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Transmembrane Domain (TM) 9 Represents a Novel Site in P-Glycoprotein That Affects Drug Resistance and Cooperates with TM6 to Mediate [^{125}I]Iodoarylazidoprazosin Labeling

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ABSTRACT

The multidrug resistant cell line DC-3F/ADII was obtained by stepwise selection for growth in actinomycin D (ActD). Compared with parental cells, it displays high resistance to ActD and vincristine and low resistance to colchicine and daunorubicin. These cells overexpress a form of P-glycoprotein (Pgp1) containing a double mutation, I837L and N839I, in transmembrane domain (TM) 9; when transfected into DC-3F, this mutation confers the DC-3F/ADII phenotype. We have shown previously that another cell line, DC-3F/ADX, also displays this phenotype and overexpresses a mutant form of Pgp1 containing a double mutation in TM6 (G338A, A339P). Hence, mutations in TM9 and TM6 are independently capable of conferring the same cross-resistance phenotype. The TM6 mutations inhibit the ability of cyclosporin A to reverse cross-resistance and to block labeling of the protein by [^{125}I]iodoarylazidoprazosin (IAAP), whereas the TM9 mutations do not show similar effects. A chimeric protein containing both pairs of mutations confers twice the level of resistance to ActD than expected from the sum of the individual mutations, but it cannot be labeled to detectable levels with [^{125}I]IAAP. Thus, TM9 represents a novel site that cooperates with TM6 to mediate drug resistance and [^{125}I]IAAP labeling.

P-glycoprotein (Pgp1) belongs to the superfamily of ATP binding cassette transporters and contains two homologous halves, each composed of six putative transmembrane α-helical domains and one ATP binding site (Endicott and Ling, 1989; Beck and Danks, 1991; Sugimoto and Tsuruo, 1991; Higgins, 1992). It is a glycosylated membrane-bound protein that functions as an ATP-dependent efflux transporter with broad substrate specificity. Its activity can be reversed by agents such as verapamil (VRP) and cyclosporin A (CsA) (Yusa and Tsuruo, 1989; Tamai and Safa, 1990). Despite intensive study, the nature and number of its substrate and reversal agent binding sites remain poorly understood. Putative drug binding regions have been identified on the fifth, sixth, eleventh, and twelfth transmembrane segments and their nearby regions either by photoaffinity labeling with drug analogs (Greenberger, 1993) or by studying the effects of site-directed mutagenesis on drug recognition and transport (Devine et al., 1992; Loo and Clark, 1993a, 1994b, 1997b; Ma et al., 1997; Hafkemeyer et al., 1998). The results have also shown that amino acid residues capable of affecting drug specificity are scattered across the entire length of the protein (Loo and Clark, 1993b, 1994a, 1996b; Hanna et al., 1996; Taguchi et al., 1997a,b; Kwan and Gros, 1998), consistent with the notion that a higher-order structure is required for substrate recognition and function. Although the number of individual binding sites remains unknown, the bulk of the existing data argue for the existence of both individual and overlapping sites located, perhaps, within at least one complex binding pocket formed by association of the N- and C-terminal halves of the protein (Bruggemann et al., 1989; Greenberger, 1993; Dey et al., 1997; Pascaud et al., 1998).

Although a great deal of the information indicating which amino acid residues can affect the interaction of drugs and reversal agents with Pgp1 has come from the use of site-directed mutagenesis, we have used CHL cell lines derived by long-term selection in the presence of actinomycin D (ActD), overexpressing naturally occurring mutant transporters, to identify such residues (Devine et al., 1992). The study of cell line DC-3F/ADX led to the finding of a double mutation, G388A/A339P, in TM6 of Pgp1 that mediated a novel drug cross-resistance pattern to four different drugs and inhibited the ability of cyclosporin A (CsA) to reverse drug resistance (Devine et al., 1992; Ma et al., 1997). During the course of that work, we noted that the cell line from which DC-3F/ADX had been selected, DC-3F/ADIV, and its immediate precursor

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ABBREVIATIONS: Pgp, P-glycoprotein; VRP, verapamil; CsA, cyclosporin A; TM, transmembrane domain; PCR, polymerase chain reaction; IAAP, iodoarylazidoprazosin; ActD, actinomycin D; COLC, colchicine; DAUN, daunorubicin; VCR, vincristine; PAGE, polyacrylamide gel electrophoresis.

DC-3F/ADII as well, exhibited the same pattern of cross-resistance (Devine and Melera, 1994a). However, deoxynucleotide sequencing of several Pgp1 cDNAs indicated that although the same mutations found in TM6 did exist in DC-3F/ADIV, they were not present in DC-3F/ADII (Troyer et al., 1996). Subsequently, it was determined that the original DC-3F/ADII subline had not been cloned before the reselection that led to the establishment of DC-3F/ADIV; hence, it probably contained a mixed population of cells (J. Biedler, personal communication). Sequencing of 24 reverse transcription-PCR generated cDNAs that overlapped the entire coding region of Pgp1 showed that the transporter expressed in a cloned subline of DC-3F/ADII contained two point mutations, an A-to-C transversion at nucleotide position 2509 and a A-to-T transversion at position 2516. Sequencing of 31 PCR-generated genomic clones from parental DC-3F cells representing this same region indicated that all were wild-type, whereas sequencing of 34 genomic clones from DC-3F/ADII cells yielded 15 mutant and 19 wild-type sequences (D. T. Chung and P.W.M. unpublished observations). Hence, it is unlikely that these nucleotide sequence differences are caused by genetic polymorphisms.

The mutations are located within TM9 (Devine et al., 1991) and result in amino acid changes, Ile^{837}→Leu (I837L), and Asn^{839}→Ile (N839I). In this report, we provide evidence that these mutations are responsible for the DC-3F/ADII drug cross-resistance phenotype. We also show that in contrast to TM6, this region does not affect the reversal activity of CsA but that together with TM6 does mediate [^{125}]IAAP labeling.

