Processing Mutations Disrupt Interactions between the Nucleotide Binding and Transmembrane Domains of P-glycoprotein and the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)*

Received for publication, July 30, 2008, and in revised form, August 13, 2008 Published, JBC Papers in Press, August 16, 2008, DOI 10.1074/jbc.M805834200

Tip W. Loo, M. Claire Bartlett, and David M. Clarke 1

From the Department of Medicine and Department of Biochemistry, University of Toronto, Toronto, Ontario M5S 1A8, Canada

P-glycoprotein (P-gp, ABCB1) is an ATP-dependent drug pump. Each of its two homologous halves contains a transmembrane domain (TMD) that has six transmembrane (TM) segments and a nucleotide-binding domain (NBD). Determining how the two halves interact may provide insight into the folding of P-gp as the drug-binding pocket and nucleotide-binding sites are predicted to be at the interface between the two halves. Here, we present evidence for NBD1-TMD2 and NBD2-TMD1 interactions. We also show that TMD-NBD interactions in immature and mature P-gp can be affected by the presence of a processing mutation. We found that the NBD-TMD mutants L443C(NBD1)/S909C(TMD2) and A266C(TMD1)/F1086C(NBD2) could be cross-linked at 0 °C with oxidant (copper phenanthroline). Cross-linking was inhibited by vanadate-trapping of nucleotide. The presence of a processing mutation (G268V/L443C(NBD1)/S909C(TMD2) and A266C(TMD1)/F1086C(NBD2)) resulted in the synthesis of the immature (150 kDa) protein as the major product and the mutants could not be cross-linked with copper phenanthroline. Expression of the processing mutants in the presence of a pharmacological chaperone (cyclosporin A), however, resulted in the expression of mature (170 kDa) protein at the cell surface that could be cross-linked. Similarly, CFTR mutants A274C(TMD1)/L1260A(NBD2) and V510C(NBD1)/A1067C(TMD2) could be cross-linked at 0 °C with copper phenanthroline. Introduction of ΔF508 mutation in these mutants, however, resulted in the synthesis of immature CFTR that could not be cross-linked. These results suggest that establishment of NBD interactions with the opposite TMD is a key step in folding of ABC transporters.

* This work was supported by grants from the Cystic Fibrosis Foundation (Grant CLARKE08GO), Canadian Institutes for Health Research (Grants 62832 and 25043), and the National Cancer Institute of Canada through the Canadian Cancer Society (Grant 19074). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Recipient of the Canadian Research Chair in Membrane Biology. To whom correspondence should be addressed: Dept. of Medicine, University of Toronto, 1 King’s College Circle, Rm. 7342, Medical Sciences Bldg., Toronto, Ontario M5S 1A8, Canada. Tel.: or Fax: 416-978-1105; E-mail: david.clarke@utoronto.ca.

2 The abbreviations used are: P-gp, P-glycoprotein; TM, transmembrane; NBD, nucleotide-binding domain; TMD1, NH2-terminal transmembrane domain containing TM segments 1–6; TMD2, COOH-terminal transmembrane domain containing TM segments 7–12; HEK, human embryonic kidney; CFTR, cystic fibrosis transmembrane conductance regulator; corr-4a, (N-[2-(5-chloro-2-methoxy-phenylamino)-2′-yl]-benzamide.

© 2008 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
tacts identified in the P-gp studies were that the use of crosslinkers and thermal motion of the protein allowed cross-linking to occur between distant cysteines or that both studies involved different structures of P-gp. The cross-linking study was performed on mature P-gp delivered to the cell surface (16), whereas the co-immunoprecipitation study on domains of P-gp expressed as separate polypeptides utilized immature forms of the protein (14). Mature and immature forms of P-gp show differences in structure (18). In this study we performed crosslinking studies on immature and mature forms of P-gp to test if they showed differences in NBD-TMD interactions. We also tested whether the immature and mature forms of the P-gp sister protein, the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel, showed differences in NBD-TMD interactions. Knowledge about folding of CFTR is important because processing mutations in CFTR such as deletion of Phe-508 are the major cause of cystic fibrosis (19).

