Superfolding of the Partially Unfolded Core-glycosylated Intermediate of Human P-glycoprotein into the Mature Enzyme Is Promoted by Substrate-induced Transmembrane Domain Interactions*

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Misprocessed mutants of human P-glycoprotein accumulate as core-glycosylated intermediates in the endoplasmic reticulum and are rapidly degraded. Trypsin digestion was used to test for structural differences between mature and core-glycosylated forms of P-glycoprotein. We found that the core-glycosylated wild-type and mutant P-glycoproteins were both 100-fold more sensitive to trypsin compared with the mature form of the wild-type enzyme. This result suggested that the core-glycosylated forms of both wild-type and mutant P-glycoproteins have similar unfolded structures, whereas the mature enzyme is folded into a more compact structure. The core-glycosylated mutant P-glycoproteins could be converted to the mature trypsin-resistant form by synthesis in the presence of drug substrate. Addition of proteasome inhibitor MG-132 to stabilize the core-glycosylated intermediate resulted in the accumulation but not maturation of the mutant protein. Further analysis showed that the second transmembrane domain TMD2 also became more resistant to trypsin digestion only after coexpression with TMD1 in the presence of substrate. Taken together, these results suggest that simply stabilizing the core-glycosylated intermediate is not sufficient to promote maturation of the processing mutants and that drug substrates induce maturation by promoting superfolding of the transmembrane domains.

P-glycoprotein, the product of the human multidrug resistance (MDR1) gene, is an ATP-dependent drug pump that extrudes a broad range of cytotoxic agents from the cell (reviewed in Ref. 1). Its physiological role is probably to protect the organism from endogenous and exogenous cytotoxic compounds (2, 3). The protein plays an important role in the phenomenon of multidrug resistance during cancer chemotherapy (4).

P-glycoprotein belongs to the ABC (ATP-binding cassette) superfamily of transport proteins. Its 1280 amino acids are organized into two tandem repeats; each repeat consists of a hydrophobic domain followed by an ATP-binding domain. Both tandem repeats are required for activity (5), and inactivation of either ATP-binding site by mutagenesis or chemical modification inhibits activity (6, 7).

The composition of the primary amino sequence of P-glycoprotein is important for synthesis of the native protein, because point mutations throughout the molecule can cause misprocessing. The misprocessed mutants accumulate in the endoplasmic reticulum as core-glycosylated intermediates in association with molecular chaperones such as calnexin and Hsc 70 (8–12). These defects in processing, however, can be overcome by carrying out synthesis in the presence of drug substrates (9).

Our aim was to understand why mutations in P-glycoprotein cause misprocessing and how drug substrates induce correct folding. We used trypsin digestion of P-glycoprotein to detect structural differences in the wild-type and mutant P-glycoproteins. We show that the core-glycosylated forms of both wild-type and mutant P-glycoproteins were 100-fold more sensitive to trypsin compared with the mature enzyme. Conversion of the mutant P-glycoproteins into compact trypsin-resistant forms was mediated by drug-induced superfolding of the transmembrane domains.

The results suggest that the core-glycosylated forms of wild-type and mutant P-glycoproteins are structurally similar and in a "loosely folded" conformation, compared with the mature enzyme. It appears that the misprocessing mutations have to overcome a "hurdle" in the superfolding pathway to the native enzyme, a barrier that is lowered by synthesis in the presence of drug substrate.

EXPERIMENTAL PROCEDURES

Generation of Constructs—The cDNAs were constructed, mutated, and inserted into the mammalian expression vector pMT21 as described previously (5, 13). For purification purposes a full-length MDR1 cDNA and the cDNAs coding for the half-molecules were modified to encode for 10 histidine residues at the COOH-ends of the proteins (14).

Expression and Nickel-Chelate Chromatography of P-glycoprotein Mutants—HEK 293 cells were transfected with the mutant cDNA constructs, and the media were replaced 24 h later with fresh media containing 10 μM CsA.1 After another 24 h, the cells were harvested. For nickel-chelate chromatography, HEK 293 cells expressing the histidine-tagged proteins were first solubilized with 1% (w/v) n-dodecyl-β-D-maltoside as described previously (15).

