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Amino acid substitutions in the sixth transmembrane domain of P-glycoprotein alter multidrug resistance

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ABSTRACT **Eukaryotic cells can display resistance to a wide range of natural-product chemotheraputic agents by the expression of P-glycoprotein (pgp), a putative plasma membrane transporter that is thought to mediate the efflux of these agents from cells. We have identified, in cells selected for multidrug resistance with actinomycin D, a mutant form of pgp that contains two amino acid substitutions within the putative sixth transmembrane domain. In transfection experiments, this altered pgp confers a cross-resistance phenotype that is altered significantly from that conferred by the normal protein, displaying maximal resistance to actinomycin D. These results strongly implicate the sixth transmembrane domain in the mechanism of pgp drug recognition and efflux. Moreover, they indicate a close functional homology between pgp and the cystic fibrosis transmembrane regulator in which the sixth transmembrane domain has also been shown to influence substrate specificity.**

Multidrug resistance (mdr) remains a major obstacle in the treatment of neoplasia. Resistance to vinca alkaloids, colchicine, anthracyclines, and actinomycin D can be conferred to eukaryotic cells by the expression of P-glycoprotein (pgp), a plasma membrane transporter that is thought to cause the efflux of these agents by an ATP-dependent mechanism (1). Pgp contains 12 putative transmembrane and 2 ATP binding domains and is a member of a superfamily of membrane transport proteins that have recruited this pump architecture to move vastly diverse substrates across membranes (1, 2). Although pgp substrates are generally lipophillic, they are structurally and functionally dissimilar, and the mechanism by which pgp can mediate the efflux of these different compounds remains unknown (3, 4). We have identified an altered form of the hamster pgp gene (pgp1) and its transcripts in multidrug-resistant DC-3F/ADX cells that results in two adjacent amino acid substitutions within the predicted sixth transmembrane (tm6) domain of the encoded pgp. This version of pgp confers an mdr phenotype that is very different from cells expressing normal pgp, implicating the tm6 domain in the mechanism of drug recognition and efflux.

MATERIALS AND METHODS

PCR. The PCR was performed using a Cetus PCR kit and thermal cycler. Primers used to amplify the 102-base-pair (bp) genomic DNA segment containing codons 338 and 339 had the following sequences: SD40, 5′-GTCTTCTTTGCTG-TATTAATT-3′; SD25, 5′-GTTGAAGATTTCATAG-GCTG-3′. These primers were synthesized on an Applied Biosystems PCR-mate oligonucleotide synthesizer as described (5). Genomic DNA from cell lines was extracted and purified as described (6).

Construction of pgp1 Expression Vectors and Stable Transfectants. Stable DC-3F transfectant cell lines were constructed as described (ref. 7, pp. 9.1.1–9.1.3) with the dual promoter vector pHβ Apr-1-neo (pLK444) (8) containing the full-length pgp1 cDNA inserted at the *Bam*HI site adjacent to the β-actin promoter. The full-length pgp1 cDNA (5) was engineered in pGEM-4Z (Promega) to have *Bam*HI sites adjacent to bases 1 and 4304. The mutant cDNA was converted into the normal sequence by replacement of a unique *Nsi* I–*Bgl* II fragment. These inserts were placed into the *Bam*HI site of pLK444, producing plasmids pLK212S (normal) and pLK110S (mutant). All constructs were confirmed by sequence analysis and mapping. G418-resistant colonies emerging from calcium phosphate transfection and subsequent exposure to G418 at three times the ED_{90} were cloned, expanded, and screened for resistance to vincristine. Cell clones capable of displaying resistance to both G418 and vincristine were chosen for further analysis. Clonal stocks were not exposed to vincristine.

ED_{50} Determinations. DC-3F cells and derivatives were maintained as described (9). ED_{50} values were generally determined for each cell line on at least three occasions, essentially as described (9), with modifications. Briefly, 4×10^4 cells were plated in 60-mm culture plates, and the cells were allowed to attach for 24 h, at which time drugs were added. The cells were trypsinized 72 h after drug addition and counted using either a Coulter counter or hemocytometer.

