The Dileucine Motif at the COOH Terminus of Human Multidrug Resistance P-glycoprotein Is Important for Folding but Not Activity*

Received for publication, October 8, 2004, and in revised form, November 12, 2004 Published, JBC Papers in Press, November 12, 2004 DOI 10.1074/jbc.M411483200

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P-glycoprotein (P-gp, ABCB1) actively transports a broad range of cytotoxic compounds out of the cell. The COOH terminus of P-gp contains a dileucine motif (Leu1260-Leu1261) and a conserved phenylalanine (Phe1268). Similar residues in SUR1 (ABCC8) were reported to be important plasma membrane-targeting signals (Sharma, N., Crane, A., Clement, J. P. t., Gonzalez, G., Babenko, A. P., Bryan, J., and Aguilar-Bryan, L. (1999) J. Biol. Chem. 274, 20628–20632). Here, we used alanine-scanning mutagenesis to test whether these residues were essential for trafficking of P-gp to the cell surface. Mutant L1260A expressed a 150-kDa immature protein that did not reach the cell surface and was sensitive to digestion by Endo H. By contrast, mutants L1261A, F1268A, and wild-type P-gps expressed the 170-kDa mature proteins at the cell surface. Mutation of Leu1260 to Gly, Ile, Trp, Lys, or Glu also resulted in the expression of the 150-kDa immature protein. All of the mutants, however, expressed the 170-kDa protein in the presence of the drug substrate/specific chemical chaperone cyclosporin A. Mutant L1260A P-gp exhibited drug-stimulated ATPase activities similar to that of wild-type enzyme after rescue with cyclosporin A. Deletion of the last 22 amino acids (Q1259-Q1280) also caused misprocessing. The mutant, however, was rescued by expression in the presence of cyclosporin A and conferred resistance to colchicine in transfected cells. These results show that the dileucine motif is not a plasma membrane targeting signal. The COOH terminus is required for proper folding of P-gp but not for activity.

There are many clinical problems associated with over- or underexpression of ATP-binding cassette (ABC) transporters at the cell surface. Overexpression of ABC-type multidrug transporters such as the multidrug resistance P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP1), or the breast cancer resistance protein interferes with drug delivery to target organs and/or contribute to the development of multidrug resistance during chemotherapy (1).

Underexpression due to defective folding and trafficking to the cell surface of mutant ABC transporters such as the cystic fibrosis conductance regulator (CFTR) and the sulfonylurea receptor (SUR1) cause cystic fibrosis (2, 3) and persistent hyperinsulinemic hypoglycemia (4, 5), respectively. Therefore, understanding the mechanism of folding and trafficking of these proteins would contribute to the development of novel treatment strategies.

P-gp is an ideal model system for studying processes involved in folding and trafficking of ABC transporters, because these processes can be manipulated by specific chemical chaperones. P-gp is a typical ABC transporter. Its 1280 amino acids are organized in two repeating halves of 610 amino acids that are joined by a linker region of about 60 amino acids (6). Each half has a transmembrane domain (TMD) containing six transmembrane (TM) segments and a hydrophilic domain containing an ATP-binding site (7, 8). The minimum functional unit is a monomer (9), but the two halves of the molecule do not have to be covalently linked for function (10, 11). Drug substrates within the lipid bilayer (12) diffuse into the common drug-binding pocket formed at the interface between TMD1 and TMD2 (13–15). Then, hydrolysis of ATP at the nucleotide binding domains (NBDs) via an alternating mechanism (16) leads to extrusion of drug substrate into the extracellular medium.

Mutations located throughout P-gp can cause protein misfolding such that the mutant proteins are retained in the endoplasmic reticulum in inactive conformations (17, 18) in association with molecular chaperones such as calnexin and Hsc 70 (19, 20). Expression of misprocessed P-gp mutants in the presence of a drug substrate, however, promotes maturation of the protein so that the protein is trafficked to the cell surface in an active form (21). Drug substrates appear to rescue misprocessed mutants by promoting domain-domain interactions (22, 23).

The COOH-terminal end appears to be important for trafficking of CFTR (24) and SURI (25). Mutation of the COOH-terminal dileucine motif of CFTR increased the amount of CFTR at the cell surface (26). In SURI, mutational analysis of the COOH-terminal residues indicated that the dileucine motif and a downstream Phe residue formed important plasma membrane-targeting signals (25). In this study, we tested whether the dileucine motif in the COOH-terminal end of P-gp was essential for trafficking of P-gp to the cell surface.