TPCK-trypsin Digestion—HEK 293 cells were transfected as described above and grown in the presence of drug substrate for 24 h before harvesting. Membranes were prepared from transfected HEK 293 cells as described previously (14) and suspended in Tris-buffered saline. The membranes (5 mg/ml protein) were treated with 5 min at 22 °C with various amounts of TPCK-trypsin (Sigma, 12,000 BAEE units per mg), and the reaction was stopped by the addition of lima bean trypsin inhibitor (Worthington).

1 The abbreviations used are: CsA, cyclosporin A; NBD, nucleotide-binding domain; TM, transmembrane segment; TMD1, transmembrane domain (residues 1–379) encompassing transmembrane segments 1–6; TMD2, transmembrane domain (residues 681–1025) encompassing transmembrane segments 7–12; TPCk, t-1-tosyalilamido-2-phenethyl chloromethyl ketone; CFPTR, cystic fibrosis transmembrane regulator.

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Endoglycosidase Digestion and Immunological Procedures—Digestion with endoglycosidase H, or endoglycosidase F (PNGase F, New England Biolabs) was carried out as described previously (12). Immunoblot analysis with monoclonal antibody A52 or with a rabbit polyclonal antibody against P-glycoprotein followed by enhanced chemiluminescence (Amersham Pharmacia Biotech) (11).

RESULTS

During structure-function analysis of P-glycoprotein, we found that changes to 49 of 401 residues (12.2%) resulted in misprocessing of the enzyme. The major product of the misprocessed mutants expressed in HEK 293 cells is the 150-kDa core-glycosylated intermediate. Misprocessing mutations are located throughout the molecule; these include the transmembrane domains (e.g. A123L), intracellular (e.g. E243A) and extracellular (e.g. Y853C) loops, the linker region (e.g. E707A), and both nucleotide-binding domains (e.g. G427C, P1194A).

Regardless of the location of these mutations, all could be converted to the fully mature enzyme (170 kDa) when the transfected cells were incubated with CsA. We used CsA because it was the most potent compound to affect protein kinesis (9). An example is shown in Fig. 1A. When mutant G268V was expressed in HEK 293 cells (no drug, lane 1), the major product was a 150-kDa core-glycosylated protein that was sensitive to digestion with endoglycosidase H (data not shown). In the presence of 10 μM CsA (+ drug, lane 1), the major product was the fully mature enzyme (170 kDa) that was resistant to digestion with endoglycosidase H (data not shown).

We attempted to purify the core-glycosylated intermediate of mutant G268V to determine whether it was still active. A polyhistidine tag was attached at the COOH-end of the mutant P-glycoprotein to facilitate purification by nickel-chelate chromatography (14). These attempts were unsuccessful because the core-glycosylated intermediate did not bind to the resin. The inability of the core-glycosylated intermediate to bind to the resin was not due to problems with solubilizing the intermediate with detergent or with aggregation. The mutant core-glycosylated intermediate could be readily solubilized with mild detergents such as digitonin (10) and that it remained in the soluble fraction after high speed centrifugation (100,000 × g, 1 h). The mutant protein was recovered by the nickel column only after its conversion to the mature form of the enzyme in the presence of drug substrate (data not shown). All other mutants gave similar results. Similarly, the mature 170-kDa wild-type protein bound the nickel column more efficiently than the core-glycosylated 150-kDa protein (data not shown), suggesting that thecore-glycosylated and mature enzyme may be structurally different.