Northern Blot Analysis, Dot Blots, and Primer-Extension Analysis of RNA. Ten micrograms of total cellular RNA (ref. 7, pp. 4.0.1–4.10.9) was analyzed by Northern blot hybridization as described (ref. 7, pp. 4.0.1–4.10.9) using the 4.3-kilobase (kb) pgp1 cDNA probe (5) labeled by random priming (10). Dot blot hybridizations (11) and primer extension (ref. 7, pp. 4.0.1–4.10.9) were also performed as described.

Immunoprecipitation. Cells were incubated with [^{35}S]methionine for 14 h and lysed, and samples of lysate containing equal amounts of labeled total protein were subjected to pgp immunoprecipitation (ref. 7, pp. 10.16.6–10.16.7) with the monoclonal antibody C219 (12). Immunoprecipitated proteins were analyzed on 4.5 M urea/SDS polyacrylamide gels after a 5-min denaturation at 100°C in SDS sample buffer (ref. 7, p. 10.2.17) containing 5 M urea. Gels were fixed, treated with autoradiographic enhancer, dried, and exposed to x-ray film.

RESULTS

Comparison of pgp1 cDNAs cloned from a multidrug-resistant Chinese hamster lung cell line, DC-3F/ADX (5), with those cloned from a normal hamster liver (13), revealed

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Abbreviations: pgp, P-glycoprotein; tm6, sixth transmembrane domain; mdr, multidrug resistance; CFTR, cystic fibrosis transmembrane regulator.

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two nucleotide differences between the transcript sequences (Fig. 1*A*). cDNAs from the multidrug-resistant DC-3F/ADX line have two nearly adjacent G → C transversions relative to the normal cDNA at nucleotide positions 1123 and 1125 that lead to amino acid substitutions at their respective codons: Gly → Ala at codon 338 and Ala → Pro at codon 339. Examination of the pgp1 genomic sequence at these positions by PCR amplification (Fig. 1*B*) revealed that the parental DC-3F cell line contains only normal alleles encoding glycine at codon 338 and alanine at 339, in agreement with the normal hamster liver cDNA sequence. The multidrug-resistant subline DC-3F/ADX, on the other hand, was found to possess mutated pgp1 genes, as predicted from its pgp1 cDNAs. Hence, DC-3F/ADX cells contain both the pgp1 genes and transcripts necessary to express pgp with the Ala/Pro double substitution. Moreover, since this change is present in the multidrug-resistant DC-3F/ADX subline but not parental DC-3F cells, it must have been acquired by somatic mutation of the pgp1 gene during the course of selection with actinomycin D.

Transfection experiments utilizing expression constructs containing the pgp1 cDNA (Fig. 2) in either the normal or mutant form demonstrated that each confers a distinctive mdr phenotype (Table 1). Although stable G418-resistant clones expressing a normal construct displayed highest resistance to colchicine, those clones expressing a mutant pgp1 cDNA had highest resistance to actinomycin D. Northern blot hybridization analysis of transfectants revealed the expression of

