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Mutations in the Sixth Transmembrane Domain of P-Glycoprotein that Alter the Pattern of Cross-resistance Also Alter Sensitivity to Cyclosporin A Reversal

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SUMMARY

The expression of a P-glycoprotein (Pgp1) cDNA encoding two amino acid substitutions in the sixth transmembrane domain of the protein (G338A339 to A338P339) confers a unique cross-resistance profile that displays preferential resistance to actinomycin D and diminished resistance to colchicine and daunorubicin. We report here that this multidrug-resistant phenotype is also insensitive to reversal by cyclosporin A (CsA) but not verapamil (VRP). However, the ability of VRP to increase the accumulation of [^{3}H]vincristine is poor in both wild-type and mutant transfectants. In contrast, the accumulation of [^{3}H]vincristine in wild-type versus mutant transfectants in the presence of CsA is dramatically increased. It is the substitution of the alanine residue at position 339 with proline that is primarily responsible for the lowered sensitivity to CsA and for the altered drug accumulation levels. Both substitutions are required to confer the unique cross-resistance profile of the double mutant, although each independently confers a specific profile of its own. These results indicate that alterations in Pgp1 structure can differentially affect the activity of CsA and VRP to mediate drug accumulation in multidrug-resistant cells and support the conclusion that the sixth transmembrane domain of the Pgp1 transporter plays important roles, in both the specificity of drug efflux and the sensitivity of the transporter to reversal agents.

An MDR phenotype is conferred to mammalian cells on overexpression of Pgp, a member of the ATP binding cassette superfamily of membrane transporters that share a basic structure that has been conserved from bacteria to humans (1). More than 50 members of the family have been described and been shown to be associated with movement, across the plasma membrane, of a wide variety of substances ranging from the influx of ions, in the case of the cystic fibrosis transporter (2, 3), to the extracellular transport of protein by the HYL B gene product of *Escherichia coli* (4, 5). Therefore, the "2 × 6" helix paradigm (1), which refers to each of the two halves of the Pgp molecule as being composed of six transmembrane α-helical segments and an ATP binding fold, has evolved to accommodate the transport of a wide variety of substances, including many natural-product anticancer drugs. Much attention, therefore, has been directed toward understanding the mechanism of action of Pgp, and other members of the family as well, with the intent of determining those regions responsible for substrate recognition and transport.

One of the approaches used for these studies has been to identify and characterize naturally occurring or site-directed mutant forms of the protein (6). Results to date have shown that mutations able to affect the cross-resistance phenotype mediated by Pgp1 and its homologs in human and mouse (MDR1 and mdr1a/MDR3, respectively; Ref. 7) are scattered throughout the protein (8–12), suggesting that the specificity for drug recognition and transport requires a higher order structure. Similar conclusions have been drawn from studies of the effectiveness of some of these mutations on the activity of reversal agents (13) that have been reported to act as competitive inhibitors of drug efflux, thereby enhancing intracellular drug accumulation and toxicity (14–16). Finally, photoaffinity labeling studies have identified multiple sites in the protein that may act either in combination to form a single complex binding site or independently to form multiple sites (17–20).

Alterations to the TM6 region of the protein have been shown to affect function, not only in Pgp1 but also in the cystic fibrosis conductance regulator transporter (4, 21). We initially reported a naturally occurring double mutation within TM6 in Chinese hamster Pgp1 that converts the wild-type sequence G338A339 to A338P339, resulting in an alter-

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ABBREVIATIONS: MDR, multidrug resistance; Pgp, P-glycoprotein; neo, neomycin; ActD, actinomycin D; CsA, cyclosporin A; VRP, verapamil; COLC, colchicine; VCR, vincristine; DAUN, daunorubicin.

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ation of the cross-resistance profile in transfectants and an enhancement of ActD resistance compared with the wild-type (1, 22). The importance of TM6 to Pgp1 function was further emphasized in a site-directed mutagenesis study of human MDR1 in which alterations to 5 of the 21 amino acid residues thought to compose TM6 altered or incapacitated Pgp activity (23). In another study, conversion of Arg347 to aspartic acid in TM6 of the cystic fibrosis conductance regulator protein has been shown to alter the selectivity of ion transport (21), whereas photoaffinity labeling studies have identified TM6, or a region immediately carboxyl-terminal to it, as a primary labeling site for the drug analog iodoarylazidoprazosin (20). Taken together, these date strongly suggest that TM6 and regions adjacent to it may serve a key role in the mechanism of action of Pgp1.