Materials and Methods

Cell Lines. The Chinese hamster lung fibroblast cell line DC-3F, maintained in Dulbecco's modified Eagle's medium/Ham's F12 medium containing 5% fetal calf serum, was used as the drug-sensitive control cell line and as the host for all transfections. The G338A, A339P, and G338A/A339P double mutant transfectants have been described previously (Devine et al., 1992; Ma et al., 1997). All chemicals and drugs, including actinomycin D, colchicine (COLC), daunorubicin (DAUN), and VRP were purchased from Sigma Chemical Co. (St. Louis, MO), unless specified otherwise. Vincristine (VCR) and CsA were generous gifts from Eli Lilly (Indianapolis, IN) and Novartis (East Hanover, NJ), respectively. VRP and CsA were dissolved in 15% and 100% ethanol, respectively. All other drugs were dissolved in 0.9% NaCl.

Site-Directed Mutagenesis and the Development of Stable Transfectants. Site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA). pGEM-4Z (Promega, Madison, WI) containing a wild-type Pgp1 cDNA insert obtained by *Bam*HI digestion of pGA-1 (Devine and Melera, 1994b) was used as the template. Mutant cDNAs were synthesized by PCR, using pairs of complementary mutant primers (prepared by Invitrogen, Carlsbad, CA). The primers had the following sequences (the underlined codons are altered from the wild-type): 5′-CAGAATCTAGCAAATCTTGGGACAGG-3′ (converts I837 to leucine), 5′-CAGAATATAGCAATTCTTGGGACAGG-3′ (converts N839 to isoleucine), and 5′-CAGAATCTAGCAATTCTTGGGACAGG-3′ (forms the double mutant I837L/N839I). To confirm that the desired nucleotide alterations were the only ones present, each of the mutant cDNAs was sequenced from the *Bsi*W1 restriction site at nucleotide position 440 to the *Xba*I site at position 3387 (Devine et al., 1991). Once confirmed, the mutant *Bsi*W1-*Xba*I fragments were removed and used to replace the corresponding fragments in wild-type Pgp1 cDNA before insertion into the eukaryotic expression vector pHβneo (Gunning et al., 1987). To obtain a cDNA containing both the TM6 and TM9 double mutations, plasmid p4.3** (Devine and Melera, 1994b), containing a full-length TM6 double mutant insert was digested with *Bsi*WI and *Bgl*II and the fragment containing the G338A/A339P mutation swapped with a similar fragment taken from the TM9 double mutant construct developed for this study by the method outlined above. The resulting clone was confirmed by DNA sequencing. The final constructs were transfected into drug-sensitive DC-3F cells using a lipofectin-based method according to instructions provided by the vendor (Invitrogen). Stable transfectants were obtained by selection with G418 (800 ng/ml; Invitrogen). Pgp1 expression levels were determined by Western blot analysis of total cell lysates, using anti-hamster P-glycoprotein monoclonal antibody MC-215 (Kamiya Biomedical Co., Thousand Oaks, CA) at a dilution of 1:250 as the primary antibody and goat anti-mouse IgG conjugated with peroxidase at a dilution of 1:50,000 as the secondary antibody. An enhance chemiluminescence ECL^+ immunoblot kit from Amersham Pharmacia Biotech (Piscataway, NJ) was used to detect the signal. Clones expressing similar levels of Pgp1 were used for the experiments reported here.

Drug Resistance and the Reversal Experiments. The ED_{50}, defined as the drug dose required to reduce cell growth to 50% of controls over a 72-h growth period, was used as the measure of drug resistance for all cell lines. The RD_{50}, defined as the dose of reversal agent required for reducing drug resistance to 50% of control values, was used to measure the reversal efficiency of CsA and VRP. The methods used to determine these values were as described previously (Devine and Melera, 1994a). All experiments were performed using the same batch of drugs and reversal agents, and the results are reported as the average of three independent experiments.

[^{125}I]IAAP Labeling of Pgp1. [^{125}I]IAAP labeling of Pgp1 was carried out as described by Dey et al. (1997), with slight modifications. Crude membranes were prepared from stable transfectants. Approximately 50 μg of membrane proteins were incubated with 6 μM [^{125}I]IAAP (NEX219, PerkinElmer Life Science Products, Boston, MA) in 50 mM Tris·HCl, pH 7.5, 50 mM NaCl, 50 mM KCl, and 10 mM $MgCl_2$ at room temperature for 15 min under subdued light. They were then illuminated with a UV lamp (366 nm) at room temperature for 15 min. The resulting labeled samples were solubilized by incubation in 1× SDS-PAGE sample buffer at room temperature for 30 min. Equal amounts of Pgp1, as measured by Western blot analysis, were then loaded into the wells of an agarose gel and analyzed by SDS-PAGE. To measure the ability of CsA to compete with [^{125}I]IAAP for labeling of Pgp1, membrane proteins were incubated with varying concentrations of CsA before incubation with the radiolabeled compound.

ATPase Activity. Crude membranes were prepared by centrifugation of total cell lysates at 100,000*g* (35,000 rpm) in a Beckman Ti 80 rotor at 4°C for 1 h (Beckman Coulter, Fullerton, CA). Pellets were resuspended in buffer and assayed for ATPase activity as described by Hrycyna et al. (1998) with some modifications. Basal activity was determined by incubation of 100 μg of crude membrane protein in a reaction mixture containing 50 mM Tris·HCl, pH 7.5, 5 mM sodium azide, 2 mM EGTA, pH 7.0, 2 mM ouabain, 2 mM dithiothreitol, 50 mM KCl, and 10 mM MgCl at 37°C for 5 min. Drug-stimulated activity was determined by the addition of verapamil to a final concentration of 250 nM from a stock solution prepared in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the reaction mixture was less than 1%, which had been shown not to affect ATPase activity. Parallel experiments were performed in the presence and absence of 300 μM vanadate. After incubation at 37°C for 3 min, the reaction was started by the addition of ATP to a final concentration of 5 mM and continued at 37°C for 20 min. The total volume of the reaction was 100 μl and was terminated by the addition of 100 μl of 5% SDS. The amount of inorganic phosphate released was measured by a colorimetric reaction as described previously (Hrycyna et al., 1998).