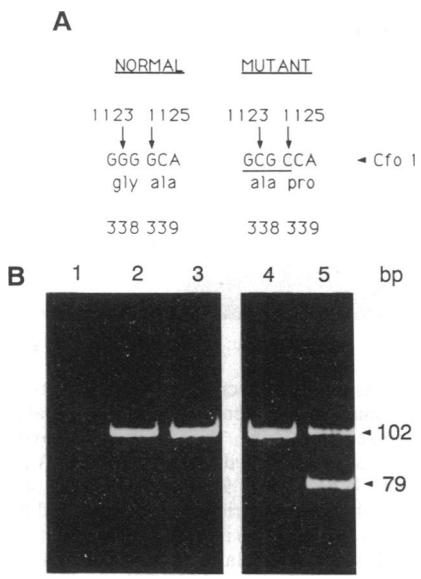

FIG. 1. (*A*) Nucleotide and amino acid sequences at codons 338 and 339 of the normal (13) and the mutant (5) pgp1. (*B*) PCR amplification and analysis of a 102-base segment of genomic DNA containing codons 338 and 339. A *Cfo* I restriction site (underlined in *A*) is created by the double mutation and hence *Cfo* I should digest mutant but not normal PCR products. Lanes: 1, negative control; 2, DC-3F uncut; 3, DC-3F cut with *Cfo* I; 4, DC-3F/ADX uncut; 5, DC-3F/ADX cut with *Cfo* I. The PCR product from the parental DC-3F cell line is not cut by *Cfo* I (lane 3, 102 bp), whereas about half from the DC-3F/ADX cell line is cut by *Cfo* I (lane 5, 79 bp). The other expected fragment of 23 bp was also observed (data not shown). Populations of PCR molecules were further characterized by cloning the products into the *Sma* I site of pGEM-4Z (Promega) and sequencing individual clones. Out of 28 individual pGEM-4Z/PCR clones analyzed from DC-3F, 27 had the normal sequence and 1 had sequence equivalent to the analogous region of the pgp2 gene (13). Of 34 individual clones analyzed from the DC-3F/ADX cell line, 18 were mutant, 6 were normal, and 10 represented the pgp2 sequence (data not shown). The pgp2 gene has no *Cfo* I sites in the 102-bp PCR product. The PCR/Cfo assay was repeated four times using different primer and genomic DNA preparations.

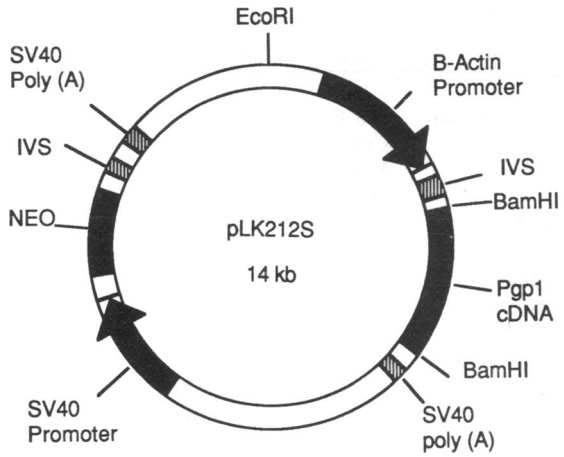

FIG. 2. pgp1 expression construct pLK212S. The full-length pgp1 cDNA from base 1 to base 4304 was placed into the *Bam*HI site of the eukaryotic expression vector pHβ Apr-1-neo (pLK444) (8), in both sense and antisense orientations. This places the pgp1 cDNA adjacent to the human β-actin promoter, which directs its expression. The neomycin phosphotransferase (NEO) gene is also present on the construct with its expression being driven by the simian virus 40 (SV40) early promoter, thus providing G418 resistance to cells containing a construct. Polyadenylylation signals and intervening sequences (IVS) accompany each transcription unit for maximal expression of mature transcripts.

4.3-kb and 2.3-kb pgp RNA transcripts (Fig. 3*A*); and primer-extension analysis (Fig. 3*B*) suggests that these transcripts were generated from the expression construct rather than endogenous pgp genes. Elevated expression of pgp mRNA and pgp was confirmed in transfectant cell lines (Fig. 4) and found to parallel the level of drug resistance observed; only small increases were detected in cell lines displaying low levels of resistance, and higher expression was observed in those with more resistance.

It is of interest that the drug-resistance profile conferred by the altered pgp1 cDNA, while not identical, is similar to that of the cell line DC-3F/ADX from which the mutant cDNA was cloned (Table 1). It is also notable that DC-3F/ADX cells emerged from the parental population while under selective pressure imposed by actinomycin D (9) and that the altered pgp confers highest resistance to that agent. Hence, the altered form is likely to have conferred a survival advantage to the cells expressing it during the stepwise selection process (9). Interestingly, however, DC-3F/ADX cells display an even higher level of resistance to actinomycin D relative to other drugs when compared to transfectant cell lines, even though pgp RNA measurements suggest that DC-3F/ADX cells exclusively express mutated pgp1 transcripts (S.E.D. and P.W.M., unpublished data). This suggests the involvement of an additional non-pgp-mediated mechanism for actinomycin D resistance in the DC-3F/ADX line.