MATERIALS AND METHODS

Construction of Mutants—The seven endogenous cysteines at positions 137, 431, 717, 956, 1074, 1125, and 1227 were replaced with alamines to create cysteine-less (Cys-less) P-gp (6). None of the cysteines were important for function (6). Single cysteine residues were re-introduced at positions Leu-443(NBD1), Ser-474(NBD1), Arg-905(TMD2), or Ser-909(TMD2) in Cys-less P-gp. Double cysteine mutants L443C(NBD1)/S909C(TMD2) and S474C(NBD1)/R905C(TMD2) were also constructed as these cysteines were previously shown to cross-link when treated with maleimide-type cross-linkers with lengths of 6–16 Å (16). These results suggested that TMD1-NBD2 and the opposite TMD of the dimer (17). A subsequent cross-linking study with P-gp showed that cysteines introduced at positions 443 and 474 in NBD1 could be cross-linked to cysteines introduced at positions 909 and 905, respectively, in TMD2 using maleimide-type cross-linkers with lengths of 6–16 Å (16). These results suggested that TMD1-NBD2 interactions were important for P-gp folding (Fig. 1A). A previous study of the domains expressed as separate polypeptides utilized immature forms of P-gp in Western blots of extracts from transfected human embryonic kidney (HEK) 293 cells.

Because P-gp mutants L443C(NBD1)/S909C(TMD2) and A266C(TMD1)/F1086C(NBD2) showed cross-linking in the presence of copper phenanthroline, we constructed CFTR mutants with cysteines at equivalent positions in a Cys-less version of CFTR (22) to yield mutants E474C(NBD1)/A1067C(TMD2) and A274C(TMD1)/L1260C(NBD2), respectively. We also constructed the V510C(NBD1)/A1067C(TMD2) CFTR mutant because position V510C or V510A promoted maturation of Cys-less CFTR (22).

Expression of Mutants, Purification, and Measurement of ATPase Activity—The mutant P-gps or CFTRs were transiently expressed in HEK 293 cells as described previously (20). HEK 293 cells expressing mutant P-gps were also grown in the presence or absence of 10 μM cyclosporin A for 24 h because it acts as a pharmacological/specific chemical chaperone to increase the yield of mature enzyme (23). Whole cell extracts of A52-tagged P-gps were subjected to immunoblot analysis using 6% (w/v) acrylamide gels and monoclonal antibody A52. Mutants of CFTR were expressed in the presence or absence of 20 μM corr-4a ([N-2-(5-chloro-2-methoxy-phenylamino)-2′-yl]-benzamide) as this compound promotes maturation of CFTR (24, 25).

Histidine-tagged P-gp was isolated by nickel-chelate chromatography as described previously (20). A sample of the isolated histidine-tagged P-gp was mixed with an equal volume of 10 mg/ml sheep brain phosphatidylethanolamine (Type II-S, Sigma) that had been washed and suspended in TBS (Tris-buffered saline: 10 mM Tris/HCl, pH 7.4 and 150 mM NaCl). The sample was sonicated and ATPase activity measured in the absence of drug substrate or in the presence of 0.5 mM verapamil. The samples were incubated for 30 min at 37 °C, and the amount of inorganic phosphate released was determined.

Disulfide Cross-linking Analysis—The P-gp or CFTR double cysteine mutants were transiently expressed in HEK 293 cells. The membranes were prepared as described previously (26) and suspended in TBS. Membrane samples were then treated with oxidant (1 mM (Cu2+/0.10-phenanthroline)3). The reactions were stopped by addition 5 mM EDTA followed by addition of 2× SDS sample buffer (125 mM Tris-HCl, pH 6.8, 20% (w/v) glycerol, and 4% (w/v) SDS) containing 50 mM EDTA and no reducing agent. The reaction mixtures were then subjected to SDS-PAGE (7.5% polyacrylamide gels) and immunoblot analysis with a rabbit polyclonal antibody against P-gp (14) or CFTR (27). Intramolecular disulfide cross-linking between domains of P-gp or CFTR can be detected because the cross-linked product migrates with a slower mobility on SDS-PAGE gels (27, 28).