* This work was supported by grants from the National Cancer Institute of Canada through the Canadian Cancer Society and from the Canadian Institutes for Health Research. 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.

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† The abbreviations used are: ABC, ATP-binding cassette; P-gp, P-glycoprotein; NBD, nucleotide-binding domain; NBD1, NH2-terminal NBD; NBD2, COOH-terminal NBD; TM, transmembrane; TMD, transmembrane domain; HEK, human embryonic kidney; MRP1, multidrug resistance-associated protein; CFTR, cystic fibrosis conductance regulator; SUR1, sulfonylurea receptor; PNGase F, peptide N-glycosidase F; PBS, phosphate-buffered saline; ER, endoplasmic reticulum; PHHI, persistent hyperinsulinemic hypoglycemia of infancy.
A sample was subjected to immunoblot analysis with rabbit polyclonal andase Hf, a 100-Endo Hf (1000 units, New England Biolabs) was incubated for 15 min at incubated with 0.1 mM sodium acetate buffer, pH 5.5, containing 2 mM the dark, the cells were washed four times with PBS buffer and then

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... such as human P-gp, human MDR3, hamster P-gp1, mouse MDR1, mouse MDR3, human CFTR, hamster SUR1, and human MRP1 are aligned. The residues at the COOH-terminal end of ABC proteins such as human P-gp, human MDR3, hamster P-gp1, mouse MDR1, mouse MDR3, human CFTR, hamster SUR1, and human MRP1 are aligned. The positions of the residues in the molecule are numbered. The dileucine motifs and the downstream Tyr residue in P-gp are in bold and are enclosed in boxes.

MATERIALS AND METHODS

Construction of Mutants—Wild-type P-gp cDNA was inserted into the mammalian expression vector pMT21 (27). The wild-type P-gp cDNA was also modified to encode for a 10-histidine tag (28) or to encode for the epitope for monoclonal antibody A52 (27). Mutations were introduced into P-gp as described previously (27). Deletion muta
tants were constructed by inserting stop codons at residues Thr1277, Val1277, or Glu1277 resulting in deletion of the last 4, 8, and 22 residues, respectively. The mutations were verified by sequencing the fragment used for mutagenesis by the dideoxynucleotide chain termination method (29).

Drug Rescue of Processing Mutations—Wild-type or mutant P-gp cDNAs were transfected into HEK293 cells. After 24 h, the medium was replaced with fresh medium containing no drug or 10 μM cyclosporin A. The cells were then incubated for another 24 h, harvested, and whole cell SDS extracts were subjected to SDS-PAGE and immunoblot analysis with rabbit polyclonal antibody against P-gp (20) followed by enhanced chemiluminescence (Pierce).

Digestion with Endoglycosidase Hf and PNGase F—Cells expressing wild-type or mutant P-gp were suspended in PBS and solubilized by addition of 0.1 volume of denaturation buffer (5% (v/v) SDS, 10% (v/v) 2-mercaptoethanol, and 500 mM EDTA). For digestion with endoglycosidase Hf, a 100-μl reaction mixture containing 10 μl of 0.5 μM sodium citrate buffer, pH 5.5, 79 μl of denatured cell extract, 10 μl of water, and 1 μl of Endo Hf (1000 units, New England Biolabs) was incubated for 15 min at 21 °C. An equal volume of 2× SDS sample buffer was then added, and the sample was subjected to immunoblot analysis with rabbit polyclonal antibody against P-gp followed by enhanced chemiluminescence.

For digestion with PNGase F, a 100-μl reaction mixture containing 10 μl of 0.5 μM phosphate buffer, pH 7.5, 10 μl of (v/v) PNGase F, 0.4 μl of water, and 1 μl of PNGase F (500 units, New England Biolabs) was incubated for 15 min at 37 °C. An equal volume of 2× SDS sample buffer was then added, and the sample was subjected to immunoblot analysis.