Results

Pgp1 Expression in Stable Transfectants. Stable transfectants were screened by Western blot analysis for Pgp1 expression. Only those transfectants that expressed comparable amounts of the protein were used for further study (Fig. 1). Crude plasma membrane preparations from each of these were also analyzed for Pgp levels (data not shown) to ensure that none of the mutations affected the targeting of Pgp to the membrane. Hence, the phenotypic differences observed between the transfectants can be attributed to the various forms of Pgp1 expressed.

Drug Cross-Resistance Profiles. To demonstrate that the TM9 double mutation was responsible for the cross-resistance phenotype displayed by DC-3F/ADII and to evaluate the contribution of each mutation separately, appropriate expression constructs were made and clones expressing each were tested for drug resistance. The ED_{50} values are presented in Tables 1 and 2. The numbers in parentheses in Table 1 are the relative resistance levels compared with those of DC-3F and are plotted as histograms in Fig. 2. Those in Table 2 indicate resistance levels relative to wild-type transfectants. All of the values shown in Table 1, except that for the N839I mutation with COLC, are significantly different (i.e., $P < 0.05$).

The cross-resistance profile conferred by the wild-type protein (Fig. 2) is very similar to that reported previously (Devine et al., 1992; Devine and Melera, 1994a; Ma et al., 1997), with maximal resistance to COLC and VCR and lower levels to ActD and DAUN. Although the absolute levels of resistance cannot be compared because DC-3F/ADII cells express >20 fold more Pgp1 than the transfectants (data not shown), the drug cross-resistance pattern displayed by the I837L/N839I transfectant was very similar to that of the DC-3F/ADII cell line (Fig. 2). In both cases, elevated resistance to ActD is accompanied by a decrease in resistance to both DAUN and COLC, with more moderate effects on VCR. Based upon the similarities of the cross-resistance profiles, it is clear that the double mutation in TM9 is responsible for the multidrug resistance phenotype displayed by DC-3F/ADII. Remarkably, this phenotype is very similar to that displayed by two other cell lines that were also selected for resistance to ActD, DC-3F/ADIV and DC-3F/ADX (Divine and Melera, 1994a), and shown to overexpress a double mutant form of Pgp1. In both of those cases, however, the mutation, G338A/A339P, occurs in TM6. Hence, TM9 and TM6 represent two different regions within Pgp1 the amino sequences of which, when altered, affect cross-resistance patterns in similar ways. When the two mutant regions are expressed together in a chimeric protein, the level of ActD resistance, relative to DC-3F, is 10-fold higher than that observed from the TM9 mutations alone. Resistance to VCR is increased 5-fold, whereas that to COLC and DAUN is increased by approximately 30 and 65%, respectively (Table 1).

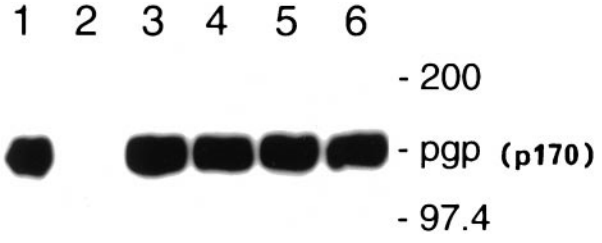

Fig. 1. Western blot analysis of Pgp1 expression in wild-type and mutant transfectants. Total cellular lysate (30 μg) was analyzed by 7.5% SDS-PAGE without boiling and transferred to Immobilon-P membranes (Millipore, Bedford, MA) for analysis. Anti-hamster P-glycoprotein monoclonal antibody at a dilution of 1:250 was used as a probe. Lane 1, wild-type transfectant; lane 2, drug sensitive DC-3F control cell line; lane 3, I837L; lane 4, N839I; lane 5, I837L/N839I (TM9 double mutant); lane 6, TM6/TM9 chimera (G338A/A339P/I837L/N839I). Mutant and wild-type transfectants expressed Pgp-1 to similar levels.

The contribution of each of the mutations to the phenotype conferred by the double mutation was also determined. When compared with DC-3F cells, expression of either of the individual mutations significantly increased resistance to each of the four drugs ($P < 0.05$), with the exception of N839I, which did not alter resistance to COLC (Table 1). However, neither of the single mutations independently conferred a cross-resistance pattern similar to that of the double mutation (Fig. 2). When the ED_{50} values for each of the mutant transfectants were compared with those for the wild-type (Table 2), it was found that neither had significantly increased resistance to ActD. Indeed, the I837L mutation slightly lowered resistance to VCR and had no effect on the other three drugs, whereas the N839I mutation reduced resistance to three of the drugs but did not significantly alter that to ActD. By contrast, the double mutation did significantly increase resistance to ActD by 1.5-fold ($P < 0.05$). Therefore, the combination of I837L and N839I is required to confer the DC-3F/ADII phenotype.

Reversal of Drug Toxicity with CsA and VRP. The data presented in Table 3 show the effects of the TM9 mutations on the ability of CsA and VRP to reverse drug resistance. Neither of the single mutations nor the double mutation affected the overall effectiveness of CsA as a reversal agent, although a modest 2-fold increase in the RD_{50} value for DAUN reversal was noted for the I837L mutation and the double mutant (P values < 0.05). The 2.3-fold increase shown for ActD in the double mutant is not significant because of the large variations in the values obtained. None of the TM9 mutations had a significant effect on the activity of VRP. These results are opposite those previously reported for TM6, which showed that the single A339P mutation and the G338A/A339P double mutant dramatically increased the RD_{50} values of CsA for all drugs and significantly increased the RD_{50} value of VRP for COLC as well (Ma et al., 1997). When the TM9/TM6 chimeric protein was analyzed (Table 3), the results were very similar to those observed for the TM6 mutations alone. Hence, in contrast to TM6, mutations in TM9 that affect drug cross-resistance have a minimal effect on the reversal activity of CsA or VRP.

