the transfected cells. Cross-linking seen in mutant L332C/L975C in whole cells (9) varied considerably if the cells were starved before cross-linking (data not shown). A possible explanation was that cross-linking was most efficient with freshly isolated cells and decreased with the metabolic state of the transfected cells. Cross-linking seen in mutant L332C/L975C in whole cells (9) varied considerably if the cells were starved before cross-linking (data not shown). A possible explanation was that cross-linking was most efficient with freshly isolated cells and decreased with the metabolic state of the transfected cells. Cross-linking seen in mutant L332C/L975C in whole cells (9) varied considerably if the cells were starved before cross-linking (data not shown). A possible explanation was that cross-linking was most efficient with freshly isolated cells and decreased with the metabolic state of the transfected cells. Cross-linking seen in mutant L332C/L975C in whole cells (9) varied considerably if the cells were starved before cross-linking (data not shown). A possible explanation was that cross-linking was most efficient with freshly isolated cells and decreased with the metabolic state of the transfected cells. Cross-linking seen in mutant L332C/L975C in whole cells (9) varied considerably if the cells were starved before cross-linking (data not shown). A possible explanation was that cross-linking was most efficient with freshly isolated cells and decreased with the metabolic state of the transfected cells. Cross-linking seen in mutant L332C/L975C in whole cells (9) varied considerably if the cells were starved before cross-linking (data not shown). A possible explanation was that cross-linking was most efficient with freshly isolated cells and decreased with the metabolic state of the transfected cells. Cross-linking seen in mutant L332C/L975C in whole cells (9) varied considerably if the cells were starved before cross-linking (data not shown). A possible explanation was that cross-linking was most efficient with freshly isolated cells and decreased with the metabolic state of the transfected cells. Cross-linking seen in mutant L332C/L975C in whole cells (9) varied considerably if the cells were starved before cross-linking (data not shown). A possible explanation was that cross-linking was most efficient with freshly isolated cells and decreased with the metabolic state of the transfected cells. Cross-linking seen in mutant L332C/L975C in whole cells (9) varied considerably if the cells were starved before cross-linking (data not shown). A possible explanation was that cross-linking was most efficient with freshly isolated cells and decreased with the metabolic state of the transfected cells.
Inhibition of ATPase Activity by Cross-linking

Pressing mutant L332C/L975C were cross-linked in the presence of nucleotides. Fig. 2A (lane 3) shows that cross-linking of the mutant occurred in the presence of ATP. No cross-linked product was observed in the presence of ATP plus vanadate, ADP, ADP plus vanadate, or with the nonhydrolyzable ATP analog, AMP-PNP (Fig. 2A, lanes 4–7). These results suggest that cross-linking between L332C and L975C occurred during ATP hydrolysis.

The effect of nucleotides on cross-linking was also tested on mutants F336C/M986C, G346C/G989C, and P350C/S993C. For these mutants, cross-linking was carried out with 10-fold less oxidant (0.2 mM) to detect for any subtle effects of the nucleotides. Fig. 2 (panels B, C, and D) shows that the presence of nucleotides had little detectable effect on cross-linking.

To test the effect of drug substrates, cross-linking of mutants L332C/L975C, F336C/M986C, G346C/G989C, and P350C/S993C was done in the presence of verapamil, cyclosporin A, vinblastine, or colchicine. No cross-linked product was observed for mutant L332C/L975C (Fig. 3A). By contrast, all the drug substrates were effective in blocking cross-linking of mutants F336C/M986C and G346C/G989C (Fig. 3, B and C), but were less effective in preventing cross-linking of mutant P350C/S993C (Fig. 3D). In mutant P350C/S993C, verapamil, cyclosporin A, and vinblastine were more effective than colchicine in inhibiting cross-linking. Mutants S979C/F336C or L339C/V982C did not yield any cross-linked product even in the presence of ATP or drug substrates (data not shown).

**DISCUSSION**

The results of cross-linking experiments suggest that TM6 and TM12 are close to each other along the entire lengths of the helices and are likely to exist in a left-handed coiled-coil arrangement. In this arrangement four of the six pairs of amino acids predicted to lie close to one another could be cross-linked. Cross-linking was not observed between F336C/S979C or L339C/V982C, even in the presence of ATP or drug substrates.
Inhibition of ATPase Activity by Cross-linking

hydrolysis promoted cross-linking between L332C/L975C suggests that inhibition of cross-linking of L332C/L975C in whole cells by verapamil or vinblastine occurred indirectly through depletion of intracellular ATP. Vinblastine and verapamil had a greater effect than colchicine since these two compounds are more efficient in stimulating the ATPase activity of P-glycoprotein, thereby depleting the ATP more rapidly.

Drug substrates inhibited cross-linking of mutants F343C/M986C, G346C/G989C, and P350C/S993C. These residues either lie close to the binding site(s) for these substrates or drug-binding results in large conformational changes in TM6, TM12, or in both transmembrane segments. Conformational changes occurring globally in P-glycoprotein during substrate binding or during ATP hydrolysis have also been detected indirectly (21, 30).

The results of this study show that there is “cross-talk” between the transmembrane domains and the ATP-binding domains of P-glycoprotein. ATP hydrolysis causes conformational changes in the transmembrane domains, while introduction of a cross-link between TM6 and TM12 inhibits drug-stimulated ATPase activity. Therefore, conformational changes occurring between TM6 and TM12 appear to be essential for coupling drug binding to stimulation of ATPase activity. A similar mechanism may exist in other ABC transporters.

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FIG. 4. Effect of oxidative cross-linking on verapamil-stimulated ATPase activity of mutant P350C/S993C. A, membranes from cells expressing Cys-less P-glycoprotein or mutant P350C/S993C were treated with or without oxidant and then incubated for 5 min at room temperature in the presence (± DTT) or absence (No DTT) of 5 mM dithiothreitol prior to immunoblot analysis. The cross-linked product is indicated by an asterisk. The positions of the mature (170-kDa) and core-glycosylated (150-kDa) forms of P-glycoprotein are indicated. B, immunoblot analysis of purified P-glycoprotein(His)10 from membranes of Cys-less and P350C/S993C mutants that were treated with (+) or without (−) oxidant before purification by nickel chromatography. C, equivalent amounts of purified P-glycoprotein(His)10 from oxidant-treated or mock-treated Cys-less and mutant P350C/S993C were mixed with lipid, and verapamil-stimulated (1 mM) ATPase activity was determined in the presence or absence of 5 mM DTT. The activities are expressed relative to the sample that was mock-treated with oxidant and is the average of two different experiments.

(data not shown). One possibility is that these residues are not close. Another possibility is that they are close, but in a non-reactive environment or that they are inaccessible to oxidant.

Cross-linking was significantly influenced by the presence of ATP or drug substrates. ATP hydrolysis rather than nucleotide binding was responsible for cross-linking between L332C/L975C. In a previous study (22), we observed in intact cells that vinblastine or verapamil, but not colchicine, could inhibit cross-linking between these two residues. The observation that ATP