Cell Surface Labeling—Cells expressing A52-tagged wild-type or mutant P-gp and grown with or without cyclosporin A were suspended in PBS buffer, pH 7.4, containing 10 mM NaIO4. After 30 min at 21 °C in the dark, the cells were washed five times with PBS and then solubilized with PBS buffer containing 1% (w/v) n-dodecyl-β-o-maltoside. Insoluble material was removed by centrifugation at 14,000 × g for 10 min. The supernatant was transferred to a fresh tube, and P-gp-A52 was immunoprecipitated with monoclonal antibody A52 and Protein A-Sepharose CL-4B beads. The immunoprecipitates were washed 5 times with PBS buffer containing 0.1% (w/v) n-dodecyl-β-o-maltoside. SDS sample buffer was then added to the beads, and the supernatant was subjected to SDS-PAGE and Western blot analysis with horseradish peroxidase conjugated to streptavidin followed by enhanced chemiluminescence.

Isolation of Histidine-tagged P-gp and Measurement of Drug-stimu
lated ATPase Activity—Briefly, 50 (10-cm diameter) plates of HEK293 cells were transfected with wild-type or mutant P-gp cDNA. After 24 h, the medium was replaced with fresh medium containing 10 μM cyclosporin A. After another 24 h at 37 °C, the cells were harvested and solubilized with buffer containing 1% (w/v) n-dodecyl-β-o-maltoside. Insoluble material was removed by centrifugation at 14,000 × g for 20 min, and histidine-tagged P-gp was isolated by nickel-chelate chromatography (28). The isolated P-gps were mixed with an equal volume of 10 mg/ml crude sheep brain lipid (Sigma Type II) and sonicated. Ali
quots of the P-gp-lipid mixture were assayed for drug-stimulated ATPase activity by addition of an equal volume of buffer containing 100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM MgCl2, 10 mM ATP and various concentrations of drug substrates: vinblastine (0–0.4 mM) or verapamil (0–2 mM). The samples were incubated for 30 min at 37 °C, and the amount of inorganic phosphate liberated was determined (30).

Measurement of Drug Resistance—Cell lines stably expressing wild-
type or mutant P-gps were generated using the Flp-In system (Invitro
gen, Canada) as described previously (31). The cells were first grown for 24 h in the presence or absence of 10 μM cyclosporin A. The cells were then washed twice with PBS to remove cyclosporin A and then incubated for 3 days at 37 °C in the presence of various concentrations (0–4000 nM) colchicine. The concentration of colchicine that inhibited growth by 80% (D50) was determined by using the 3-(4,5-dimethylthia
zol-2-yl)-2,5-diphenyl tetrazolium bromide assay (32).

RESULTS

The SUR1 receptor forms a complex with the Kir potassium channel at the cell surface of pancreatic β cells to generate an ATP-sensitive potassium (KATP) channel (25). SUR1 is similar to P-gp in that it has two nucleotide-binding domains (NBDs) and six transmembrane segments (TMs) in each of the two transmembrane domains (TMDs). It also contains an additional NH2-terminal domain of consisting of five TMs. It was postulated that trafficking of SUR1 to the cell surface depends on the presence of a dileucine motif and a down
stream Phe at the COOH terminus of the protein. Mutation of the first Leu residue, the downstream Phe residue, or deletion of seven or more residues at the COOH terminus resulted in retention of the mutant SUR1 within the cell (25). Therefore, these residues were postulated to form the plasma membrane-targeting signals.

Comparison of the COOH-terminal sequences of P-gp and other ABC proteins such as mouse and hamster P-gps, human MDR3, human CFTR, hamster SUR1, or human MRP1 (Fig. 1) shows the presence of a conserved dileucine motif (Leu1260, Leu1261) that is often followed by a downstream phenylalanine (Phe1268). Other residues in the COOH-terminal region that are highly conserved are Lys1264 and Tyr1267 (Phc1268). Accordingly, we tested whether residues Leu1260, Leu1261, Lys1264, Tyr1267, and Phe1268 are important for folding and trafficking of P-gp to the cell surface. The codons for residues Leu1260, Leu1261, Lys1264, Tyr1267, and Phe1268 in the wild-type P-gp cDNA were each mutated to alanine. The mutant cDNAs were then expressed in HEK293 cells.
cells, and then samples of whole cell SDS extracts were subjected to immunoblot analysis. P-gp contains three glycosylation sites at residues Asn91, Asn94, and Asn99 (33). Mutant P-gps that are retained in the endoplasmic reticulum (ER) are only core-glycosylated and migrate on SDS-PAGE gels with an apparent mass of 150 kDa. The carbohydrate on P-gp are modified when the protein passes through the Golgi, and as a result the protein migrates with an apparent molecular mass of 170 kDa in SDS-PAGE gels (19). Fig. 2A shows that the major product in mutants L1260A or Y1267A was the 150-kDa protein. By contrast, the major product in wild-type P-gp and in mutants L1261A, K1264A, and F1268A was the 170-kDa protein.