In an effort to further develop an understanding of the functional role played by TM6, we prepared through the use of site-directed mutagenesis individual full-length Pgp1 cDNAs that contain single mutations corresponding to each of those found in the hamster TM6 double mutant and, for the current study, analyzed the contribution made by each to the overall cross-resistance pattern. We also determined the effects of the double and single mutants on the sensitivity of hamster Pgp1 to the reversal agents CsA and VRP. Our results show that although each of the single mutations confers its own unique cross-resistance pattern in transfectants, both are required for expression of the pattern originally observed in the double mutant. We also show that TM6 is likely to be a major site for the interaction for CsA because for three of the four drugs tested, mutations in this domain render the protein insensitive to reversal by this agent but not VRP.

Materials and Methods

Cell lines and drugs. The Chinese hamster lung fibroblast cell line DC-3F and the transfected cell lines 212S17 and 110S23, which express equivalent levels of the wild-type, G338A339, and double mutant, A338P339, forms of Pgp1, respectively, have been previously described (1) and are referred to as DC-3F/G338A339, and DC-3F/A338P339, respectively. ActD, COLC, and VRP were purchased from Sigma Chemical (St. Louis, MO). VCR, DAUN, and CsA were generous gifts from Eli Lilly (Indianapolis, IN), Wyeth-Ayerst (Princeton, NJ), and Sandoz (East Hanover, NJ). VRP and CsA were dissolved in 15% and 100% ethanol to final concentrations of 8 and 1 mg/ml, respectively, and diluted directly into the media to the desired concentrations. Other drugs were dissolved in 0.9% NaCl and filter-sterilized. All assays were conducted using a single batch of drug that had been frozen in aliquots and calibrated for potency before each use. Control assays were performed on all cell lines to demonstrate that the amounts of CsA and VRP that were used were not toxic to cells and that the maximal amount of ethanol introduced with the drugs did not in itself affect the level or pattern of cross-resistance.

Site-specific mutagenesis and development of transfectants. Site-directed mutagenesis was accomplished using the Altered Sites *In Vitro* Mutagenesis System from Promega (Madison, WI). Oligonucleotides were generated from an Applied Biosystems (Norwalk, CT) PCR Mate 391 DNA Synthesizer according to the manufacturer's instructions. A 2.1-kb *Bam*HI/*Hin*dIII DNA fragment from the 5′ end of the wild-type Pgp1 cDNA that contains the TM6 domain (8) was used as the starting template. The single mutant forms corresponding to a conversion of G338 to alanine and A339 to proline were prepared with the use of GCTGTATTAATTGCGGCATTCAGTATTGGAC and TTGCTGTATTAATTGGGCCATTCAGTATTGG, respectively (underlined sequences indicate the codons that are changed from the wild-type). After the desired nucleotide sequence alterations were confirmed by DNA sequencing to be the only alterations present, the mutant 2.1-kb fragments were used to replace the corresponding wild-type fragments in full-length Pgp1 cDNAs, and the resulting single mutant forms of the cDNA were subsequently inserted into the eukaryotic expression vector pHBneo (24). These were then transfected into parental drug-sensitive DC-3F cells as previously described (1). Stable transfectants were selected with G418 (800 ng/ml, GIBCO, Grand Island, NY), and neo^+ clones were expanded and analyzed for Pgp1 expression levels via Western blot analysis of total cell protein using the C219 monoclonal antibody and the ECL system (Amersham, Arlington Heights, IL) as previously described (25). Two clones, DC-3F/A338 and DC-3F/P339, that express the respective single mutant forms of Pgp1 to levels equivalent to those of DC-3F/G338A339 and DC-3F/A338P339 were chosen for further study.

Drug resistance measurements. The method has been previously described for determination of the ED_{50} value, which is defined as the drug dose required to reduce cell numbers to 50% of control values over the course of a 72-hr growth period (1). The relative resistance of the transfected cell lines to each of the four drugs tested was determined by comparison of the individual ED_{50} values with those of parental DC-3F cells. The dose of reversal agent required to reduce resistance to 50% of that observed in the absence of such an agent is defined as the RD_{50}; this value was determined by linear regression analysis of data obtained from plots that compared the percentage of the original resistance versus the reversal agent concentration. Changes in the RD_{50} values were used to evaluate the effects of the various mutations on the ability of both CsA and VRP to reverse resistance.