RESULTS

P-gp consists of two NBDs and two TMDs containing 6 TM segments each (Fig. 1A). A previous study of the domains expressed as separate polypeptides showed that TMD1 co-immunoprecipitated with NBD1 whereas TMD2 co-immunoprecipitated with NBD2 (14). These results predicted that TMD1-NBD1 and TMD2-NBD2 interactions were important for P-gp folding (Fig. 1A). The recent crystal structure of Sav1866 showed potential interactions between NBD and the opposite TMD of the dimer (17). A subsequent cross-linking study with P-gp showed that cysteines introduced at positions 443 and 474 in NBD1 could be cross-linked to cysteines at positions 909 and 905, respectively, in TMD2 using maleimide-type cross-linkers with lengths of 6–16 Å (16). These results suggested that TMD1-NBD2 interactions could also be important in P-gp (Fig. 1B). A potential problem with the cross-linking study, however, was that the use of cross-linkers at elevated temperatures and long incubation times (15 min at 26 °C) might have allowed distant cysteines to be cross-linked due to thermal motion of the protein. Therefore, to determine if the L443C(NBD1)/S909C(TMD2) and S474C(NBD1)/R905C(TMD2) pairs of cyst-
Effect of Processing Mutations on P-gp and CFTR NBD-TMD Interactions

FIGURE 1. Schematic models of NBD-TMD interactions in P-gp. A, model of NBD-TMD interactions of immature P-gp based on co-immunoprecipitation studies. The NH2- and COOH-halves of P-gp are colored white and light gray, respectively. The drug-binding pocket (DRUG) is shown as a dark gray oval. Co-immunoprecipitation studies of domains of P-gp expressed as separate polypeptides showed NBD1-TMD1 and NBD2-TMD2 interactions (14). The NBDs are depicted as ovals while the TM segments are depicted as numbered cylinders. Curved lines indicate the positions of intracellular loops (ICL) that connect the TM segments. Regions in the NH2- and COOH-halves of P-gp are shaded in white or gray, respectively. B, alternative model of NBD-TMD interaction in P-gp. The model is based on the observations that cysteines introduced at positions Leu-443 and Ser-474 could be cross-linked to cysteines at positions Ser-909 or Arg-905, respectively (16) (indicated by filled circles). The NBDs, TMDs, and TM segments are labeled as described above. The locations of positions equivalent to cysteines S473 and R905 in the other half of P-gp (A266C/F1086C) are indicated. The locations of processing mutations G268V and L1260A that were used to inhibit maturation of P-gp are indicated as squares.

These results show that cysteines at positions 443(NBD1) and 909(TMD2) lie close enough in the native structure to be directly linked by a disulfide bond when treated at 0 °C when thermal motion of the protein is low. Cysteines at positions 474(NBD1) and 905(TMD2) may lie further apart as they showed no disulfide bond formation even when treated with oxidant at 25 °C.

To test if cross-linking between Cys-443(NBD1) and Cys-909(TMD2) affected the activity of P-gp, we assayed for drug-stimulated ATPase activity. Drug-stimulated ATPase activity is a useful assay because it has been shown that the turnover numbers for transport and drug-stimulated ATPase activity are comparable (31). In addition, there is good correlation between drug-resistance and ATPase assays (32). The L443C(NBD1)/S909C(TMD2) mutant was previously demonstrated to be functional as its drug transport activity was similar to the Cys-less parent (16). Accordingly, histidine-tagged Cys-less and Cys-443(NBD1)/Cys-909(TMD2) mutants of P-gp were assayed in HEK 293 cells followed by isolation by nickel-chelate chromatography. The P-gps were mixed with lipid and assayed for verapamil-stimulated ATPase activity. Verapamil was used because it is one of the most potent activators of P-gp ATPase activity (33). In the presence of saturating levels of verapamil (0.5 mM) it was found that mutant L443C(NBD1)/S909C(TMD2) showed ATPase activity that was similar to the Cys-less parent. To test for the effect of cross-linking on activity, isolated mutant L443C(NBD1)/S909C(TMD2) and Cys-less P-gps were treated with or without 1 mM copper phenanthroline for 15 min at 20 °C. The reactions were stopped by addition of EDTA to 5 mM. The proteins were then assayed for verapamil-stimulated ATPase activity. Fig. 3 shows that cross-linking of mutant L443C(NBD1)/S909C(TMD2) caused more than 85% decrease in activity while treatment of Cys-less P-gp with copper phenanthroline had little effect. The activity of cross-linked mutant L443C(NBD1)/S909C(TMD2) could be restored after treatment with the thiol-reducing agent dithiothreitol (Fig. 3). These results suggest that conformational changes at the NBD1-TMD2 interface may be critical for activity.