[^{125}I]IAAP Labeling of Mutant Pgp1 and Competition with CsA. The effect of TM9 and TM6 mutations on the direct interaction of Pgp1 with substrate was evaluated by photolabeling of plasma membranes with [^{125}I]IAAP. As shown in Fig. 3 the Pgp1 expressed by the wild-type transfectant (Fig. 3A, lane 1) and by the I837L, N839I, and I837L/N839I 9 mutant transfectants (Fig. 3, B-D, lane 1, respectively), and by DC-3F/ADII cells (Fig. 3E, lane 1) all labeled to a similar extent when exposed to [^{125}I]IAAP. Incubation of the membranes with 0.8 μM CsA before exposure was sufficient to eliminate the majority of labeling in all cases, although some residual signal did remain in the N839I, I837L/N839I, and DC-3F/ADII, samples (Fig. 3, C-E, lane 2), that was removed when the CsA concentration was increased to

4.0 μM (Fig. 3, C-E, lane 3). Hence, the TM9 mutations do not interfere with the ability of IAAP to label Pgp1 and have minimal effect on the ability of CsA to inhibit that labeling.

Figure 4 shows the result of a similar experiment carried out with Pgp1 expressed by the G338A, A339P, and G338A/A339P mutant transfectants and by DC-3F/ADX cells. In all cases, the extent of labeling in the absence of CsA was similar to that observed for the wild-type protein (Fig. 4, A-E, lane 1). Hence, the TM6 mutations do not affect the ability of IAAP to label Pgp1. However, although 0.8 μM CsA inhibited labeling of the wild-type protein, it required at least 4.0 μM to prevent labeling of the G338A and the G338A/A339P mutant proteins as well as those expressed by DC-3F/ADX cells (Fig. 4, B, D, and E). Labeling of the A339P mutant could not be prevented at concentrations as high as 16 μM (Fig. 4C). Hence, mutations in TM6 strongly affect the ability of CsA to compete with IAAP for labeling of Pgp1. When similar experiments were attempted with the TM9/TM6 chimeric protein, no IAAP labeling was observed in the absence of CsA (Fig. 4F, lane 3), indicating that when expressed in combination, these two double mutations disrupt the IAAP labeling site.

Overall these results are consistent with the RD_{50} data presented in Table 3 showing that mutations in TM9 do not affect the ability of CsA to reverse drug resistance and with observations published previously (Chen et al., 1997; Ma et al., 1997) showing that TM6 represents a major CsA interaction site.

ATPase Activity. The results presented in Fig. 5, A and B, show the effects of the TM9 double mutation and of each of the single mutants, as well as the TM6 double mutation and the TM6/TM9 chimera, on both basal and VRP stimulated ATPase activity in transfectants. Both the I837L and N839I had small but significant ($P < 0.03$) effects on the basal ATPase activity. The former increased activity by 18%, whereas the latter decreased it by 20%. When expressed together in the TM9 double mutant, however, ATPase activity was restored to wild-type levels. Although the TM6 double mutant seemed to increase basal ATPase activity by 53%, the

TABLE 1

ED_{50} values for all cell lines relative to DC-3F

The values shown are the mean ± S.E.M. from three separate determinations. The numbers in parentheses indicate the fold resistance relative to the DC-3F parental cell line.

	ActD	COLC	VCR	DAUN
		ng/ml		
DC-3F	1.3 ± 0.1 (1)	35.2 ± 0.9 (1)	14.1 ± 0.8 (1)	23.8 ± 2.1 (1)
WT	3.5 ± 0.5 (2.7)	185.5 ± 13.4 (5.3)	71.9 ± 8.0 (5.1)	82.0 ± 3.3 (3.4)
I837L/N839I	5.3 ± 0.1 (4.0)	44.1 ± 0.5 (1.3)	45.7 ± 3.4 (3.2)	38.5 ± 2.6 (1.6)
I837L	3.5 ± 0.3 (2.7)	154.5 ± 1.5 (4.4)	45.7 ± 2.3 (3.2)	76.3 ± 2.6 (3.2)
N839I	4.7 ± 0.6 (3.6)	36.0 ± 0.6 (1.0)	17.6 ± 0.4 (1.2)	36.0 ± 0.5 (1.5)
T6/T9 Chimera	50.8 ± 5.0 (39.0)	61.7 ± 2.2 (1.8)	216.8 ± 8.7 (15.3)	60.0 ± 4.6 (2.5)
DC-3F/ADII	112.5 ± 10.9 (86.0)	40.3 ± 14.0 (6.8)	494.5 ± 19.2 (35.1)	187.0 ± 9.0 (7.8)

WT, wild-type.

TABLE 2

ED_{50} values of the mutant relative to the wild-type transfectants

The values shown are the mean ± S.E.M. from three separate determinations. The numbers in parentheses indicate the fold resistance of each mutant compared with wild-type transfectants.

	ActD	COLC	VCR	DAUN
		ng/ml		
WT	3.5 ± 0.5 (1)	185.5 ± 13.4 (1)	71.9 ± 8.0 (1)	82.0 ± 3.3 (1)
I837L/N839I	5.3 ± 0.1 (1.5)	44.1 ± 0.5 (0.2)	45.7 ± 3.4 (0.7)	38.5 ± 2.6 (0.5)
I837L	3.5 ± 0.3 (1)	154.5 ± 1.5 (0.8)	45.7 ± 2.3 (0.6)	76.3 ± 2.6 (0.9)
N839I	4.7 ± 0.6 (1.4)	36.0 ± 0.6 (0.2)	17.6 ± 0.4 (0.2)	36.0 ± 0.5 (0.4)
T6/T9 Chimera	50.8 ± 5.0 (15.0)	61.7 ± 2.2 (0.3)	216.1 ± 8.7 (3.0)	60.0 ± 4.6 (0.7)

WT, wild-type.