We then tested whether the difference between the 150- and 170-kDa proteins was due to glycosylation and not proteolysis. The mutants were digested with Endo H and PNGase F. P-gp that is retained in the ER is only core-glycosylated and is sensitive to digestion with Endo H. Mature 170-kDa P-gp, however, contains complex carbohydrates and is resistant to digestion with Endo H. Fig. 2B shows that treatment of wild-type or mutant L1260A P-gps with Endo H resulted in the disappearance of the 150-kDa protein and the appearance of a 140-kDa protein (Fig. 2B). By contrast, the 170-kDa mature protein of wild-type P-gp was resistant to digestion with Endo H. The 170- and 150-kDa proteins were both sensitive to digestion by PNGase F. Similar results were observed with mutant Y1267A (data not shown).

We then determined whether mutations to Leu1260 or Tyr1267 inhibited maturation of P-gp, because they were membrane-targeting signals or because they inhibited folding of the protein. To distinguish between these two possibilities, the mutants were subjected to a “drug-rescue” assay. We previously showed that mutants defective in folding could be rescued by carrying out expression in the presence of a drug substrate or modulator of P-gp. The drug substrate or modulator acts as a specific chemical chaperone to correct the folding defect (21, 34). Mutations that cause the P-gp to be misprocessed and retained in the ER appear to trap the protein in a pre-folded state where there are incomplete or abnormal domain-domain interactions (22, 23). Expression of the misprocessed mutants in the presence of the drug substrate/modulator, however, allows the protein to overcome the thermodynamic hurdle for folding by promoting interactions between the TMDs. As a result, the misprocessed mutants mature and escape the quality control mechanism in the ER and are trafficked to the cell surface as an active protein (21). If a mutation disrupts a membrane-targeting signal, then maturation should still be inhibited even after the folding defect is corrected by the presence of a chemical chaperone. Accordingly, wild-type P-gp and mutants L1260A, L1261A, K1264A, Y1267A, and F1268A were expressed in HEK293 cells in the presence or absence of the chemical chaperone/drug substrate cyclosporin A. Whole cell SDS extracts were then subjected to immunoblot analysis. Fig. 2A shows that the cyclosporin A was able to rescue mutants L1260A and Y1267A, because a 170-kDa protein was then detected (Fig. 2A). These results show that mutation of Leu1260 and Tyr1267 affected folding and were not essential plasma membrane-targeting signals.

We then tested the effects of other amino acid substitutions at positions Leu1260 and Tyr1267. Residue Leu1260 was mutated to Gly, Ile, Trp, Lys, or Glu, whereas residue Tyr1267 was mutated to Leu, Trp, Phe, or Lys. The mutants were expressed in HEK293 cells in the presence or absence of cyclosporin A and whole cell extracts were subjected to immunoblot analysis. Fig. 2C shows that residue Leu1260 was very sensitive to change, because replacement with Gly, Ile, Trp, Lys, or Glu, inhibited maturation of P-gp. The major product in these mutants in SDS-PAGE gels was the 150-kDa protein. Maturation of all of the mutants, however, was restored in the presence of cyclosporin A. Residue Tyr1267 was more tolerant to change. Mutants Y1267L, Y1267W, and Y1267F were still able to mature, because the major product was
the 170-kDa protein (Fig. 2C). Mutant Y1267K, however, showed inhibited maturation that could be overcome by expression in the presence of cyclosporin A.

We then determined whether the 170-kDa rescued mutant proteins were actually at the cell surface. For cell surface labeling, the A52-epitope-tagged wild-type and mutant L1260A P-gps were used. The presence of the epitope tag allowed us to distinguish the mutant proteins from any endogenous P-gp present in HEK293 cells. Accordingly, HEK293 cells expressing A52-tagged wild-type or mutant L1260A were grown for 24 h in the presence or absence of 10 \mu M cyclosporin A. Whole cell SDS extracts were then subjected to immunoblot analysis with monoclonal antibody A52. Fig. 3A shows that the major products of A52-tagged mutant L1260A P-gp were the 150- and 170-kDa proteins when expression was carried out in the absence or presence of cyclosporin A, respectively.