Comparison of computer-modeled structures of the normal and mutant tm6 domains (Fig. 5) revealed the likely basis for the functional differences observed between the two versions of the protein; i.e., the α-helical structure often assigned to transmembrane domains was predicted to be unwound by $\approx$30° in the mutant relative to the normal tm6 domain (Fig. 5). Pro-339 in the mutant tm6 is thought to cause this alteration, and its presence changes the relative positions of many amino acid side chains within the helix. Thus with the transfection results, these modeling studies suggest several possibilities including that the tm6 domain exerts its influence (*i*) as a binding site common to all or most drugs, in which structural changes directly influence interactions with multiple substrates; (*ii*) as a region within the folded protein that, when altered, indirectly influences function; or (*iii*) as part of a

Table 1. Drug-resistance properties of transfectants

Cell line	Drug	ED_{50}, ng/ml	Fold resistance
Control			
DC-3F (parental)	Act D	3.0	—
	Colc	30.0	—
	Vinc	100.0	—
	Daun	60.0	—
LK1.5A	Act D	4.2 ± 0.1	1.4
(antisense	Colc	27.9 ± 0.3	0.9
negative	Vinc	109.1 ± 0.5	1.1
control)	Daun	47.9 ± 0.9	0.8
Transfectant			
Normal pgp1 (Gly-Ala)			
212S-17	Act D	38.0 ± 2.9	12.7
	Colc	812.0 ± 30	27.0
	Vinc	745.0 ± 8.7	7.5
	Daun	867.0 ± 116	14.4
220S-5	Act D	23.3 ± 4.3	7.8
	Colc	699.0 ± 26	23.3
	Vinc	480.0 ± 80	4.8
	Daun	723.0 ± 64	12.1
212S-10.2	Act D	6.3 ± 0.4	2.1
	Colc	130.8 ± 25	4.4
	Vinc	99.7 ± 7.4	1.0*
	Daun	ND	ND
Mutant pgp1 (Ala-Pro)			
110S-23	Act D	91.1 ± 2.1	30.4
	Colc	211.7 ± 7.1	7.1
	Vinc	563.0 ± 37	5.6
	Daun	268.3 ± 5.9	4.5
110S-26	Act D	38.4 ± 0.5	12.8
	Colc	127.6 ± 0.5	4.3
	Vinc	238.6 ± 7.3	2.4
	Daun	16.6 ± 10	0.3
110S-5	Act D	22.1 ± 0.9	7.4
	Colc	68.4 ± 1.4	2.3
	Vinc	91.9 ± 7.6	0.9*
	Daun	51.7 ± 7.2	0.9
Other			
DC-3F/ADX†	Act D	14,514 ± 2165	4834
	Colc	3,764 ± 223	126
	Vinc	9,118 ± 128	91
	Daun	1,548 ± 112	26

ED_{50} values for stably transfected DC-3F cell lines harboring pgp1 expression constructs are shown. Six clonal pgp1 cell lines were analyzed, three containing the normal and three the mutant construct, each set representing a range of drug resistances. Each value shown is the average from three experiments carried out in parallel on a single day. Resistance to the four drugs was evaluated simultaneously. Some variability in resistance was noted that seemed to be related to the state of the cells at the time of the assay. The observed relative differences in drug resistances were not altered, however, and representative experiments are reported. DC-3F values represent the consensus of several measurements made in our laboratory over several months. Baseline measurements of DC-3F can vary by as much as about ±0.5-fold. The cell line LK444.10.2 (contains expression vector without pgp1 insert) had ED_{50} values similar to DC-3F and LK1.5A (data not shown). ED_{50}, drug dose that reduces viability to 50% of control; Act D, actinomycin D; Colc, colchicine; Vinc, vincristine; Daun, daunorubicin; ND, not done.

*Although most clones in Table 1 scored highly positive in initial vincristine screens of G418-resistant clones, an attempt was made to also study several clones that were marginally positive, and hence, a low level of stringency was applied in the initial screen to ensure that no positives would be missed. Several clones included initially were found to be mdr-negative upon further examination (data not shown).

†See refs. 9, 11, and 14–16 for description of the DC-3F/ADX cell line; the values shown were independently determined for this work.