TABLE 3

RD_{50} values of CsA and VRP for all transfectants

The numbers in parentheses represent the ratios of the RD_{50} values normalized to those of the wild-type for each drug. The RD_{50} values for COLC and VCR could not be reliably obtained with the N839I transfectant or with the I837L/N839I transfectant because of the low resistance conferred by these mutations.

	ActD	COLC	VCR	DAUN
		ng/ml		
CsA				
WT	101 ± 8 (1.0)	70 ± 5 (1.0)	64 ± 1 (1.0)	90 ± 9 (1.0)
I837L	88 ± 5 (0.9)	67 ± 4 (1.0)	81 ± 4 (1.3)	195 ± 16 (2.2)
N839I	95 ± 8 (0.9)			97 ± 3 (1.1)
I837L/N839I	234 ± 96 (2.3)		106 ± 17 (1.7)	174 ± 22 (1.9)
TM6/TM9 Chimera	504 ± 19 (5.0)	883 ± 87 (12.6)	872 ± 35 (9.7)	757 ± 79 (8.4)
VRP				
WT	480 ± 41 (1.0)	169 ± 69 (1.0)	429 ± 14 (1.0)	277 ± 6 (1.0)
I837L	340 ± 39 (0.7)	189 ± 17 (1.1)	377 ± 2 (0.9)	347 ± 10 (1.3)
N839I	403 ± 8 (0.8)			361 ± 37 (1.3)
I837L/N839I	344 ± 12 (0.7)		429 ± 23 (1.0)	393 ± 49 (1.4)
TM6/TM9 Chimera	858 ± 84 (1.8)	>2000 (>12)	462 ± 20 (1.1)	1648 ± 92 (6.0)

WT, wild-type.

significance of this observation is questionable because of large variation in the data set ($P > 0.09$). The TM6/TM9 chimera, however, did increase the basal ATPase activity by 46% ($P < 0.05$). Overall, the effects of the TM9 mutations on basal ATPase activity were marginal and probably caused by small structural perturbations in the protein. However, the I837L and N839I mutations compliment each other to restore basal ATPase activity, suggesting that TM9 may play a role in the inherent catalytic activity of the protein.

Whereas the single TM9 mutations marginally affected basal ATPase activity, neither of them nor the TM9 double mutation had an effect on verapamil-stimulated ATPase activity (Fig. 5B). Hence, these mutations apparently do not affect the conformational changes that are thought to occur in the protein during drug-stimulated ATP hydrolysis. Both the TM6 double mutation and the TM6/TM9 chimera decreased verapamil stimulated ATPase activity by 20% ($P < 0.05$). The effect of the TM6 double mutation on verapamil-stimulated ATPase activity is consistent with the fact that insertion of a proline residue in TM6 (A339P) reduces verapamil stimulated ATPase activity, whereas the single G338A mutation does not (J.S. and P.W.M., submitted).

Discussion

The TM9 and TM6 mutants are both composed of two point mutations that change two amino acid residues within transmembrane spanning domains (Devine et al., 1992; Troyer et al., 1996). In the case of TM6, a rationale can be developed for the existence of both mutations and for the order in which they emerged. One of the TM6 mutations (A339P) increases resistance to the selective agent ActD, whereas the other (G338A) does not. When both are present, resistance is increased to a level greater than that conferred by the single