Drug accumulation assays. Cells (1×10^4) were plated in minimal essential medium/Ham's F-12 medium supplemented with 5% fetal bovine serum and antibiotics (GIBCO BRL, Gaithersburg, MD) onto 35-mm tissue culture dishes as previously described (22). After 72 hr at 37° in a 5% CO_2 atmosphere, the original media was replaced with 1 ml of fresh media containing 0.25 μCi of [^{3}H]VCR (8.6–17.9 Ci/mmol; Amersham) to a final concentration of 19 nM and the desired amount of reversal agent. The cells were then incubated at 37° with 5% CO_2 for varying periods of time, washed with room-temperature phosphate-buffered saline followed by immersion in ice-cold phosphate-buffered saline for 5 min, and then removed from the dishes by rigorous pipetting. An aliquot of the resulting cell suspension was removed for determination of cell number via the use of a hemocytometer, and the remainder was spun briefly in a microcentrifuge. The resulting pellets were drained and then solubilized in 100 μl of 1 N NaOH and neutralized by the addition of 100 μl of 1 N HCl, and the entire sample was mixed with 10 ml of scintillation cocktail and counted in a Packard TriCarb Scintillation Spectrometer. The final cpm were normalized to that of 1×10^6 cells.

Results

Cross-resistance profiles of the single mutants. To identify transfectants with expression levels of the single mutant forms of hamster Pgp1 (Fig. 1A) equivalent to the levels of the wild-type and double mutant forms expressed by DC-3F/G338A339 and DC-3F/A338P339, respectively, we screened populations of DC-3F cells that had been transfected with the vector phBneo into which had been cloned a full-length copy of a hamster Pgp1 cDNA that contained either the single A338 or the single P339 mutation. The neo-resistant colonies were identified and after expansion

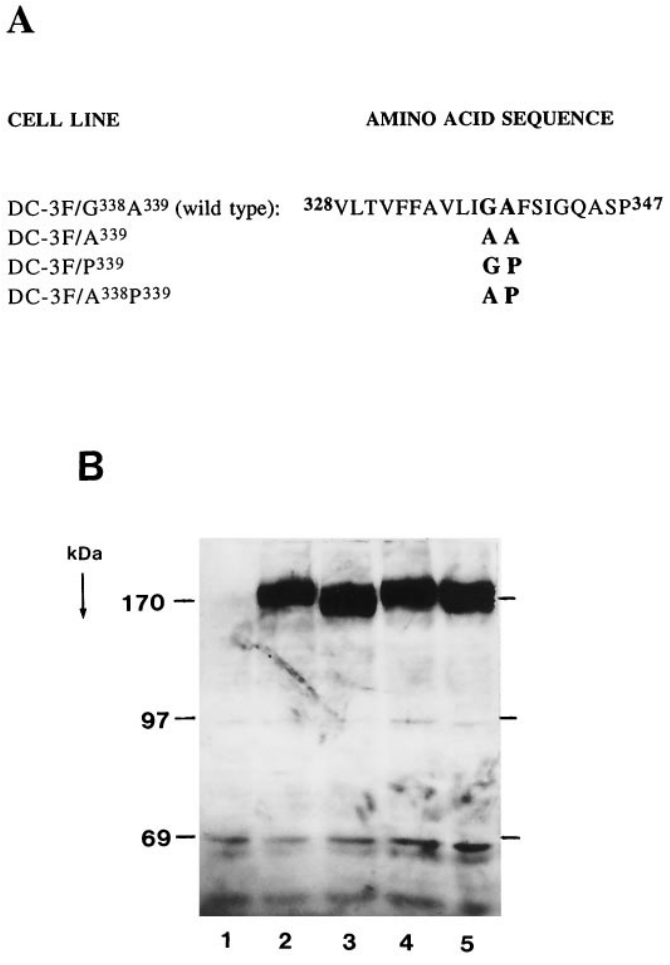

Fig. 1. Amino acid sequence of the wild-type and mutant forms of the putative TM6 domain of Pgp1 and Western blot analysis of wild-type and mutant Pgp1 expression. A, Amino acid sequences of the putative wild-type TM6 domain of the Chinese hamster Pgp1 gene and its mutant forms. B, Western blot analysis of cell lysates from wild-type and mutant transfectants. Protein (50 μg) was applied to a 7.5% sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane, and Pgp was detected using the ECL method (Amersham) with the monoclonal antibody C219 (Signet) as probe. *Lane 1*, parental cell line DC-3F. *Lane 2*, DC-3F/A338P339. *Lane 3*, DC-3F/G338A339. *Lane 4*, DC-3F/P339. *Lane 5*, DC-3F/A338.

analyzed for the level of expression of Pgp via Western blotting using the monoclonal antibody C219. Because prior analysis (22) had shown that no significant differences in the level of Pgp detected in DC-3F cells or transfectants could be demonstrated by using plasma membrane versus whole-cell preparations, we chose to use the latter for these studies. As shown in Fig. 1B, two transfectants, DC-3F/A338 and DC-3F/P339 that expressed the appropriate levels of the single A338 and P339 mutant forms of Pgp1 were identified and used for the remainder of the study.