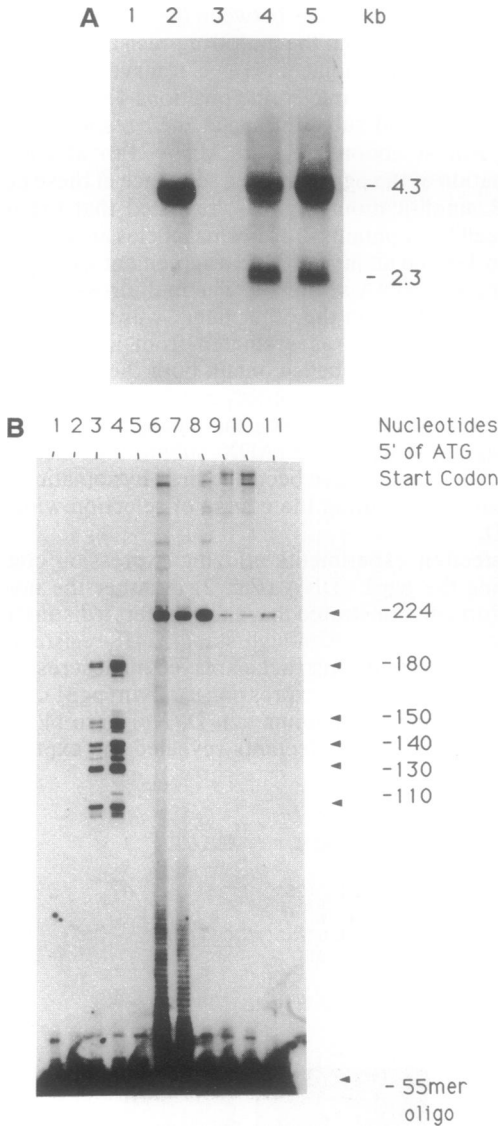

FIG. 3. Analysis of pgp transcripts in transfectants. (*A*) Northern blot analysis of transfectants. Ten micrograms of total cellular RNA was electrophoresed in a 1.2% agarose/formaldehyde gel, transferred to nitrocellose, and probed with a pgp1 cDNA probe. Lanes: 1, DC-3F; 2, DC-3F/ADII (5, 9); 3, LK444-10.2; 4, 212S-17; 5, 110S-23. Note the 4.3-kb transcript in lanes 2, 4, and 5 and a 2.3-kb transcript in lanes 4 and 5, which is likely to represent a pgp1 splicing variant (5). Ethidium bromide staining of duplicate samples electrophoresed on the same gel showed relatively uniform loading (data not shown). (*B*) Primer-extension analysis of transfectants. An oligonucleotide that is the complement to bases 51–106 of the pgp1 cDNA (5) was hybridized to 40 μg of total RNA and extended, as described (ref. 7, pp. 4.0.1–4.10.9). Lanes: 1, tRNA; 2, DC-3F; 3, DC-3F/AD II; 4, DC-3F/ADX; 5, LK444-10.2; 6, 212S-17; 7, 220S-5; 8, 110S-23; 9, 212S-10.2; 10, 110S-5; 11, 110S-26. The extension product noted in the transfectants corresponds to the length expected for transcripts originating from the vector construct (220-base extension product, equivalent to position −224 upstream of the ATG start codon). The identity of this extension product was further confirmed by amplification and analysis of the extension product by using the PCR (data not shown). The PCR was also used to confirm the presence of mutant transcripts in cell lines transfected with a mutant construct and the absence of mutated pgp1 transcripts in those transfected with the normal construct (data not shown).

hydrophobic gate or channel through which drugs transit the cell membrane and whose hydrophobic moment may be changed by mutation in amino acid sequence, namely, Gly^{338}-Ala^{339} to Ala^{338}-Pro^{339}. Interestingly, the tm6 domain of another member of the pgp superfamily, the cystic fibrosis