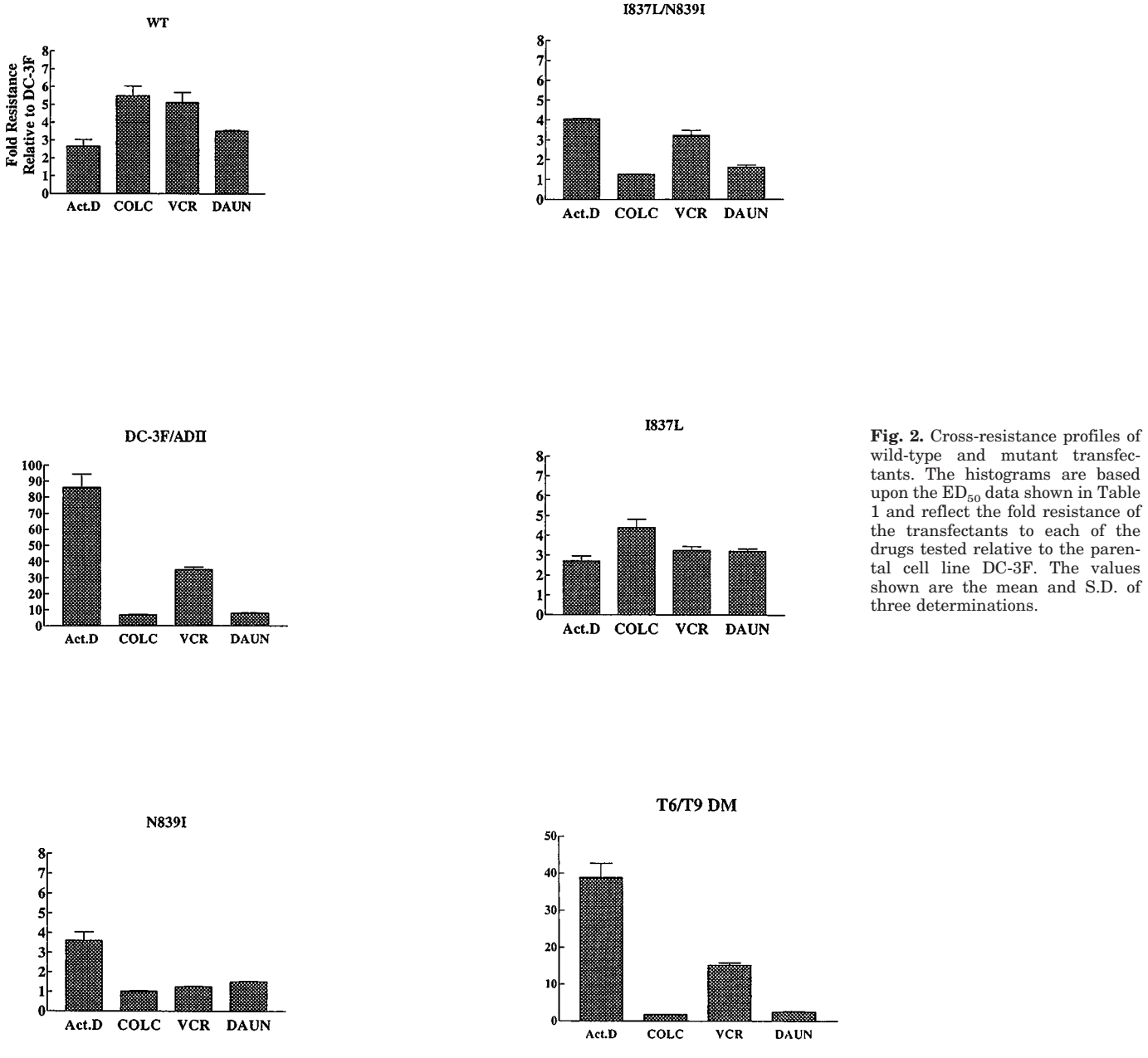

Fig. 2. Cross-resistance profiles of wild-type and mutant transfectants. The histograms are based upon the ED_{50} data shown in Table 1 and reflect the fold resistance of the transfectants to each of the drugs tested relative to the parental cell line DC-3F. The values shown are the mean and S.D. of three determinations.

mutations alone (Ma et al., 1997). Because G338A itself provides no selective advantage, it is likely that the A339P mutation preceded it and that G338A emerged during the selection process as the drug concentration was increased. It is more difficult to make a similar argument for TM9. Compared with wild-type, the I837L mutation clearly does not enhance resistance to ActD (Table 1) and provides no apparent selective advantage. Although the N839I mutation does increase resistance to ActD by 1.4-fold (Table 1), this value lies outside the 95% confidence window and is not significantly different ($P > 0.05$) than the wild-type. However, it may reflect a small increase that, in vivo, provides sufficient advantage to maintain the cell long enough to acquire an additional mutation(s) that ensures survival. In the case of DC-3F/ADII cells, that mutation is I837L, which, combined with N839I, does yield a significant ($P < 0.05$) 1.5-fold increase in resistance to ActD. Alternatively, because both of these mutations individually may be considered to be silent with respect to ActD resistance, either one or both may have pre-existed in the population, the other being acquired during the early stages of selection.

The finding that alterations in TM9 and TM6 can confer the same cross-resistance profile to four structurally different drugs suggests that both either directly or indirectly affect the ability of the transporter to recognize the structural determinants of each drug in similar ways. Because these determinants are fixed, it is difficult to visualize how both of these regions could recognize them at the same time, which would be required if the two regions were part of the same binding site. Although one could imagine that, during the conformational changes that are thought to occur during the transport process (Zhang et al., 1993; Loo and Clark, 1996a, 1997a; Mechetner et al., 1997; Ramachandra et al., 1998; Wang et al., 1998), residues within a common site could become rearranged to allow different combinations to bind drugs, recent reports based upon disulfide cross linking studies (Loo and Clark, 2000) have indicated that TM9 is not

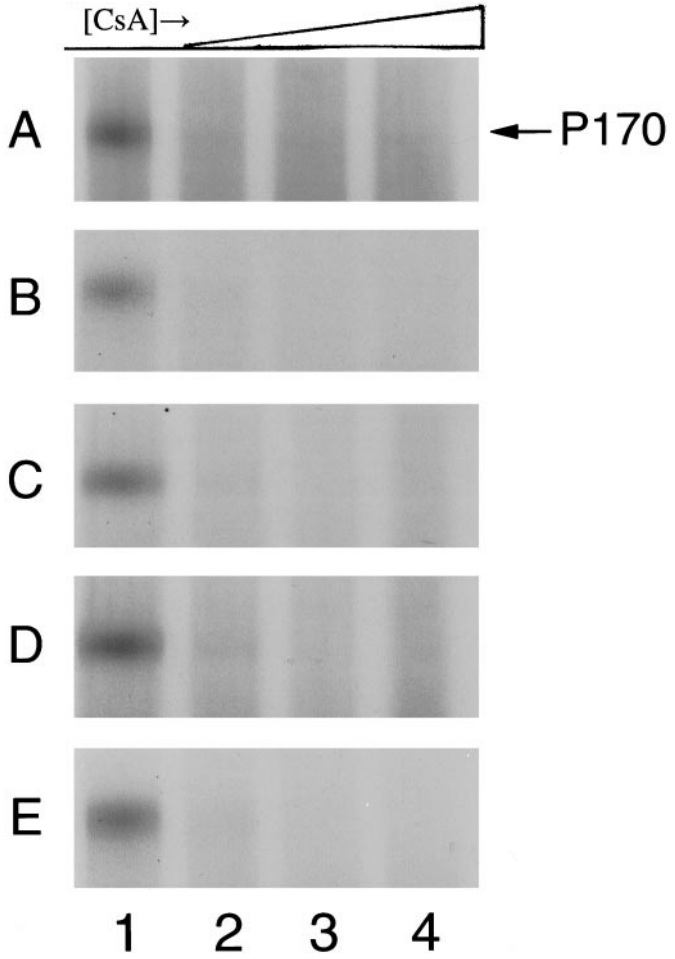

Fig. 3. [I^{125}]IAAP labeling of TM9 mutant transfectants and reversal by CsA. Membrane protein samples (50 μg) containing equivalent amounts of Pgp1 as indicated by Western blot analysis were incubated with either no CsA (lane 1), 0.8 μM CsA (lane 2), 4.0 μM CsA (lane 3), or 16 μM (lane 4) CsA before being labeled by [I^{125}]IAAP and analyzed by SDS-PAGE on 7.5% gels. A, wild-type; B, I837L; C, N839I; D, I837L/N839I; and E, DC-3F/ADII.