We then tested whether cross-linking of mutant L443C(NBD1)/S909C(TMD2) was sensitive to conformational

FIGURE 2. Cross-linking analysis of P-gp NBD1/TMD2 cysteine mutants. Mutants S474C(NBD1)/R905C(TMD2) and L443C(NBD1)/S909C(TMD2) were expressed in HEK 293 cells in the absence of drug substrates. Membranes were prepared, and samples were treated with (+) or without (−) 1 mM copper phenanthroline (CuP) at 0 or 25 °C for 15 min. The reactions were stopped by addition of SDS sample buffer containing 50 mM EDTA and no reducing agent. Samples were subjected to immunoblot analysis. The positions of mature (170 kDa) and cross-linked (X-link) forms of P-gp are indicated.
changes induced by drug substrates. For example, it was previously shown that drug substrates such as vinblastine, cyclosporin A, and rhodamine B or vanadate trapping of nucleotide could block cross-linking of mutant L339C(TMD1)/F728C(TMD2) (34). Accordingly, we first tested if vinblastine, cyclosporin A, or rhodamine B would affect cross-linking of mutant L443C(NBD1)/S909C(TMD2). Membranes prepared from cells expressing mutant L443C(NBD1)/S909C(TMD2) were preincubated with or on drug or with saturating concentrations of vinblastine (0.1 mM), cyclosporin A (0.01 mM), rhodamine B (1 mM), or verapamil (1 mM) for 15 min at 20 °C. The samples were then cooled on ice, treated with copper phenanthroline, and then subjected to immunoblot analysis. Fig. 4A shows that the drug substrates did not inhibit cross-linking of mutant L443C(NBD1)/S909C(TMD2). The results suggest that residues Cys-443(NBD1) and Cys-909(TMD2) lie outside the drug-binding sites and that binding of drug substrates do not cause any major conformational changes at this part of the NBD1/TMD2 interface.

We then tested whether vanadate-trapping of nucleotide affected cross-linking of mutant L443C(NBD1)/S909C(TMD2). P-gp can be trapped at a transition state during ATP hydrolysis by including vanadate in the reaction mix (35). Vanadate traps ADP at either NBD by mimicking the transition state of the γ-phosphate of ATP during hydrolysis and trapping at one NBD inhibits ATP hydrolysis at the second site (35). Vanadate-trapping of nucleotide causes conformational changes in P-gp and this can be detected by disulfide cross-linking analysis (36). Accordingly, membranes prepared from cells expressing mutant L443C(NBD1)/S909C(TMD2) were preincubated with or without ATP plus vanadate for 15 min at 37 °C. Samples were then cooled on ice, treated with copper phenanthroline, and subjected to immunoblot analysis. Fig. 4B shows that vanadate trapping of nucleotide blocked cross-linking. These results suggest that ATP-dependent conformational changes at the NBD1-TMD2 interface may be critical for function.

The observation that cysteines at positions 443(NBD1) and 909(TMD2) can form a disulfide bond when exposed to oxidant at low temperature suggests that mature P-gp has a structure that resembles Sav1866 (17). When domains of P-gp are expressed as separate polypeptides however, co-immunoprecipitation assays detected little evidence for interactions between the NBD1 and TMD2 quarter-molecules (14). Instead, NBD1-TMD1 and NBD2-TMD2 interactions were detected. A potential problem in expressing half-molecule or quarter-molecule forms of P-gp, however, is that the proteins do not mature (14). In the absence of one or more domains, P-gp truncation mutants appear to be incompletely folded because they are only core-glycosylated and more sensitive to protease digestion (37). The mature protein contains complex carbohydrate and is relatively resistant to protease digestion. Therefore, establishment of NBD1-TMD2 interfaces.