The results of experiments designed to determine the cross-resistance profiles mediated by the single mutant forms of Pgp1 are shown in Fig. 2. The histograms indicate the fold resistance expressed by the transfectants to the four drugs tested (ActD, VCR, COLC, and DAUN) compared with drug-sensitive DC-3F cells. Table 1 presents these data in tabular form but shows the relative changes in resistance levels of the mutant transfectants to those of the wild-type. The single A338 mutation has a strong effect on VCR resistance, increasing it 3-fold, but little impact on resistance to ActD. In comparison, the single P339 mutation, although having very little effect on the level of VCR resistance, increases resistance to ActD by 2.7-fold. Expression of the double mutant results in a level of resistance to ActD that is greater (6.6-fold) than the sum of that observed from the single mutants, whereas the level of resistance to VCR (2.1-fold) reflects their average.

For COLC and DAUN, the single A338 mutation enhances resistance to both drugs by 2–3-fold compared with the wild-type, whereas the P339 mutation has little, if any, effect on resistance to either one (Table 1). When expressed together in the double mutant, however, P339 suppresses the positive effect of the A338 mutation and reduces resistance to both drugs to levels below those of the wild-type. Hence, although the amino acid residues present at positions 338 and 339 individually play a role in establishing the cross-resistance profile conferred by Pgp1 and alterations to either of them can influence that profile, it is the combination of mutations that dictates the unique profile displayed by the double mutant.

Mutations in TM6 alter the sensitivity of Pgp1 transfectants to reversal agents. To establish whether mutations that alter cross-resistance also have an impact on the sensitivity of the transporter to reversal agents, we determined the RD_{50} value for VRP and CsA in the wild-type, double mutant, and single mutant transfectants; the results of these experiments are shown in Fig. 3 and Table 2.

As shown in Fig. 3, the level of resistance mediated by the transfected Pgp1 cDNAs decreased in a dose-dependent fashion in response to either reversal agent. However, in the case of CsA (Fig. 3), introduction of the P339 mutation (C) shifted the curve for each of the four drugs tested, far to the right, indicating that this mutation rendered the transporter relatively insensitive to CsA. Introduction of the A338 mutation (B) had no such effect, but the double mutation gave results similar to those observed with P339 alone (D). Interestingly, similar studies with VRP (Fig. 3, E–H) indicated that for three of the four drugs tested (ActD, DAUN, and VCR), none of the TM6 mutations substantially affected the sensitivity of the transporter. In the case of COLC, however, both the single A338 and P339 mutations, as well as the double mutation, shifted the curve to the right (Fig. 3, F–H).

To further evaluate these results, the data from Fig. 3 were subjected to linear regression analysis to determine the RD_{50} values for each combination of drug, mutation, and reversal agent (Table 2). These values were then normalized to those of the wild-type transfectants. The relative differences are shown within parentheses.

Introduction of the A338 mutation has only a minor effect on the sensitivity of the transporter to reversal by CsA, increasing the RD_{50} from 0-fold for DAUN to 2.6-fold for ActD. The P339 mutation, however, severely disables the effectiveness of this reversal agent for all four drugs, elevating the RD_{50} value from 13-fold for DAUN to 29.3-fold for VCR. The double mutant displays a similar insensitivity, but the effect of the proline residue is apparently moderated in all cases by the presence of A338.

Both of the single mutations, as well as the double mutation, however, are much less effective in their ability to

