Correction of Defective Protein Kinesis of Human P-glycoprotein Mutants by Substrates and Modulators*

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There is growing evidence that abnormal protein folding or trafficking (protein kinesis) leads to diseases. We have used P-glycoprotein as a model protein to develop strategies to overcome defects in protein kinesis. Misprocessed mutants of the human P-glycoprotein are retained in the endoplasmic reticulum as core-glycosylated biosynthetic intermediates and rapidly degraded. Synthesis of the mutant proteins in the presence of drug substrates or modulators such as capsaicin, cyclosporin, vinblastine, or verapamil, however, resulted in the appearance of a fully glycosylated and functional protein at the cell surface. These effects were dose-dependent and occurred within a few hours after the addition of substrate. The ability to facilitate processing of the misfolded mutants appeared to be independent of the cell lines used and location of the mutation. P-glycoproteins with mutations in transmembrane segments, extracellular or cytoplasmic loops, the nucleotide-binding domains, or the linker region were processed to the fully mature form in the presence of these substrates. These drug substrates or modulators acted as specific chemical chaperones for P-glycoprotein because they were ineffective on the ΔF508 mutant of cystic fibrosis transmembrane conductance regulator. Therefore, one possible strategy to prevent protein misfolding is to carry out synthesis in the presence of specific substrates or modulators of the protein.

Abnormal protein folding or trafficking is associated with a growing number of diseases (1–3). Diseases such as Alzheimer’s and prion-related diseases are characterized by the presence of high levels of insoluble protein aggregates in brain tissue. These plaques appear to be aggregates of misfolded β-amyloid protein in Alzheimer’s disease or aggregates of misfolded prion protein in the prion-associated diseases such as Creutzfeldt-Jacob disease or Scrapie (mad cow) (4, 5). In cystic fibrosis, the major defect is due to deletion of a single amino acid (ΔF508) in the cystic fibrosis transmembrane conductance regulator (CFTR)1 resulting in abnormal trafficking to the plasma membrane. The mutant CFTR protein is misfolded, retained in the endoplasmic reticulum, and rapidly degraded (6). Potential therapy for diseases involving folding and/or trafficking defects in the target protein is to prevent misfolding during protein biogenesis.

We have used the human multidrug transporter (P-glycoprotein) as a model system for studying ways to prevent protein misfolding. P-glycoprotein appears to be an excellent model system, because we have identified many misprocessed P-glycoprotein mutants. These temperature-sensitive or -insensitive mutant proteins are misfolded, retained in the endoplasmic reticulum as core-glycosylated biosynthetic intermediates in association with molecular chaperones such as calnexin and Hsp70, and rapidly degraded (7–10).

P-glycoprotein, the product of the human MDR1 gene, is an energy-dependent pump located at the plasma membrane that interacts with a wide variety of structurally diverse cytotoxic agents that do not have a common intracellular target (11). This protein has clinical importance because it may be one of several mechanisms whereby cancer cells become resistant to chemotherapy.

The protein consists of 1280 amino acids organized in two tandem repeats of 610 amino acids, joined by a linker region of 60 amino acids. Each repeat consists of an NHE-containing hydrophobic domain containing six potential transmembrane sequences followed by a hydrophilic domain containing a nucleotide-binding site. The organization of the domains is characteristic of members of the ABC superfamily of ATP-binding cassette) transporters, the best known member being CFTR.

Our goal was to develop a strategy to specifically rescue the misfolded mutants of P-glycoprotein so that they could exit the endoplasmic reticulum and reach the plasma membrane in a functional form. Nonspecific low molecular weight compounds such as glycerol (12, 13) have been shown to nonspecifically affect protein kinesis. Therefore, we wished to determine whether substrates or modulators of P-glycoprotein could act as specific chemical chaperones and have a more rapid effect on processing of misfolded proteins. We show that biosynthesis of the processing mutants in the presence of substrates or modulators of P-glycoprotein results in the relatively rapid appearance of a fully mature and functional transporter at the cell surface.

EXPERIMENTAL PROCEDURES

Generation of Constructs—Wild-type and mutant MDR1 cDNAs, modified to encode the epitope for monoclonal antibody A52 at the COOH-terminal ends of the proteins, were inserted into the mammalian expression vector pMT21 as described previously (7). Oligonucleotide-directed mutagenesis was carried out as described previously (7). For purification purposes wild-type and mutant MDR1 cDNAs were modified to encode for 10 histidine residues at the COOH ends of the proteins (14). The sequence at the COOH terminus of P-glycoprotein that would normally end as TKRQ became TKR(ΔLDPR)Q.