**FIGURE 3.** Effect of cross-linking on verapamil-stimulated ATPase activity of P-gp. Equivalent amounts of histidine-tagged Cys-less (C-less) or mutant L443C(NBD1)/S909C(TMD2) P-gps were assayed for drug-stimulated ATPase activity in the presence of 0.5 mM verapamil after treatment with (+) (black bars) or without (−) (gray bars) 1 mM copper phenanthroline (CuP) followed by treatment with 5 mM dithiothreitol (DTT) (white bars).

**FIGURE 4.** Effect of drug substrates or ATP plus vanadate on cross-linking of P-gp mutant L443C(NBD1)/S909C(TMD2). Membranes prepared from cells expressing mutant L443C(NBD1)/S909C(TMD2) were treated with (A) no drug (None), 0.1 mM vinblastine (Vin), 0.01 mM cyclosporin A (Cyclo), 1 mM rhodamine B (Rhod), or 1 mM verapamil (Ver) for 15 min at 20 °C or incubated in the presence of 5 mM ATP plus 0.2 mM sodium vanadate (ATP/VO4) for 15 min at 37 °C. Samples were then treated with (+) or without (−) 1 mM copper phenanthroline (CuP) for 15 min at 0 °C. The reactions were stopped by addition of SDS sample buffer containing 5 mM EDTA and no reducing agent. The samples were then subjected to immunoblot analysis. The positions of mature (170 kDa) and cross-linked (X-link) forms of P-gp are indicated.
To test if NBD1-TMD2 interactions differ in the mature and immature forms of P-gp, the G268V (26) or L1260A (38) processing mutations were introduced into mutant L443C(NBD1)/S909C(TMD2) to inhibit its maturation. Processing mutations are amino acid changes that appear to trap P-gp as an incompletely folded protein in the endoplasmic reticulum (reviewed in Ref. 39). In addition, processing mutations G268V(TMD1) and L1260A(TMD2) were selected because they are located outside the NBD1 and TMD2 domains to be cross-linked (see Fig. 1B) and appear to trap the protein in an incompletely folded conformation rather than cause gross misfolding as expression of either processing mutation in the presence of a drug substrate promotes maturation and delivery of active P-gp to the cell surface (18, 38). The G268V mutation is located in the second intracellular loop in TMD1 whereas the L1260A mutation is located at the COOH-end of NBD2. Mutant G268V(TMD1)/L443C(NBD1)/S909C(TMD2) was transiently expressed in HEK 293 cells in the presence or absence of the drug substrate cyclosporin A. Membranes were prepared and samples were treated with (+) or without (−) 1 mM copper phenanthroline (CuP) for 15 min at 0 °C. The reactions were stopped by addition of SDS sample buffer containing 5 mM EDTA and no reducing agent and subjected to immunoblot analysis. The positions of cross-linked (X-link), mature (170 kDa) or immature (150 kDa) forms of P-gp are indicated.

We then tested if the mature and immature forms of P-gp showed structural differences at the TMD1-NBD2 interface. Because no cross-linking studies have identified cysteines that could be cross-linked at the TMD1-NBD2 interface, we constructed a series of double cysteine mutants between a cysteine in TMD1 (A266C or F267C) and another in NBD2 (R1085C, Y1087C or D1088C) that would be equivalent to L443C(NBD1) and S909C(TMD2), respectively, in each half of P-gp. Membranes were prepared from cells expressing these double cysteine mutants and samples were treated with or without 1 mM copper phenanthroline at 0 °C followed by immunoblot analysis. Cross-linking product was observed only in mutant A266C(TMD1)/F1086C(NBD2) (Fig. 6A). It was observed that the mutant yielded mature 170-kDa P-gp as the major product and about 50% of the protein was cross-linked in mutant A266C(F1086C) containing the L1260A processing mutation expressed in HEK 293 cells in the absence (no drug) or presence of 10 μM cyclosporin A (+ Cyclo). Membranes were prepared and samples were treated with (+) or without (−) 1 mM copper phenanthroline (CuP) for 15 min at 0 °C. The reactions were stopped by addition of SDS sample buffer containing 5 mM EDTA and no reducing agent and subjected to immunoblot analysis. The positions of cross-linked (X-link), mature (170 kDa) or immature (150 kDa) forms of P-gp are indicated.