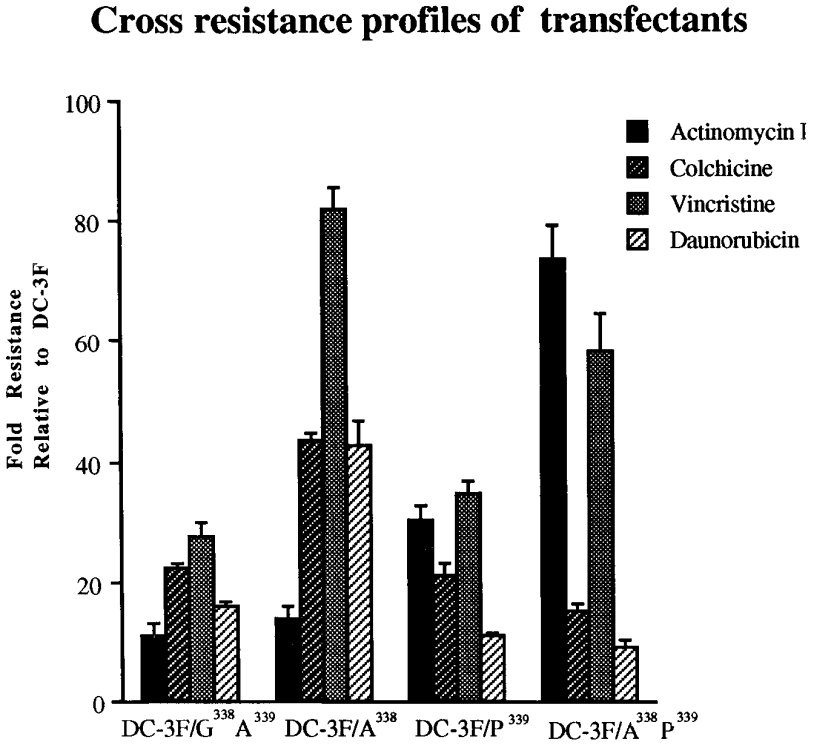

Fig. 2. Drug-resistance patterns of wild-type and mutant transfectants. ED_{50} values for four drugs were determined as previously described (22). Fold resistance is determined by dividing the ED_{50} value of the respective transfected cell line by that of parental drug sensitive DC-3F cells. The values shown are the mean of three determinations for each of the drugs tested. *Error bars,* standard deviation. Transfectants analyzed include DC-3F/G338A339 (wild-type), DC-3F/A338 (single mutant), DC-3F/P339 (single mutant), and DC-3F/A338P339 (double mutant).

TABLE 1

Relative changes in the ED_{50} values between mutant and wild-type transfectants

The ratios shown are derrived from the respective drug resistance values plotted in Fig. 2. The values reported for the mutant tranfectants are normalized to those of the wild-type.

	DC-3F/$G^{338}A^{339}$	DC-3F/A^{338}	DC-3F/P^{339}	DC-3F/$A^{338}P^{339}$
ActD	1.0	1.2	2.7	6.6
VCR	1.0	3.0	1.3	2.1
COLC	1.0	2.2	1.0	0.7
DAUN	1.0	2.7	0.8	0.6

desensitize the transporter to VRP (Table 2). Although introduction of the A338 or P339 mutation does elevate the RD_{50} value for VRP reversal of COLC resistance by 3.8- and 5.2-fold, respectively, and the double mutant elevates it by 8.1-fold, the ability of VRP to reverse resistance to the other three drugs is not substantially affected by any of the mutations tested.

It is unlikely that the effects seen here are due to the potential cytotoxic side effects of the reversal agents themselves because growth of the individual transfected cell lines was not affected by the highest concentrations of the agents used in each case (data not shown). We conclude, therefore, that the A338P339 double mutant yields an MDR phenotype that is clearly insensitive to reversal by CsA and that the main contributor to that insensitivity is the P339 substitution.

The effect of TM6 mutations on the accumulation of [^{3}H]VCR. Because the TM6 mutations differentially affect the ability of CsA and VRP to reverse resistance, we sought to determine whether in the presence of either of these agents, there were commensurate changes in the ability of the cells to accumulate [^{3}H]VCR.

Different amounts of CsA or VRP, varying in concentration from 0.01–2.0 μg/ml, which corresponds to the range of values determined to be effective in the RD_{50} studies (Table 2), were used in the drug accumulation assays. Under the conditions used here, DC-3F cells accumulate 5000 cpm of [^{3}H]VCR (~0.4 pmol) during a 5-hr exposure to the drug (Fig. 4A). Hence, the very low level of endogenous Pgp1 thought to be present in these cells (22) is insufficient to prevent drug accumulation in the presence of 19 nM VCR (see Materials and Methods). Interestingly, the addition of CsA or VRP, at 0.2 μg and 0.4 μg/ml, respectively, enhances accumulation to 0.80 and 0.56 pmol, whereas at higher concentrations (2.0 μg and 4.0 μg/ml, which are the equivalent of 1.6 and 8.0 μM, respectively), these agents increase accumulation to 1.0 pmol. Hence, VRP is five times less effective than CsA in its ability to enhance accumulation of VCR in DC-3F cells. However, it is not clear to what extent these results are dependent on the very low levels of Pgp1 present in these cells.