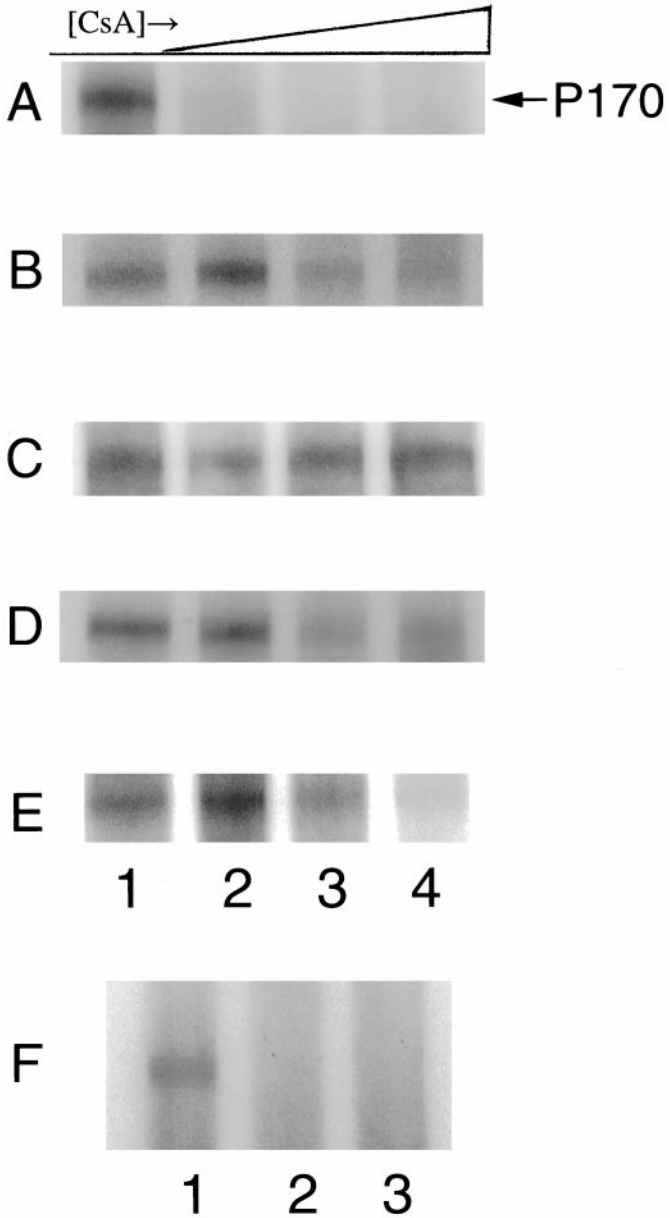

Fig. 4. [I^{125}]IAAP labeling of TM6 and TM9/TM6 mutant transfectants and reversal by CsA. Experiments were carried out as described in Fig. 3. A, wild-type; B, G338A; C, A339P; D, G338A/A339P double mutant; E, DC-3F/ADX cells. In F, no CsA was used. Lane 1, wild-type transfectant; lane 2, DC-3F cells; lane 3, TM9/TM6 chimera.

located within the proposed drug binding pocket of MDR1 that is predicted to be formed between TMs 4, 5, and 6 and 10, 11, and 12. Therefore, how TM9 would impose its affects upon drug recognition as a component of a common site is unclear, although it is possible that interactions with one or more of the TMs that form the pocket could in turn alter its configuration and therefore the ability of the transporter to recognize substrates. A similar argument has been proposed (Loo and Clark, 1999) to explain how the G185V mutation in MDR1, which also lies outside of the proposed drug binding pocket, alters the cross-resistance phenotype compared with the wild-type (Choi et al., 1989). The results presented here, however, indicate that mutations in TM9 are able to confer the same cross-resistance phenotype as mutations in TM6. This would require that the alterations to TM9 indirectly cause a structural change, presumably through interactions with one of the TMs forming the drug binding pocket, that mimic those imposed by alteration to TM6 itself. Although this might be the case, it is also possible that TM9 represents a drug recognition site different from that located within the proposed drug binding pocket. This site, perhaps through interactions with other membrane proteins, might be involved with facilitating passage of substrates from the membrane to the transporter or in directing them from the transporter to the outside of the cell.