Expression, Purification, and Measurement of Mg2+-ATPase Activity of P-glycoprotein Mutants—HEK 293 cells were transfected with the mutant cDNA constructs. After 24 h, the medium was replaced with

1 The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; PAGE, polyacrylamide gel electrophoresis.

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FIG. 1. Expression of wild type and mutants of P-glycoprotein-A52 (A) or wild-type and mutant (ΔF508) CFTR (B) in the absence or the presence of drug substrates. 24 h after transfection of HEK 293 cells with various cDNAs, the medium was replaced with fresh medium containing various concentrations (μM) of capsaicin, cyclosporin A, verapamil, or vinblastine. After another 24 h at 37 °C, the cells were harvested and lysed with SDS sample buffer, and the cell extracts were subjected to immunoblot analysis with monoclonal antibody M3A7 (B), followed by chemiluminescence. The positions of the mature (170 kDa) and core-glycosylated (150 kDa) forms of P-glycoprotein, as well as the mature (→ C) and core-glycosylated (← C) forms of CFTR are indicated.

Results

Effect of Drug Substrates on Processing of Misfolded Mutants—The effect of substrates and modulators of P-glycoprotein on the biosynthesis of two processing mutants were initially studied; G268V in the NH2-terminal transmembrane domain (16) and ΔY490 in the NH2-terminal nucleotide-binding domain (17). Mutant G268V is a temperature-insensitive processing mutant, whereas mutant ΔY490 contains a deletion at an equivalent position to the ΔF508 mutation in CFTR. The cDNAs coding for these mutant P-glycoproteins were modified to encode for the epitope of monoclonal antibody A52. The presence of the epitope provided us with an important tool for following the synthesis of the misfolded mutants. It allowed us to distinguish the mutant protein from any endogenous P-glycoprotein. In the absence of drug substrates (Fig. 1A), the majority of the mutant P-glycoproteins had an apparent mass of 150 kDa compared with 170 kDa for the wild-type enzyme. The 170- and 150-kDa forms of the enzyme represent mature and core-glycosylated biosynthetic intermediate, respectively. In the presence of capsaicin, cyclosporin A, verapamil, or vinblastine, however, the amount of mature enzyme (170 kDa) for both mutants increased in a dose-dependent manner. The ability to “rescue” misprocessed mutants appeared to be independent of the location of the mutation. In addition to the mutants G268V and ΔY490, we were able to facilitate processing of P-glycoproteins with mutations in the predicted transmembrane segments (TM1, G54V; TM5, G300V; TM7, A718L; and TM9, A841L), in the extracellular loops between transmembrane segments (G854V), in the cytoplasmic loops (G251V and W803A), in the nucleotide-binding domains (G427C and S434C), and in the linker region connecting the two halves of the molecule (E707A) (data not shown).

The most potent of the four compounds was cyclosporin A. More than 50% of the mutant protein was present as the mature form of the enzyme in the presence of 2–10 μM of cyclosporin A, 5–20 μM vinblastine, 12.5–50 μM verapamil, or 75–150 μM capsaicin. The highest concentration of capsaicin (300 μM) appeared to be quite toxic to the cells. Except for vinblastine, the cells continued to proliferate in the presence of the various drug substrates. Vinblastine, which is an inhibitor of microtubule assembly, did not cause immediate cell death but resulted in the detachment of the cells from the dish. Other hydrophobic compounds that are not substrates of P-glycoprotein, such as 3-methoxy-tyramine or 3-hydroxy-4-methoxyphenethyl amine, had no effect on the processing of the misfolded mutants (data not shown). The effects of drug substrates on folding appear to be specific to P-glycoprotein because the drug substrates of P-glycoprotein could not rescue the temperature-sensitive CFTR ΔF508 processing mutant (Fig. 1B).