To determine whether the mature (170-kDa) and core-glycosylated (150-kDa) forms of P-glycoprotein were structurally different, we tested the mutant P-glycoprotein-A52 for their sensitivity to digestion by trypsin. As shown in Fig. 1A (no drug), the 150-kDa protein of mutant G268V was quite sensitive to trypsin. It was completely digested with 10 μg/ml TPCK-trypsin (lane 3). By contrast, the mutant became about 100-fold more resistant to digestion by trypsin when converted to the mature form (170-kDa) after growing in the presence of CsA. Even when synthesized in the presence of CsA, the 150-kDa protein remained sensitive to 10 μg/ml trypsin. Similar results were observed with the other mutants or when expression was detected with rabbit polyclonal antibodies against either NBD (data not shown).

The sensitivity of the wild-type enzyme was similar to the processing mutant (Fig. 1B). Again, the mature enzyme was about 100-fold more resistant to trypsin than the core-glycosylated 150-kDa protein. These results suggested that the core-glycosylated intermediate of both the wild-type and mutant enzymes were in a more “open” conformation and are likely to be structurally similar. The core-glycosylated wild-type enzyme, however, is quite efficiently converted to the mature form even in the absence of drug substrate.

It was possible that rapid degradation of the core-glycosylated intermediate did not allow it time to fold to the mature form. Therefore, if the intermediate could be stabilized in the absence of drug substrate, then conversion to the mature form should occur. To test this possibility, we expressed mutant G268V in the presence of a proteasome inhibitor (MG-132). Proteasome inhibitors such as MG-132 prevent degradation of core-glycosylated wild-type P-glycoprotein, as well as that of the cystic fibrosis conductance regulator (16, 17). As shown in Fig. 1C (lanes 3 and 4), incubation of mutant G268V for 24 h in the presence of MG-132 resulted in accumulation of the core-glycosylated intermediate and a 130-kDa digestion product. There was, however, no detectable maturation of the intermediate. Increased accumulation of the core-glycosylated intermediate and the 130-kDa digestion product was also seen with the
wild-type enzyme (lanes 1 and 2) but not in the amount of the mature (170-kDa) product. These results confirm previous findings that stabilizing the core-glycosylated intermediate does not enhance maturation (16, 17).

The proteolytic digestion experiments suggested that misprocessing mutations likely cause P-glycoprotein to remain in an unfolded state, whereas the presence of drug substrates induces superfolding into the native structure. To determine how drug substrates induce superfolding, we tested their effects on association of the homologous halves of P-glycoprotein that were expressed as separate polypeptides. The two homologous halves of P-glycoprotein can be expressed as separate polypeptides, but coupling of ATPase activity to drug-binding requires the contemporaneous expression of both halves (15). This suggested that both halves are necessary for drug binding.

To test for interactions between the homologous halves we coexpressed each half of P-glycoprotein that contained either a histidine tag (N-Half-His or C-Half-His) or the epitope for monoclonal antibody A52 (G268V N-Half-A52) at the COOH-end of the molecule and tested for association using nickel-chelate chromatography. We first introduced a processing mutation (G268V) into N-Half-His. Unlike the histidine-tagged full-length G268V P-glycoprotein, the mutant (G268V) N-Half-His could be recovered by nickel-chelate chromatography, even when grown with no CsA (Fig. 2A).

Wild-type or G268V N-Half-His molecules were then coexpressed with the C-Half-A52 molecule. We reasoned that if the two homologous halves associated, then C-Half-A52 could be recovered by nickel-chelate chromatography. As shown in Fig. 2B, C-Half-A52 was not retained on the column when expressed alone or when coexpressed with wild-type G268V N-Half-His in the absence of CsA. In contrast, CsA promoted interaction between C-Half-A52 and mutant G268V N-Half-His, such that most of the C-Half-A52 was now recovered by nickel-chelate chromatography. Similarly, interaction between wild-type N-Half-His and C-Half-A52 was also enhanced by the presence of CsA. These interactions persisted even after washing the membranes several times to remove substrate, suggesting that the interactions are due to enhanced association during biosynthesis, rather than drug-induced stabilization of the complex.