In the presence of an overexpressed wild-type Pgp1 transgene, the accumulation of [^{3}H]VCR in DC-3F cells is effectively inhibited (Fig. 4B), but at a concentration of 0.1 μg/ml, which is similar to the 0.2 μg/ml dose found to increase accumulation in DC-3F cells, CsA is unable to enhance the accumulation of drug (Fig. 4B). Hence, the transfectants are more insensitive to the presence of CsA than are DC-3F parent cells, an observation that is consistent with the fact that the expression level of Pgp1 in the transfectants is likely to be on the order of ≥10-fold higher than that of the controls (22). At 1.0 μg/ml, CsA is able to enhance accumulation to DC-3F levels (to 0.4 pmol), suggesting that this dose is able to reverse the effects of the Pgp1 contributed by the transgene. At 2.0 μg/ml, accumulation in the transfectants exceeds that value and reaches 0.64 pmol in 5 hr. This value approaches that seen in DC-3F cells treated with 2 μg/ml CsA (1 pmol) (Fig. 4A), and given the apparent dose response, suggests that further increases in the CsA concentration would permit these transfectants to accumulate VCR to that level as well.

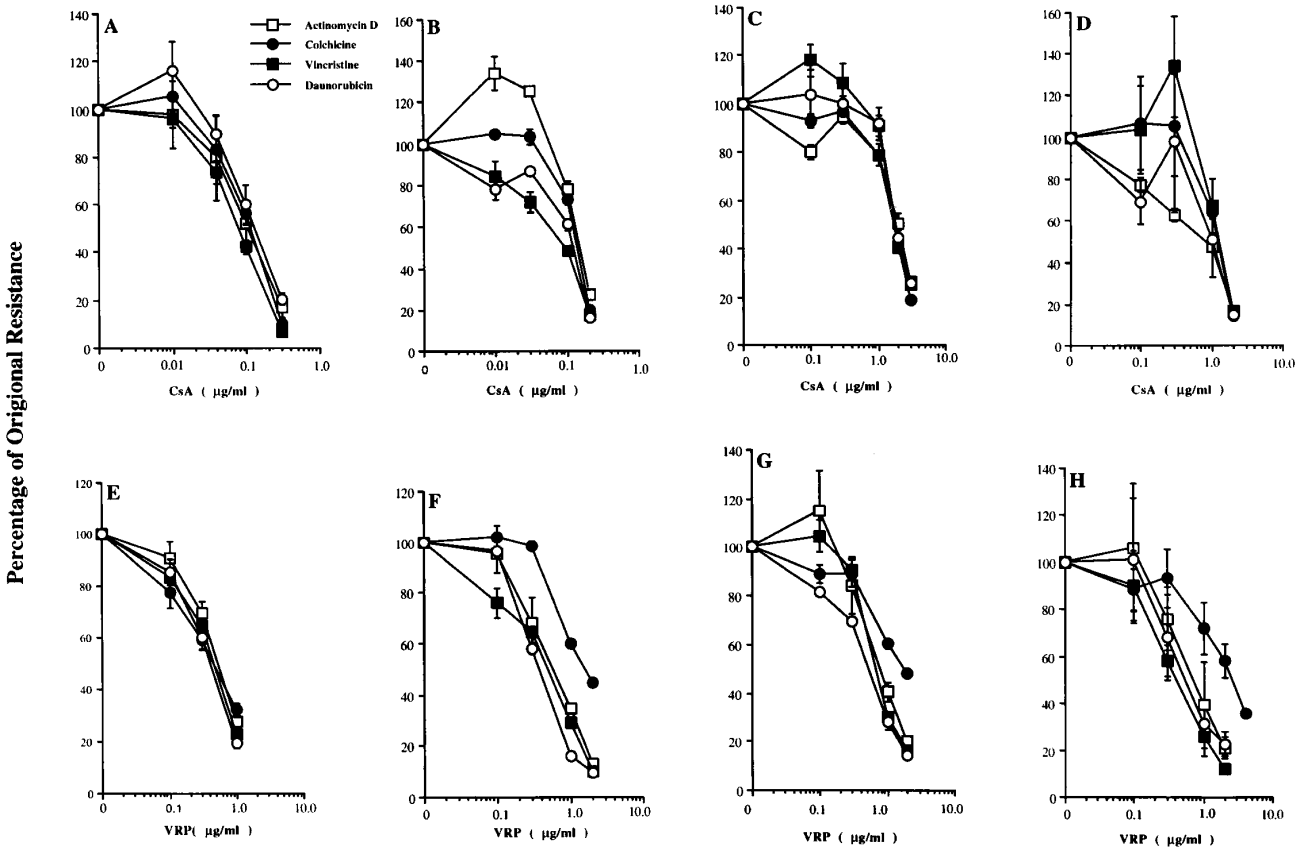

Fig. 3. Reversal of drug resistance in transfectants by CsA or VRP. The ED_{50} values for ActD, COLC, VCR, and DAUN in the presence of varying concentrations of CsA or VRP were determined by linear regression analysis of drug dose-versus-cell survival curves (data not shown). Resistance as a percentage of that observed in the absence of reversal agent is plotted against the range of concentrations of CsA (A–D) or VRP (E–H) that were tested. A and E, DC-3F/G338A339. B and F, DC-3F/A338. C and G, DC-3F/P339. D and H, DC-3F/A338P339. All assays were performed a minimal of three times, and the mean values are shown.