Because the A52-tagged P-gps have the same maturation characteristics as the untagged proteins (Fig. 2A), they could be used for cell surface labeling studies. Cells expressing the A52-tagged wild-type and mutant P-gps and grown in the absence or presence of cyclosporin A were treated with sodium periodate to oxidize the carbohydrate groups on the protein and then reacted with biotin-X-hydrazide. The cells were then washed and solubilized with n-dodecyl-\(\beta\)-D-maltoside. Insoluble material was removed by centrifugation and A52-tagged P-gps immunoprecipitated with monoclonal antibody A52. The immunoprecipitated proteins were then subjected to Western blot analysis with horseradish peroxidase conjugated to streptavidin. Fig. 3B shows that the 170-kDa protein was labeled in the wild-type P-gp grown in the absence or presence of cyclosporin A. By contrast, the 170-kDa protein was labeled in mutant L1260A only when grown in the presence of cyclosporin A. Labeling of the 150-kDa protein was not detected when mutant L1260A was grown without cyclosporin A. These results indicate that the rescued mutant is present at the cell surface.

Because residue Leu\(^{1260}\) was very sensitive to replacement with other amino acids, we determined whether mutant L1260A retained drug-stimulated ATPase activity when at the cell surface. The drug- and ATP-binding sites in P-gp are coupled such that occupation of the drug-binding site located in the TMDs (14, 15, 35) stimulates ATPase activity up to 20-fold (36). To determine drug-stimulated ATPase activity, we attached a histidine tag at the COOH-end of the wild-type and mutant L1260A P-gps. The presence of the histidine tag did not affect the maturation characteristics of mutant L1260A. When histidine-tagged mutant L1260A was expressed in the absence or presence of cyclosporin A, the major products in SDS-PAGE were the 150- and 170-kDa proteins, respectively (data not shown). Accordingly, histidine-tagged wild-type and mutant were grown in HEK293 cells in the presence of cyclosporin A and then isolated by nickel-chelate chromatography. Equivalent amounts of the isolated proteins were mixed with lipid and assayed for drug-stimulated ATPase activity. Fig. 4 shows that mutant L1260A retained more than 80% of the verapamil- or vinblastine-stimulated ATPase activity of wild-type P-gp. Half-maximal activation of wild-type and rescued mutant L1260A occurred at \(\sim 30\ \mu M\) verapamil. In the presence of vinblastine, however, both wild-type and rescued mutant L1260A showed half-maximal activity at 6 \(\mu M\) vinblastine. Therefore, residue Leu\(^{1260}\) was not essential for coupling of drug binding to ATPase activity.

To determine whether any segment of the COOH-terminal end of P-gp was essential for trafficking to the cell surface, we constructed a series of deletion mutants that lacked the last 4 (4), 8 (\(\Delta 8\)), and 22 (\(\Delta 22\)) amino acids by inserting stop codons at residues Thr\(^{1277}\), Val\(^{1278}\), or Gln\(^{1259}\). The wild-type and deletion mutants were transiently expressed in HEK293 cells in the absence or presence of 10 \mu M cyclosporin A. Samples of whole cell SDS extracts were subjected to immunoblot analysis with rabbit polyclonal antibody against P-gp. When the deletion mutants \(\Delta 4\), \(\Delta 8\), and \(\Delta 22\) were expressed in the absence of cyclosporin A, the major product in mutants \(\Delta 4\) and \(\Delta 8\) was \(\sim 170\) kDa, whereas the major product in mutant \(\Delta 22\) was the 150-kDa protein (Fig. 5). Deletion of the last 4 or 8 amino acids did not appear to affect maturation of the proteins. The maturation of mutant \(\Delta 22\), however, was restored after expression in the presence of cyclosporin A (Fig. 5). These results show that the last 22 amino acid (Gln\(^{1259}\), Gln\(^{1260}\)) of P-gp were not essential for maturation when expression was carried out in the presence of cyclosporin A.

We then determined whether the last 22 amino acids were important for activity. Because deletion of the COOH-terminal...
end of P-gp removed the histidine tag, isolation of histidine-tagged P-gp for measurement of drug-stimulated ATPase activity was not possible. An alternative method for measuring activity is to assay for the ability of the mutant protein to confer resistance in transfected cells. Accordingly, we generated stable cell lines. Untagged wild-type and mutant Δ22 P-gp were subcloned into the pcDNA5/FRT vector and cotransfected with the Flp recombinase expression pOG 44 into Flp-In-293 cells (Invitrogen) that contained a single Flp recombinase target (37). The transfected cells were then selected on hygromycin B. Resistant colonies were grown and expression of P-gp confirmed by immunoblot analysis (data not shown).