Restoration of processing of the misfolded mutants appeared to be quite rapid and occurred within a few hours after the addition of drug substrates to the medium. Fig. 2A shows that after 4 h in the presence of 15 μM cyclosporin A, about 50% of mutant G268V was present as the fully mature (170-kDa) form of the enzyme and that after 24 h, more than 80% of the mutant protein was present in the fully mature form. The total amount of P-glycoprotein also increased dramatically in the presence of cyclosporin A. These results suggested that the drug stabilized the mutant protein resulting in decreased turnover. This was confirmed by pulse-chase studies. Fig. 2B shows that in the absence of cyclosporin A, the 150-kDa P-glycoprotein of mutant G268V was not processed to the mature enzyme. The core-glycosylated protein was rapidly degraded (half-life about 2 h), and there was little product remaining after 8 h. In the presence of cyclosporin A, however, the kinetics of maturation of the P-glycoprotein of mutant G268V was similar to that of wild-type enzyme. By 4 h post-labeling, the majority of the 150-kDa protein was processed to the mature enzyme (170 kDa). The processed P-glycoprotein was stable for at least 24 h. Cyclosporin A had no detectable effect on the processing of the wild-type enzyme.

Detection of P-glycoprotein at the Cell Surface and Measurement of Drug-stimulated ATPase Activity—Cell surface labeling was performed (8) to determine whether the mutant proteins reached the plasma membrane. HEK 293 cells were transfected with the histidine-tagged mutant cDNAs and then incubated in the presence or the absence of 15 μM cyclosporin A. The transfected cells were then treated with periodate to con-
We have previously found that wild-type but not misprocessed
P-glycoproteins were present at the cell surface when expression was done without drug
substances. We tested if the mutant P-glycoproteins were active when ex-
dered by nickel-chelate chromatography (10). Apparently the processing mutants are misfolded, resulting in masking of the histidine tag. We modified the cDNAs of the mutant P-glyco-
proteins to code for 10 tandem histidine residues at the COOH end of the molecule to facilitate purification by nickel-chelate chromatography (14). The cDNAs of the mutant P-glycopro-
teins were transiently expressed in HEK 293 cells and then incubated for 24 h in the presence or the absence of 15 μM cyclosporin A. Histidine-tagged P-glycoprotein was isolated by nickel-chelate chromatography. The majority of the wild-type
P-glycoprotein was bound to the nickel column and was eluted with 0.3 M imidazole regardless of whether expression was carried out in the presence or the absence of cyclosporin A. This
was determined by loading equivalent amounts of the flow-
through and eluted fractions on SDS-PAGE followed by immu-
oblottting with a polyclonal antibody against P-glycoprotein
data not shown). By contrast, most of the P-glycoprotein of mutants G268V and ΔY490 grown without drug substrate were recovered in the flow-through fractions during nickel-chelate chromatography. In the presence of 15 μM cyclosporin A, how-
ever, the majority of the mutant P-glycoproteins were recov-
ered by nickel-chelate chromatography and had an apparent
mass of 170 kDa. Similar results were obtained when the transfected cells were incubated in the presence of capsaicin, verapamil, or vinblastine (data not shown).

Drug-stimulated ATPase activity of the wild-type and mu-
ant P-glycoproteins was measured in the presence of vera-
pamil, vinblastine, or colchicine after the addition of phospho-
lipid. Fold-stimulation indicates the ratio of the activity with drug
substrate to that found without drug substrates.

FIG. 2. Time-dependent appearance of the 170-kDa (mature)
form of mutant P-glycoprotein-A52 (G268V). A, HEK 293 cells were
transfected with A52-tagged mutant G268V cDNA and incubated for
24 h at 37 °C. The medium was then replaced with fresh medium
containing 15 μM cyclosporin A. At the indicated times (h), the cells
were harvested, lysed with SDS sample buffer, and subjected to immu-
noblot analysis with monoclonal antibody A52, followed by chemilumi-
nescence. B, HEK 293 cells were transfected with wild-type or mutant
G268V P-glycoprotein-A52 cDNAs or vector alone (Control). After 24 h,
the cells were pulse-labeled with [35S]methionine and [35S]cysteine for 20
min at 37 °C in the presence (+ Cyclosporin A) or the absence (− No Drug)
of 15 μM cyclosporin A and then chased for the indicated times (h) in the
presence or the absence of cyclosporin A. Labeled P-glycoprotein-A52
was immunoprecipitated with monoclonal antibody A52. The immuno-
precipitates were analyzed by SDS-PAGE and fluorography. The posi-
tions of the mature (170-kDa) and core-glycosylated (150-kDa) forms of P-glycoprotein are indicated.