The results with VRP are shown in Fig. 4F. As expected from the results with DC-3F cells, VRP was much less effective than CsA in enhancing the accumulation of VCR in wild-type transfectants. At 2 μg/ml (4 μM), only 0.1 pmol of drug was accumulated in 5 hr, whereas at less than half of that dose, 1.6 μM, CsA allowed an accumulation of 0.64 pmol. In the presence of the wild-type transgene, therefore, VRP is nearly 10-fold less effective than CsA in its ability to enhance accumulation within 5 hr. This is consistent with the fact that the transfectants contain substantially more Pgp1 than control DC-3F cells. Nevertheless, VCR does accumulate in the wild-type transfectants, but it does so in a much slower fashion than in the presence of CsA. Because DC-3F cells and their MDR variants are collaterally sensitive to VRP (28), we did not use doses higher than 4 μg/ml (8 μM) to evaluate its ability to enhance drug accumulation in transfectants.

Introduction of the A338 mutation reduces the ability of CsA, at 1.0 μg/ml, to enhance [^{3}H]VCR accumulation in transfectants by $\geq$5-fold (Fig. 4C). This reflects the results of the reversal studies reported in Table 2, which showed that approximately twice as much CsA is required to reach the RD_{50} for VCR in cells expressing the A338 mutation. Increasing the amount of CsA to 2.0 μg/ml does increase accumulation but not to the extent seen in the wild-type (Fig. 4B). Hence, the A338 mutation does reduce the effectiveness of CsA. Introduction of P339, on the other hand, completely eliminates the ability of CsA to enhance drug accumulation, even at 2 μg/ml (Fig. 4D), whereas cells expressing the A338 P339 double mutation regain partial sensitivity and are able to accumulate drug to modest levels. Overall, these results are consistent with those of the experiments reported in Fig. 3 and Table 2 and support the conclusion that mutations within TM6 at amino acid positions 338 and 339 have a major impact on the ability of CsA to act as a reversal agent for Pgp1-mediated MDR. They also suggest that TM6 may contain or interact with a major CsA binding/interaction site within Pgp1.

At concentrations of VRP that were sufficient to effectively reverse resistance to VCR (0.4 μg/ml; Table 2), a modest increase in the accumulation of drug in both wild-type (Fig. 4F) and double mutant (Fig. 4I) transfectants was observed, whereas very little accumulation took place in the A338 and P339 transfectants (Fig. 4, G and H) respectively. Increasing the concentration of VRP to 2 μg/ml did enhance accumulation in all cases tested, but even at the highest doses analyzed, the accumulation levels did not approach those observed in the presence of CsA. Hence, although VRP is an effective reversal agent against both the wild-type and TM6 mutant forms of Pgp1 (Table 2), its ability to enhance accumulation of VCR within the 5-hr time period analyzed here is poor compared with that of CsA.

TABLE 2

RD_{50} values of transfectants

RD_{50} values (ng/ml) of the transfectants for CsA and VRP were determined by linear regression analysis of the resistance versus reversal agent dosage curves in Fig. 3. Values shown are the mean of multiple determinations (>3). Values in parentheeis are ratios of the RD_{50} values for each drug normalized to those of the wild-type.

	DC-3F/$G^{338}A^{339}$	DC-3F/A^{338}	DC-3F/P^{339}	DC-3F/$A^{338}P^{339}$
CsA				
ActD	109 ± 56 (1)	285 ± 9 (2.6)	2012 ± 294 (18.5)	782 ± 369 (7.2)
COLC	101 ± 24 (1)	225 ± 10 (2.2)	1657 ± 70 (16.4)	1015 ± 20 (10.0)
VCR	64 ± 8 (1)	118 ± 9 (1.8)	1876 ± 104 (29.3)	1300 ± 264 (20.3)
DAUN	166 ± 60 (1)	153 ± 6 (0.9)	2154 ± 234 (13.0)	843 ± 551 (5.1)
VRP				
ActD	499 ± 59 (1)	561 ± 72 (1.1)	791 ± 39 (1.6)	731 ± 334 (1.5)
COLC	425 ± 78 (1)	1628 ± 268 (3.8)	2205 ± 294 (5.2)	3441 ± 1608 (8.1)
VCR	403 ± 20 (1)	395 ± 34 (1.0)	686 ± 48 (1.7)	437 ± 133 (0.9)
DAUN	368 ± 22 (1)	422 ± 9 (1.2)	461 ± 14 (1.3)	605 ± 267 (1.6)