Flp-In 293 cells expressing wild-type or mutant Δ22 P-gp were incubated overnight in the absence or presence of 10 μM cyclosporin A. The cells were then washed twice to remove cyclosporin A and then incubated with various concentrations of the cytotoxic P-gp substrate colchicine. The concentration of drug that caused 80% cell death (D_{50}) was then determined. Wild-type P-gp conferred a 22-fold (D_{50} of 243 nM) increase in resistance to colchicine relative to that of control cells (D_{50} of 11 nM) (Fig. 6). Exposure of the cells to cyclosporin A did not significantly alter the resistance conferred by wild-type P-gp. By contrast, mutant Δ22 conferred little resistance when it was not rescued with cyclosporin A. The concentrations of colchicine used in these experiments (up to 4 μM) were too low to rescue misprocessed P-gp mutants.2 When mutant Δ22 was first rescued by carrying out expression in the presence of cyclosporin A, it subsequently conferred a 10-fold increase in resistance to colchicine (D_{50} of 110 mM) when compared with control cells. It would be expected that the rescued mutant would confer less resistance than wild-type P-gp (10-versus 22-fold), because the level of mature transporter at the cell surface would decrease over time in mutant Δ22 after removal of the chemical chaperone. The wild-type P-gp does not have any folding defect, so it would continue to be synthesized and transported to the cell surface and therefore would be expected to show increased relative resistance. These results show that the last 22 amino acids are not essential for conferring drug resistance.

**DISCUSSION**

When the COOH-terminal mutants of P-gp were expressed in the absence of cyclosporin A, their maturation properties were very similar to those reported for the SUR1 COOH-terminal mutants (25). In P-gp, maturation of the protein was not significantly decreased when the last 4 or 8 amino acids were deleted, whereas mature 170-kDa protein was not detected when the last 22 amino acids were deleted. In SUR1 (25), there was substantial cell surface expression or channel activity in mutants lacking the last 2, 4, or 7 residues and little channel activity or maturation in mutants lacking the last 13 or more amino acids. The effects of point mutations in the COOH-terminal end of P-gp and SUR1 also showed many similarities. In both P-gp and SUR1, only mutation of the first Leu in the dileucine motif (Leu1566 in SUR1; Leu1267 in P-gp) as well as a downstream Phe/Tyr residue (Phe1574 in SUR1; Tyr1267 in P-gp) had significant effects on maturation of the protein. Therefore, Sharma et al. (25) concluded that, in SUR1, Leu1566 and Phe1574 were essential plasma membrane-targeting signals.

Although our results with the P-gp COOH-terminal mutants show similar results to that found in SUR1, we have been able to differentiate whether the dileucine motif and the downstream Tyr residue in the COOH-terminal of P-gp are bona fide plasma-membrane targeting signals or whether they contribute to folding. This is because of the ability of drug substrates to act as specific chemical chaperones for rescuing misfolded mutants. For example, the dileucine motif mutant L1296DA and the deletion mutant Δ22 in which all putative plasma-membrane targeting signals were deleted could be rescued when

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2 T. W. Loo, M. Claire Bartlett, and D. M. Clarke, unpublished observations.
expressed in the presence of cyclosporin A (Figs. 2A and 5). Apparently, cyclosporin A diffused into the cell and reached the ER where it bound to the drug-binding site that exists transiently in the misfolded protein and acts as a scaffold to induce proper folding of the mutant protein (34). Therefore, the COOH-terminal of P-gp appears to contribute to folding of the molecule but was not essential for trafficking to the cell surface. It is still possible that the dileucine motif could act as an apical membrane-targeting signal. In this study, HEK293 cells were grown under nonpolarized conditions.