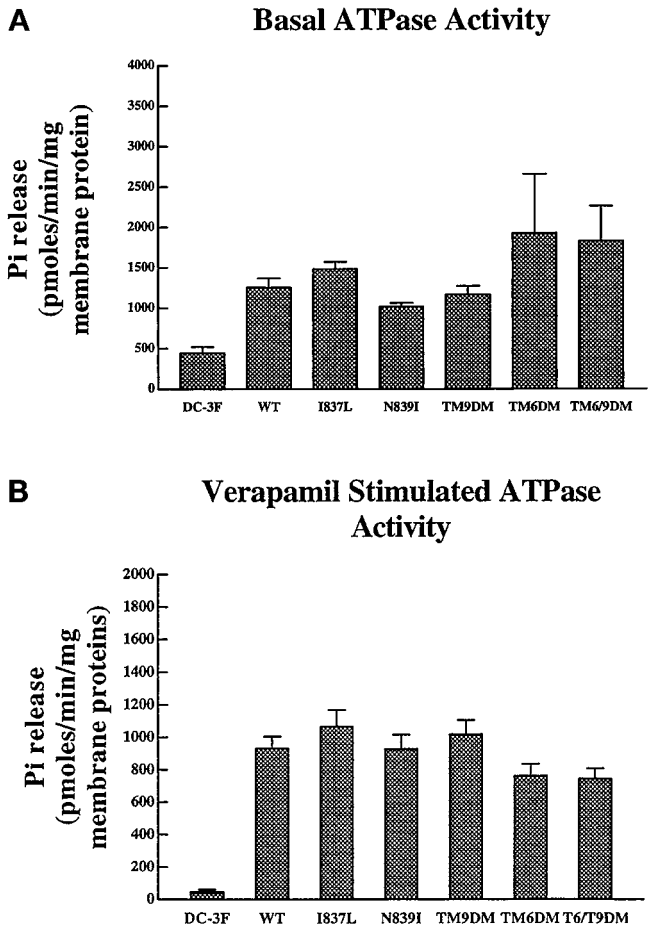

Fig. 5. Basal and verapamil-stimulated ATPase activity. A, basal ATPase activity. B, verapamil-stimulated ATPase activity is defined as the difference in phosphate release in the presence and absence of 250 μM verapamil, as described under *Materials and Methods*. Each value shown is the mean and S.D. of three determinations.

TM9 is the only transmembrane domain in Pgp1 that does not contain any aromatic amino acid residues (Pawagi et al., 1994), which supports the notion that TM9 may represent or be a component of a unique drug recognition site. Such residues are thought to form sterically compatible binding sites that allow the passage of a variety of ring-containing compounds across the membrane (Pawagi et al., 1994). In their absence, affinity for lipophilic drugs would be expected to be low, consistent with the characteristics of a site that interacts with substrates at high concentrations, as would be found in the membrane (Shapiro et al., 1997). Moreover, such a site would not necessarily be directly involved with passage of a substrate across the membrane, but rather with facilitating the movement of substrates from within the membrane to the transporter itself. Alternatively, it might be involved with releasing substrate from the transporter to the extracellular media. TM9 is unique in this regard as well in that an unconventional structure of Pgp-1 predicts that the loop region linking TM9 with TM8 may be located on the extracellular as opposed to the cytosolic side of the membrane, which would place TM9, perhaps transiently, on the surface of the cell (Skach et al., 1993; Zhang et al., 1996).

Unlike the G185V mutation, whose pleiotropic effects on MDR1 function (Ramachandra et al., 1996) were interpreted to be caused by higher order structural perturbations, the TM9 mutations displayed no such effects on Pgp1 function. In fact, little or no alteration in the effectiveness of the reversal agents CsA and VRP was found, nor was the ability of the mutant protein to label with IAAP impaired. In addition these mutations had minimal effect on the ability of CsA to inhibit IAAP labeling and only marginally affected the ATPase activity of the protein. Indeed, the major effect was to alter the drug cross-resistance phenotype by actually lowering the ability of the protein to confer resistance to COLC, VCR, and DAUN while increasing it to ActD (Table 2). This implies a direct effect on the ability of the protein to recognize substrates and suggests that TM9 is involved in such a role.

Although alterations to TM9 and TM6 can generate the same cross-resistance phenotype, they cause different effects as well. Mutations to TM9 do not affect the ability of CsA to reverse drug resistance or to interfere with [^{125}I]IAAP labeling of Pgp1, whereas mutations to TM6 greatly affect both. Moreover, mutations to TM9 do not alter [^{125}I]IAAP labeling, suggesting that this region of the C-terminal half of the protein alone does not mediate its interaction with IAAP. Similarly, mutation of the N-terminally located TM6 does not alter [^{125}I]IAAP labeling. Yet when the TM9 and TM6 mutations are expressed as a chimera, [^{125}I]IAAP labeling is abolished (Fig. 4F). Because it has been shown that TM6 in MDR1 labels with [^{125}I]IAAP, whereas TM9 does not (Greenberger, 1993), our data suggest that TM9 and TM6 act together, perhaps indirectly, to form the [^{125}I]IAAP binding site, as opposed to its labeling sites. The inability to label with [^{125}I]IAAP does not affect the ability of the chimeric protein to confer drug resistance, however; in fact, ActD and VCR resistance is greatly enhanced in chimera expressing transfectants, whereas resistance to COLC and DAUN is greatly reduced (Fig. 2). This is consistent with published observations showing that the binding site for a Bolton-Hunter derivative of DAUN differs from that for IAAP (Demmer et al., 1999).

The relative increase in ActD resistance conferred by the

TM6 mutations compared with wild-type in independent experiments in which Pgp1 expression levels vary considerably, ranges from 2.5- to 6-fold (Devine et al., 1992, 1994a; Ma et al., 1997). By comparison, the relative increase in ActD resistance in transfectants expressing the TM9 mutations is 1.5-fold (Table 2). In transfectants expressing the chimera, however, resistance to this drug is increased 15-fold more than wild-type (Table 2) and is twice that expected from adding the effects of the two mutations alone (i.e., 6 + 1.5 = 7.5). Explanations for this type of synergy include the possibility that the mutations alter residues that act cooperatively to facilitate the same process or that they cause extensive unfolding of the protein (Weber et al., 1990). Extensive unfolding of the chimeric protein is unlikely because the transporter remains active and maintains ATPase activity near that of wild-type (Fig. 5). Therefore, we interpret these results to suggest that TM9 and TM6 together are instrumental in mediating the transport process. Clarification of these and other issues concerning the role of TM9 role in Pgp1 function and in the protein's ability to recognize and transport such a wide variety of substrates remains the subject for future studies.

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