We then tested if the mature and immature forms of P-gp showed structural differences at the TMD1-NBD2 interface. Because no cross-linking studies have identified cysteines that could be cross-linked at the TMD1-NBD2 interface, we constructed a series of double cysteine mutants between a cysteine in TMD1 (A266C or F267C) and another in NBD2 (R1085C, Y1087C or D1088C) that would be equivalent to L443C(NBD1) and S909C(TMD2), respectively, in each half of P-gp. Membranes were prepared from cells expressing these double cysteine mutants and samples were treated with or without 1 mM copper phenanthroline at 0 °C followed by immunoblot analysis. Cross-linking product was observed only in mutant A266C(TMD1)/F1086C(NBD2) (Fig. 6A). It was observed that the mutant yielded mature 170-kDa P-gp as the major product and about 50% of the protein was cross-linked after treatment with oxidant. Cross-linking of mutant A266C(TMD1)/F1086C(NBD2) was relatively specific, as cross-linked product was not observed in mutants that contained only Cys-266(TMD1) or Cys-1086(NBD2), or in mutants A266C(TMD1)/R1085C(NBD2), A266C(TMD1)/ Y1087C(NBD2), A266C(TMD1)/D1088C(NBD2), F267C(TMD1)/ R1085C(NBD2), F267C(TMD1)/F1086C(NBD2), F267C(TMD1)/ Y1087C(NBD2), F267C(TMD1)/D1088C(NBD2) (data not shown). The A266C(TMD1)/F1086C(NBD2) mutant has similar properties to that of mutant L443C(NBD1)/S909C(TMD2) in that drug substrates such as vinblastine, cyclosporin A, rhodamine B or verapamil did not protect the mutant from cross-linking but cross-linking was blocked by vanadate trapping of nucleotide (Fig. 6A).
S909C(TMD2) in that it had very low verapamil-stimulated ATPase activity compared with the Cys-less parent (less than 10% activity). It appears that they may be some asymmetry at the NBD1-TMD2 and TMD1-NBD2 interfaces. The verapamil-stimulated ATPase activities of P-gp mutants containing only the Cys-266 or Cys-1086 mutation showed that the reduced activity was due to the presence of the F1086C change (data not shown).

To test if there were structural differences between the mature and immature forms of P-gp at the TMD1-NBD2 interface, the L1260A processing mutation was introduced into mutant A266C(TMD1)/F1086C(NBD2). Mutant L1260A/A266C(TMD1)/F1086C(NBD2) was expressed in the presence or absence of cyclosporin A and membranes prepared from the cells were treated with or without copper phenanthroline at 0 °C followed by immunoblot analysis. Fig. 6B shows that cross-linking was observed with mature P-gp (right panel) but not with immature P-gp (left panel). Therefore, both the NBD1-TMD2 and TMD1-NBD2 interfaces show similar structural differences in the mature and immature forms of the protein as cross-linking was observed with only mature P-gp.

CFTR is a sister protein of P-gp. To test if the mature and immature forms of CFTR also show structural differences at the NBD-TMD interfaces, we first introduced cysteines at positions equivalent to those in P-gp that showed cross-linking at the NBD-TMD interfaces. CFTR mutant A274C(TMD1)/L1260C(NBD2) contains cysteines at sites equivalent to P-gp mutant A266C(TMD1)/F1086C(NBD2). Membranes prepared from cells expressing CFTR mutant A274C(TMD1)/L1260C(NBD2) were treated with or without copper phenanthroline at 0 °C and samples subjected to cross-linking analysis. Fig. 7A shows that mutant A274C(TMD1)/L1260C(NBD2) expressed the mature protein as the major product and cross-linked product was detected after treatment with oxidant. CFTR mutant E474C(NBD1)/A1067C(TMD2) contains cysteines at positions equivalent to those in P-gp mutant L443C(NBD1)/S909C(TMD2). The CFTR mutant E474C(NBD1)/A1067C(TMD2), however, did not mature when it was expressed in plain medium (Fig. 7E, left panel).