We then tested the effect of a misprocessing mutation located in the C-half of P-glycoprotein (A841L), on the ability of the two halves to associate. In this case, the epitope tag for monoclonal antibody A52 was attached to the COOH-end of the N-half molecule (N-Half-A52), and the histidine tag was attached to the COOH-end of the C-half molecule (C-Half-His). Wild-type N-Half-A52 was coexpressed in HEK 293 cells with either wild-type C-Half-His or mutant (A841L) C-Half-His, in the presence or absence of CsA. Fig. 3A shows that CsA had a dramatic effect on the association of A841L C-Half-His and wild-type N-Half-A52. No N-Half-A52 was retained by the nickel column when coexpression was carried out in the absence of drug substrate. In the presence of CsA, however, two bands (A and B) were eluted. Band B has an apparent mass of 72 kDa, and its size corresponds to the core-glycosylated form of the N-half molecule. Band A (96 kDa) appeared to be a more extensively glycosylated form of the N-half molecule, suggesting that this form of the protein had passed through the Golgi apparatus. This was confirmed by observing that both bands A and B were sensitive to digestion with PNGase F (Fig. 3B), but only band B (72 kDa) was sensitive to endoglycosidase H.

Cell surface labeling with membrane-impermeant biotin hydroxide (10) showed that the 96-kDa N-Half-A52 was processed correctly and targeted to the cell surface (data not shown). Fig. 3A also shows that coexpression of wild-type N-Half-A52 with wild-type C-Half-His in the presence of CsA resulted in about a 2-fold increase in the amount of Band A recovered by nickel-chelate chromatography. No 96-kDa (Band A) protein was observed when only the N-Half molecule was expressed in the presence or absence of drug substrate (see Fig. 2A). This suggested that the misprocessing mutations and drug substrates...
Folding of P-glycoprotein

Folding of P-glycoprotein can be expressed as a separate polypeptide (11), for drug binding (15, 18–22). Because each domain of P-glycoprotein can be expressed with monoclonal antibody A52.

It is thought that mutants are retained in the endoplasmic reticulum because they are structurally different from the wild-type enzyme. Our results, however, suggest that rather than inducing misfolding, the mutations cause accumulation of a core-glycosylated intermediate that resembles that of the wild-type enzyme.

Our results suggest that the drug substrates promote super-folding of the enzyme by initiating interactions between TMD1 and TMD2. Indeed, increased protection of TMD2 from trypsin digestion after coexpression with TMD1 occurred only after expression in the presence of drug substrate (Fig. 4). In whole cells, drug substrates did not increase the level of expression of any of the TMDs when each was expressed separately but caused about a 5–10-fold increase in the amount of TMD1 and TMD2 only when they were coexpressed. Similarly, there was no increased expression of either TMD when each was coexpressed with either NBD (data not shown).

Most of the core-glycosylated wild-type enzyme appears to exist in a partially unfolded state in the endoplasmic reticulum (Fig. 1B) and may not be functional until just before or after it has left the endoplasmic reticulum. This may be of practical value to the cell. By keeping P-glycoprotein and possibly other plasma membrane transport proteins in a partially unfolded state until the very last moment, the cell prevents transport of various substrates into the endoplasmic reticulum. This may be accomplished with the aid of chaperones such as calnexin and Hac70 (10, 11). It is possible that when the protein is ready for trafficking to the plasma membrane, conformational changes due to super-folding of the TMDs may be able to dislodge the enzyme from the chaperones.

The results also show for the first time that the TMDs alone are sufficient to mediate drug-protein interactions in P-glycoprotein. Although, it has been observed that mutations in the NBDs can affect substrate specificity of P-glycoprotein (23, 24), their presence is not required for drug binding. The requirement of both TMDs to be present for drug-induced folding is consistent with the observation that residues in the transmembrane segments from both halves of the molecule are required for drug binding (15, 18–22).

In a broader context, the results suggests that another strategy to overcome P-glycoprotein-mediated drug resistance during cancer chemotherapy could be to identify compounds that could prevent super-folding of the core-glycosylated intermediate. These compounds could mimic the effects of mutations that cause misprocessing.

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