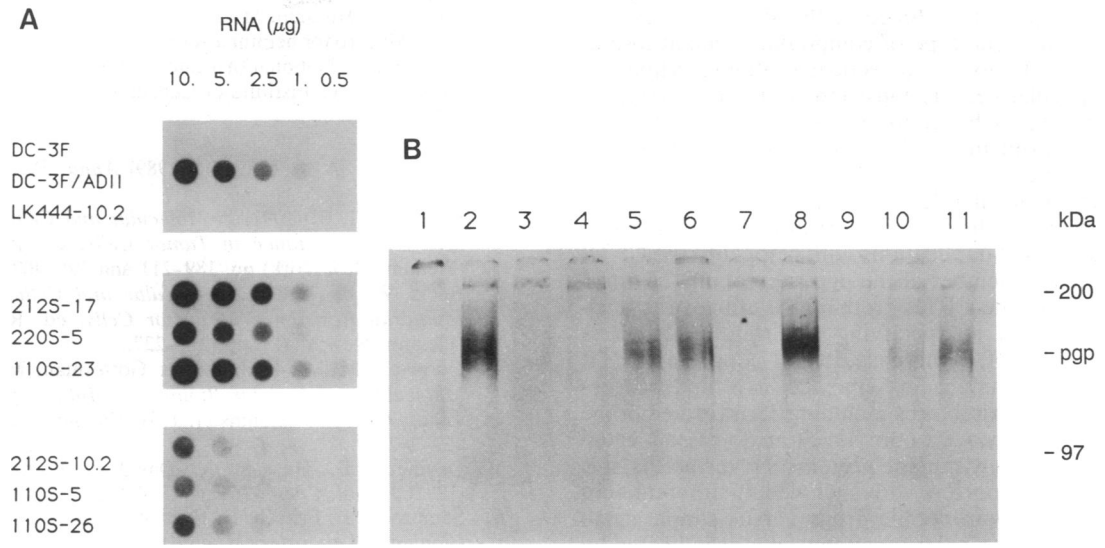

FIG. 4. Expression of pgp mRNA and pgp in transfectants. (*A*) Dot blot analysis of transfectants. Total RNA (as indicated in μg) was applied to nitrocellulose and hybridized as in Fig. 3*A*. Note that pgp mRNA levels parallel the drug-resistance and pgp expression levels observed in these cell lines (Table 1 and Figs. 3*B* and 4*B*). (*B*) Immunoprecipitation of pgp. pgp was immunoprecipitated from ^{35}S-labeled lysates using the monoclonal antibody C219 (12) and analyzed on a 4.5 M urea/5% polyacrylamide gel. Lanes: 1, DC-3F; 2, DC-3F/AD II; 3, LK444-10.2; 4, LK1.5A; 5, 212S-17; 6, 220S-5; 7, 212S-10.2; 8, 110S-23; 9, 110S-5; 10, 110S-26; 11, DC-3F/AD II (half the amount of lysate precipitated in lane 2).

transmembrane regulator (CFTR), was recently shown to help dictate the ion selectively of that protein (17). Hence, the tm6 domain of both proteins plays a role in specifying preferences for substrates within broad classes.

DISCUSSION

We have identified a mutant form of pgp that confers an altered mdr phenotype relative to the normal protein. The mutant form has two amino acid substitutions in the proposed tm6 domain of the protein as a result of the acquisition of somatic mutations in the pgp gene. This double substitution involves Gly^{338}, which is changed to Ala, and the adjacent Ala^{339}, which is changed to Pro.

Although our data strongly implicate the tm6 domain in the efflux mechanism, other single amino acid substitutions in pgp have been described that affect the mdr phenotype. These include $Gly^{185} \rightarrow$ Val (18–20) located in a predicted cytoplasmic loop linking tm2 and tm3 in human mdr1, and $Ser^{939} \rightarrow$ Phe (21) located in tm11 of mouse mdr3. The altered drug-resistance phenotypes conferred by these variant pgp molecules are different from the one described here. Nevertheless, it is tempting to speculate that even though the domains defined by these substitutions are well separated in the linear structure of the pgp molecule, they interact to form a structure capable of mediating drug resistance. Such a structure might require that these domains be proximally situated in three-dimensional space in the folded pgp molecule.

One feature of pgp that remains a mystery is its ability to mediate the efflux of many structurally unrelated compounds. Since several drugs appear to be affected by the tm6 alteration, it seems likely that this domain is somehow involved in mediating the efflux of all or most drugs. Any single site in pgp that would be capable of recognizing many different compounds would be expected to possess a number of structural features, one or several of which might be devoted to recognizing a single type of compound. Should tm6 be involved in substrate recognition, subtle alterations in the positions of amino acid side groups such as those predicted in the tm6 modeling studies (Fig. 5) could be envisioned to tailor the site for recognition of a single compound. Normally, such a drug binding site may represent a structural compromise that retains the ability to bind many compounds