It was previously shown that mutations V510C or V510A promoted maturation of Cys-less CFTR (22). It was possible that V510C could interact with TMD2. Accordingly, mutant V510C(NBD1)/A1067C(TMD2) was constructed and expressed in HEK 293 cells. Membranes were then prepared, treated at 0 °C with copper phenanthroline, and subjected to immunoblot analysis. Fig. 7B shows that mature protein was the major product and cross-linked product was detected after treatment with oxidant.

To test if immature CFTR shows structural differences from the mature protein at the NBD-TMD interfaces, we introduced the ΔF508 processing mutation into CFTR mutants A274C(TMD1)/L1260C(NBD2) and V510C(NBD1)/A1067C(TMD2). The ΔF508 processing mutation was utilized because it is the most common cystic fibrosis mutation as it is found in at least one chromosome in 90% of affected individuals (40). Introduction of the ΔF508 mutation into mutants A274C(TMD1)/L1260C(NBD2) or V510C(NBD1)/A1067C(TMD2) yielded immature CFTR protein that was not cross-linked when membranes expressing the mutants were treated with copper phenanthroline (Fig. 7, C and D). Unfortunately, attempts to promote maturation of mutants ΔF508/A274C(TMD1)/L1260C(NBD2) (C) or ΔF508/V510C(NBD1)/A1067C(TMD2) (D) were treated with (+) or without 1 mM copper phenanthroline (CuP) for 15 min at 0 °C. The reactions were stopped by addition of SDS sample buffer containing 5 mM EDTA and no reducing agent and subjected to immunoblot analysis. The positions of immature, mature, and cross-linked (X-link) forms of CFTR are indicated.

**Effect of Processing Mutations on P-gp and CFTR NBD-TMD Interactions**

**FIGURE 7. Effect of processing mutations and corr-4a on maturation of CFTR TMD-NBD cross-linking mutants.** Membranes prepared from cells expressing mutants A274C(TMD1)/L1260C(NBD2) (A) or V510C(NBD1)/A1067C(TMD2) (B) were treated with (+) or without 1 mM copper phenanthroline (CuP) for 15 min at 0 °C. Samples were treated at 0 °C with copper phenanthroline (Fig. 7B) were treated with (∓) or without 1 mM copper phenanthroline (CuP) for 15 min at 0 °C. Samples were treated at 0 °C with copper phenanthroline (Fig. 7B) or without 1 mM copper phenanthroline (CuP) for 15 min at 0 °C. The reactions were stopped by addition of SDS sample buffer containing 5 mM EDTA and no reducing agent and subjected to immunoblot analysis. The positions of immature, mature, and cross-linked (X-link) forms of CFTR are indicated.
that the presence of processing mutations disrupted TMD1-TMD2 (41) and NBD1-NBD2 (42) interactions. In the present study, we report that processing mutations can also disrupt NBD1-TMD2 and NBD2-TMD1 interactions. Processing mutations near the NH2- (G268V) or COOH-end (L1260A) of the molecule disrupted cross-linking between Cys-443(NBD1) and Cys-909(TMD2). NBD1-TMD2 interactions were restored when the processing mutants were expressed in the presence of the drug substrate cyclosporin A. Similar findings were made for TMD1-NBD2 interactions. Studies on the L1260A/A266C(TMD1)/F1086C(NBD2) mutant showed that only the mature form of the protein could undergo cross-linking (Fig. 6B). It appears that individual domains of ABC transporters can fold properly. This is expected because the crystal structure of NBD1 of CFTR expressed as a separate polypeptide (43) was found to be similar to the NBDs in the ABC transporter BtuCD (15). It appears that the individual domains of eukaryotic ABC transporters undergo cotranslational folding (44) and that establishment of domain-domain contacts may take place after synthesis. Indeed, it has been demonstrated that half-molecule forms of P-gp can be synthesized as separate polypeptides but they will associate after synthesis to form a functional complex (45). Introduction of a processing mutation into a P-gp half-molecule inhibited interactions between the half-molecules (37). These interactions however, are restored upon co-expression of the two halves of P-gp in the presence of a drug substrate. Establishment of native NBD-TMD contacts may contribute to interactions between the two halves of P-gp as shown in Fig. 8. The presence of a processing mutation such as L1260A traps P-gp as immature protein with incomplete NBD-TMD contacts (Fig. 8, left panel). Binding of drug substrate at the interface between the TMDs induces P-gp to complete the folding process to promote TMD1-NBD2 and NBD1-TMD2 interactions to yield a native structure (Fig. 8, right panel).