The crystal structures of prokaryotic ABC transporters such as MsBA (38) or BtuCD (39) suggest that the COOH-terminal end of the molecule is far away from the drug-binding domain (located in the membrane). Therefore, the question remains as to how a drug substrate can correct a defect that is quite far from the drug-binding site. One possibility is that a mutation in the COOH-terminal end of P-gp interferes with the establishment of correct domain-domain contacts during the folding process in the ER. We have previously shown that processing mutations that are relatively far away from the drug-binding pocket can disrupt interactions between the TMDs (22) and the NBDs (23). In a model of the effect of processing mutations on P-gp folding, we proposed that the processing mutation causes the protein to pause at an early folding step before all domain-domain contacts have been completed (23). Disruption of any domain-domain interactions between the TMDs, NBDs, and TMDs and NBDs would result in protein misfolding and retention of the misfolded protein in the ER. This may indeed be the case as gleaned from the crystal structure of BtuCD (39). When the sequences of P-gp and BtuCD are aligned, the crystal structure of BtuCD predicts that the COOH-terminal of P-gp would be in contact with NBD1 at the NBD1/NBD2 interface (Fig. 7). Deletion of the last 22 amino acids (∆22) or mutation of Leu1260 may alter interactions between NBD1 and NBD2 so that folding is less efficient than in wild-type enzyme. The crystal structure of BtuCD shows that the COOH-terminal end could potentially be involved in making an important contact point between the NBDs (39).

Although deleting the last 22 amino acids results in incomplete protein folding, the mutant protein can function once it has been induced to mature. This is not unexpected, because in some prokaryotic ABC proteins, the NBDs are still functional and bind ATP with relatively high affinity even though they lack the COOH-terminal region corresponding to the last 40 amino acids of P-gp (40, 41). The maturation characteristics of mutant ∆22 may also explain why Currier et al. (42) obtained very few drug-resistant NIH 3T3 cells by direct selection on colchicine when the cells were transfected with a P-gp mutant lacking the last 23 amino acids. The deletion mutant was likely misfolded and not at the cell surface.

A recent analysis of the COOH-terminal end of MRP1 showed some differences when compared with P-gp (43). Trafficking of MRP1 to the cell surface was not affected by deletion of the last 30 amino acids. Instead removal of as few as the last 4 amino acids decreased transport activity by 75%. Deletion of the last 22 amino acids reduced activity by almost 90%. Remarkably, replacement of the last 22 amino acids of MRP1 with the last 26 amino acids of P-gp yielded a chimeric protein with maturation and functional properties similar to that of wild-type MRP1, even though 18 of the 22 amino acids being replaced were different.

The COOH terminus of CFTR, however, does not appear to be required for activity or trafficking to the cell surface. A mutant lacking the last 60 amino acids showed trafficking and activity similar to wild-type CFTR (44). The major function of the COOH-terminal region of CFTR may be to modulate its interaction with other proteins (24, 45–47), mediate endocytic recycling (48), and control its mobility at the cell surface (49). Many of these functions are thought to be mediated by the PD2 binding motif of CFTR (last three amino acids, Thr-Arg-Leu), although the dileucine motif (Leu1430–Leu1431) appears to be involved in internalization. Mutation of the dileucine motif increased the amount of CFTR at the cell surface (26).

In SUR1, the importance of the COOH-terminal region was first noted in a clinical study of a patient with persistent hyperinsulinemic hypoglycemia of infancy (PHHI) that did not respond to diazoxide (4). PHHI is usually caused by mutations in the KATP channel resulting in persistent insulin secretion, because the beta cell membrane potential remains depolarized. Diazoxide can be used to treat patients with mild forms of PHHI, because it increases the open probability of the mutant KATP channels and allows inhibition of insulin release. The patient in the study by Dunne et al. (4) required a partial pancreatectomy, because there was no response to clinical treatment. This suggested that there was a lack of KATP channels in the plasma membrane. The SUR1 protein in this patient was then shown to have a deletion of 221 residues at the COOH-end of the molecule (4). The COOH terminus of SUR1 was subsequently shown to be required for trafficking of KATP channels to the plasma membrane (25). Recently, it was reported that diazoxide (50) and sulfonylurea (51) could restore cell surface expression of mutant SUR1 proteins that were defective in trafficking due to point mutations. Therefore, the ability of these compounds to act as chemical chaperones in correcting SUR1 trafficking defects indicates that SUR1 would also be a useful model system for studying trafficking of ABC proteins. The chaperone effect may explain the variable responses during clinical treatment of PHHI. Depending on the mutation, the chemical chaperone can have dual effects. It can increase the open probability of the channel and increase the number of channels in the plasma membrane.

The COOH-terminal region appears to have different roles in the various ABC transporters. It is important for trafficking of SUR1, for activity in MRP1, and for protein-protein interactions in CFTR.

In summary, the dileucine motif at the COOH-terminal end of P-gp is not a plasma membrane-targeting signal. It is not required for activity but plays an important role in protein folding.
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