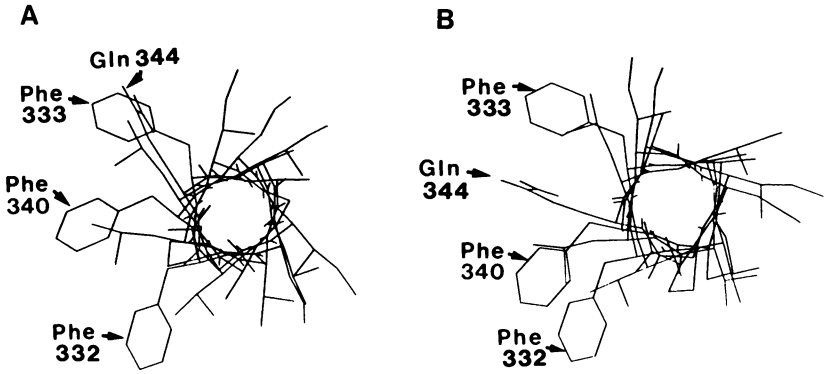

FIG. 5. Predicted structures of the normal and mutant tm6 domains. tm6 domains of the normal and mutant amino acid sequences were folded into right-handed α-helices by using the Polygen (Waltham, MA) QUANTA program and energy-minimized with the CHARMM program. (*A*) End view of normal helix. (*B*) End view of mutant helix. Note that the views were rotated such that side chains of Phe-332 and Phe-333 were positioned at the same place in each view. The relative positions of the other amino acid side chains are different in the two models (e.g., note positions of Phe-340 and Gln-344 in each helix).

but binds none optimally. Hence, if the site was altered to optimally recognize one type of compound, it might lose or be diminished in its ability to recognize others. While this depiction is speculative, it is consistent with the observation that alterations of pgp that confer increased resistance to one or more drugs result in the decreased resistance to others (refs. 18–20; Table 1).

Although the molecular details of pgp function address the basic problem of membrane transport in general, this information has additional implications with respect to the CFTR protein, which is a member of the pgp superfamily of transport proteins (22). The CFTR protein also utilizes the analogous (tm6) domain in specifying substrate preferences within broad classes of, in this case, ions (17). pgp and CFTR are related at the amino acid sequence level and possess similar molecular architectures including 12 transmembrane domains (17, 22). Moreover, both have tm6 domains located in analogous positions within the proteins. Nevertheless, it is remarkable that transporters with such widely different substrates (i.e., bulky hydrophobic drugs versus simple small ions) apparently share similar functional sites as indicated by the common effect tm6 alterations have on both.

It is now becoming clear that pgps are capable of conferring not only a single cross-resistance pattern but also a range of many different such patterns *in vitro*. cDNAs encoding several qualitatively different forms of pgp have been expressed in transfected cells. These include both the normal human mdr1 cDNA and its $Gly^{185} \rightarrow Val$ mutant (18, 20); the mouse mdr1 and mdr3 cDNAs, which encode functionally distinct pgps (21, 23); a mouse mdr3 mutant (21); and both the normal and mutant forms of hamster pgp1 described above (Table 1). In each case a distinct cross-resistance pattern has been observed. It appears, therefore, that genetic variation plays a role in determining the cross-resistance pattern that accompanies the mdr phenotype.

Transfectant cell lines 212S-10.2 and 110S-5 were found to have very low pgp mRNA and protein expression levels (Figs. 3*B* and 4) and, hence, low levels of drug resistance (Table 1). Whereas the resistance profiles of these lines were consistent with those of clones expressing the same constructs at higher levels, each pgp conferred resistance to only a subset of the drugs tested. This finding suggests a possible explanation for some forms of "atypical" mdr (3) that likewise display partial resistance phenotypes. Interestingly, the pfmdr gene, which is another member of the pgp superfamily, apparently confers resistance to chloroquine in protozoa only when certain amino acid substitutions have occurred in the protein (24). As noted, transfectants with low levels of mutant or normal pgp1 expression [i.e., 212S-10.2, 110S-5, and 110S-26 (Table 1)] likewise indicate that the isoform expressed dictates whether a cell will display any resistance at all to certain drugs.

Although knowledge of pgp sites critical for drug efflux may provide a rationale for more successful treatment of neoplasia and aid the development of reversal agents or nonsubstrate drug analogs, it should at a minimum shed light on the mechanism by which this superfamily of membrane transporters functions.

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