Establishment of native NBD-TMD interactions after synthesis also plays a critical role in forming a functional complex because immature P-gp is inactive (18). NBD-TMD contacts appear to be critical for function because cross-linking of mutants L443C(NBD1)/S909C(TMD2) (Fig. 3) or introduction of the F1086C mutation at the TMD1/NBD2 interface inhibited activity. The TMD-NBD interfaces appear to be critical for cross-talk between the ATP- and drug-binding sites as vandate trapping of nucleotide inhibited cross-linking of mutants containing cysteines at the NBD1-TMD2 (L443C(NBD1)/S909C(TMD2)) and TMD1-NBD2 (A266C(TMD1)/F1086C(NBD2)) interfaces (Figs. 4B and 6).

The observation that NBD-TMD interactions are important for folding and activity has recently been reported for CFTR (46). It was found cysteines introduced into NBD1 could be cross-linked to cysteines introduced into the fourth intracellular loop of TMD2 using cross-linkers of various lengths. Cross-linking of NBD1 to TMD2 also inhibited CFTR chloride channel activity. The Phe-508 position was shown to be close to the NBD1-TMD2 interface as a cysteine introduced at position 508 could be cross-linked to cysteines introduced at positions 1065, 1068, 1069, and 1074 in the fourth intracellular loop of TMD2. The mature and immature forms of CFTR were also found to be structurally different as only the mature form of CFTR showed cross-linking between NBD1 and TMD2.

Further cross-linking analysis of CFTR also showed that the NBDs made multiple contacts with the cytoplasmic loops of both TMDs. Cross-linking analysis provided evidence for TMD1-NBD2, TMD1-NBD1, and TMD2-NBD2 interactions (47). It was found that cross-linking between NBDs and TMDs in opposite halves of CFTR rapidly inhibited channel gating whereas cross-linking between NBDs and TMDs in the same halves had a lesser impact on activity.

Mapping of NBD1-TMD2 contacts by cross-linking analysis was also recently reported for the yeast Yor1p ABC transporter that has similar domains to CFTR (48). It was found that the structure of Yor1p also resembled the structure of Sav1866 as the cysteines introduced into the second intracellular loop of the TMDs could be cross-linked to cysteines introduced into the opposite NBD. As observed with P-gp (this study), it was found that processing mutations in Yor1p inhibited NBD1-TMD2 interactions. The native NBD1-TMD2 interactions could be restored when suppressor mutations were introduced into the mutant to promote maturation. Therefore, different ABC transporters appear to share many similarities in structure and folding pathways.

Acknowledgments—We thank the Cystic Fibrosis Foundation Therapeutics, Inc. and Dr. Robert Bridges (Rosalind Franklin University, Chicago, IL) for providing corr-4a.

REFERENCES
1. Ambudkar, S. V., Dey, S., Hrycyna, C. A., Ramachandra, M., Pastan, I., and Gottesman, M. M. (1999) Annu. Rev. Pharmacol. Toxicol. 39, 361–398
2. Schinkel, A. H., Smit, J. I., van Tellingen, O., Beijnen, J. H., Warnaar, E. van Deemter, L., Mol, C. A., van der Valk, M. A., Rohanus-Maandag, E. C., te Riele, H. P., Berns, A. J. M., and Borst, P. (1994) Cell 77, 491–502
3. Thiebaut, F., Tsuuru, T., Hamada, H., Gottesman, M. M., Pastan, I., and Willingham, M. C. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7735–7738
4. Lee, C. G., Gottesman, M. M., Cardarelli, C. O., Ramachandra, M., Jeang, K. T., Ambudkar, S. V., Pastan, I., and Dey, S. (1998) Biochemistry 37, 3594–3601
5. Chen, C. J., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M., and Roninson, I. B. (1986) Cell 47, 381–389
Effect of Processing Mutations on P-gp and CFTR NBD-TMD Interactions

OCTOBER 17, 2008 • VOLUME 283 • NUMBER 42 • JOURNAL OF BIOLOGICAL CHEMISTRY 28197