FIG. 3. Cell surface labeling of histidine-tagged wild-type and
mutant forms of P-glycoprotein after expression in the absence
(−) or the presence (+) of cyclosporin A. HEK 293 cells were
transfected with cDNAs coding for the histidine-tagged wild-type or
mutant P-glycoprotein. After 24 h, the cells were incubated in the
presence or the absence of cyclosporin A. After another 24 h,
the cells were treated with periodate and biotin-LC-hydrazide, and the biotiny-
lated enzyme was detected using horseradish peroxidase conju-
gated to streptavidin followed by chemiluminescence.

vert the carbohydrate moieties to aldehydes, followed by addition
of biotin-LC-hydrazide (Pierce). Biotin-LC-hydrazide is a
nonpermeable compound that forms covalent attachments to extracellular glycoproteins after periodate oxidation (18). The
histidine tagged P-glycoprotein mutants were then purified by nickel-chelate chromatography and immunoblotted with streptavidin-conjugated horseradish peroxidase. Fig. 3 shows
that wild-type but not the mutant P-glycoproteins, was present
at the cell surface when expression was done without drug
substrate. When the transfected cells were incubated in the presence of 15 μM cyclosporin A, however, both mutant proteins
were detected at the cell surface.

To test if the mutant P-glycoproteins were active when expressed
in the presence of drug substrates, we attempted to
purify the mutant P-glycoproteins by nickel chelate chromatog-
raphy for measurement of drug-stimulated ATPase activity.
We have previously found that wild-type but not misprocessed
P-glycoprotein containing a histidine tag can readily be recov-
of the misfolded proteins results in the appearance of a functional transporter at the cell surface.

**DISCUSSION**

Drug substrates or modulators of P-glycoprotein appear to be acting as powerful “chaperones” for processing misfolded P-glycoproteins. All misprocessed mutants of P-glycoprotein that we have tested could be converted to the fully mature form of the enzyme, even when the mutations were located in different domains of the molecule. The effects of these substrates or modulators were also independent of the cell lines because the misfolded mutants could be rescued when expressed either transiently in HEK 293 cells (this study) or stably in NIH 3T3 cells (data not shown).

The ability of drug substrates to facilitate folding appears to be specific for P-glycoprotein because these substrates were ineffective on the CFTR mutant (ΔF508). Reversal of the misfolding phenotype of the ΔF508 CFTR mutant can be accomplished by incubation at lower temperatures (19) or by exposure to nonspecific low molecular weight compounds such as glycerol (12, 13), trimethylamine N-oxide, or deuterated water (13). In our hands, the effectiveness of glycerol treatment or lowering the growth temperature to facilitate processing was cell line-dependent. For example, some misfolded P-glycoprotein mutants such as G714A are temperature- and glycerol-sensitive only when stably expressed in NIH 3T3 cells but not when expressed in HEK 293 cells (data not shown). In addition, maturation of the misfolded P-glycoproteins in the presence of glycerol or by lowering the incubation temperatures is a slow process that usually requires 24–72 h. By contrast, the appearance of the mature form of any of the misprocessed mutants of P-glycoprotein occurs within 2–4 h after addition of any drug substrate (Fig. 2). Another interesting observation is that misfolded mutants that are temperature- and glycerol-insensitive, such as G251V, G268V, and E707A could also be rescued by these drug substrates when expressed in either HEK 293 or NIH 3T3 cells (data not shown).

The exact mechanism of how these specific drug substrates or modulators facilitate processing of misfolded P-glycoproteins mutants is not known. A possible explanation is that the drug-binding site(s) in P-glycoprotein are formed early in the folding intermediates during biosynthesis. Occupation of the drug-binding site(s) stabilized the folding intermediates in a “near native” conformation, thus escaping the cell’s quality control mechanism (Fig. 2B). Recently, Qu and Thomas (20) studied the effects of the CFTR ΔF508 on the thermodynamic stability and folding yield of the nucleotide-binding domain 1 and concluded that the major deleterious effect of the mutation was to allow accumulation of a folding intermediate that was prone to self-association. The ΔF508 mutation had little effect on the thermodynamic stability of the folded nucleotide-binding domain 1. The mutation also did not appear to enhance in vivo proteolysis because inhibition of proteasomes (21, 22) did not enhance the efficiency of maturation of the full-length ΔF508 CFTR. These results suggest that mutations that cause mis-processing slow one or more folding steps, resulting in an increased concentration of the intermediate that is prone to self-aggregation. Therefore, in P-glycoprotein, it is possible that the occupation of the drug-binding site(s) in the early stages of folding may reduce the concentration of the intermediate that is prone to self-aggregation.

In summary, the results of this study demonstrate that a potential strategy in the treatment of diseases involving trafficking/misfolding of proteins would be to identify specific synthetic and natural substrates or modulators and to include these during biosynthesis.

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