Discussion

Our initial report of the TM6 double mutation in hamster Pgp1 indicated that these alterations, although enhancing the ability of the Pgp1 transporter to mediate resistance to ActD, reduced its effectiveness in conferring resistance to DAUN and COLC (1, 22). The current results are consistent with those original studies and show that under the conditions used here, the level of ActD resistance in transfectants expressing the double mutation is 6.6-fold greater than the wild-type, whereas resistance to DAUN and COLC is reduced by 40% and 30%, respectively (Table 1). The actual differences in resistance levels for the various drugs, however, are not the same as previously reported and likely reflect the fact that although the 110S23 (G338A339) and the 220S17 (A338P339) transfectants represent clones of those studied originally (1), they are from latter passage numbers in which the level of transgene expression is known to have changed.[1] Moreover, the drug batches used for the present studies are different from those used earlier.

When analyzed separately, each of the single mutants confers its own unique cross-resistance profile compared with the wild-type, and it is the combination of the two that is responsible for the phenotype displayed by the double mutant (Fig. 2 and Table 1). From the standpoint of selection, it is interesting to note that by itself, the A338 mutation does not significantly enhance resistance to ActD (Table 1), the agent used for the stepwise selection of the DC-3F/ADX cell line from which the double mutant was cloned (8). Hence, although one cannot rule out the possibility that both mutations occurred simultaneously, it is more likely that P339 was the first mutation to emerge because it enhances resistance to ActD by 2.7-fold over the wild-type (Table 1). Acquisition of the A338 mutation then followed and, when coexpressed with P339, further increased resistance by 6.6-fold over that of the wild-type, thus providing the basis of a second positive selection for ActD resistance. In addition, although overexpression of the wild-type Pgp1 gene most likely accounted for the initial survival of DC-3F cells in the presence of low drug concentrations (22), our finding that the bulk of the Pgp1 transcripts expressed in DC-3F/ADX cells contain both mutations (22) suggests that they emerged relatively early during selection.

The presence of proline is known to limit structural flexibility and induce bends in α-helices (26). Moreover, preliminary molecular modeling experiments[2] (1) predict that the insertion of P339 is also likely to alter the structure of the TM6 α-helix by changing the positions of the individual side groups relative to each other along the length of the helix. The alterations noted here enhance the ability of Pgp1 to accommodate ActD as a substrate while having minimal effects on the other drugs (Table 1). On the other hand, the replacement of G338 by alanine increases resistance to VCR, COLC, and DAUN but does not affect resistance to ActD (Table 1). When expressed together in the double mutant, the combined effect is not additive, and the resistance levels observed do not reflect the sum of the two single mutations. In addition, the cross-resistance pattern resulting from the double mutation reflected a 2.5-fold enhancement of ActD resistance over that displayed by the P339 mutation alone and a 2–4-fold decrease in resistance to COLC and DAUN over that displayed by the single A338 mutation. These results are consistent with the widely held notion that a single complex drug binding/interaction site is responsible for substrate identification by Pgp1.

The results of the reversal experiments with CsA and VRP were somewhat different. In the case of CsA, the ability to reverse resistance to three of the four drugs tested was altered somewhat by the single A338 mutation, whereas the ability to reverse DAUN resistance was not affected (Table 2 and Fig. 3). The P339 mutation, on the other hand, had a dramatic impact on the effectiveness of CsA, raising the RD_{50} values for all of the drugs tested (from 13-fold for DAUN to 30-fold for VCR) (Table 2). Clearly, the A338 to P338 alteration had a major impact on the ability of CsA to mediate reversal. Expression of the double mutant, again, did not yield additive results. For each of the four drugs, the RD_{50} levels observed for the double mutant reflected the average or near-average of the RD_{50} levels for the single mutations, perhaps suggesting that the mutations may have altered a common site or region, although a more global effect on Pgp1 secondary structure cannot be ruled out. In contrast, and with the exception of elevating the RD_{50} value for COLC, none of the TM6 mutations affected the ability of VRP to reverse resistance. This indicates that the major site for interaction of VRP with Pgp1 lies at a location other than TM6, a conclusion that is consistent with the results of Kajiji *et al.* (13), who reported that a major VRP binding site is located in TM11. The fact that VRP reversal of COLC resis-

[1] S. E. Devine, J. F. Ma, and P. W. Melera, unpublished observations.

[2] J. F. Ma, D. J. Gringrich, and P. W. Melera, unpublished observations.

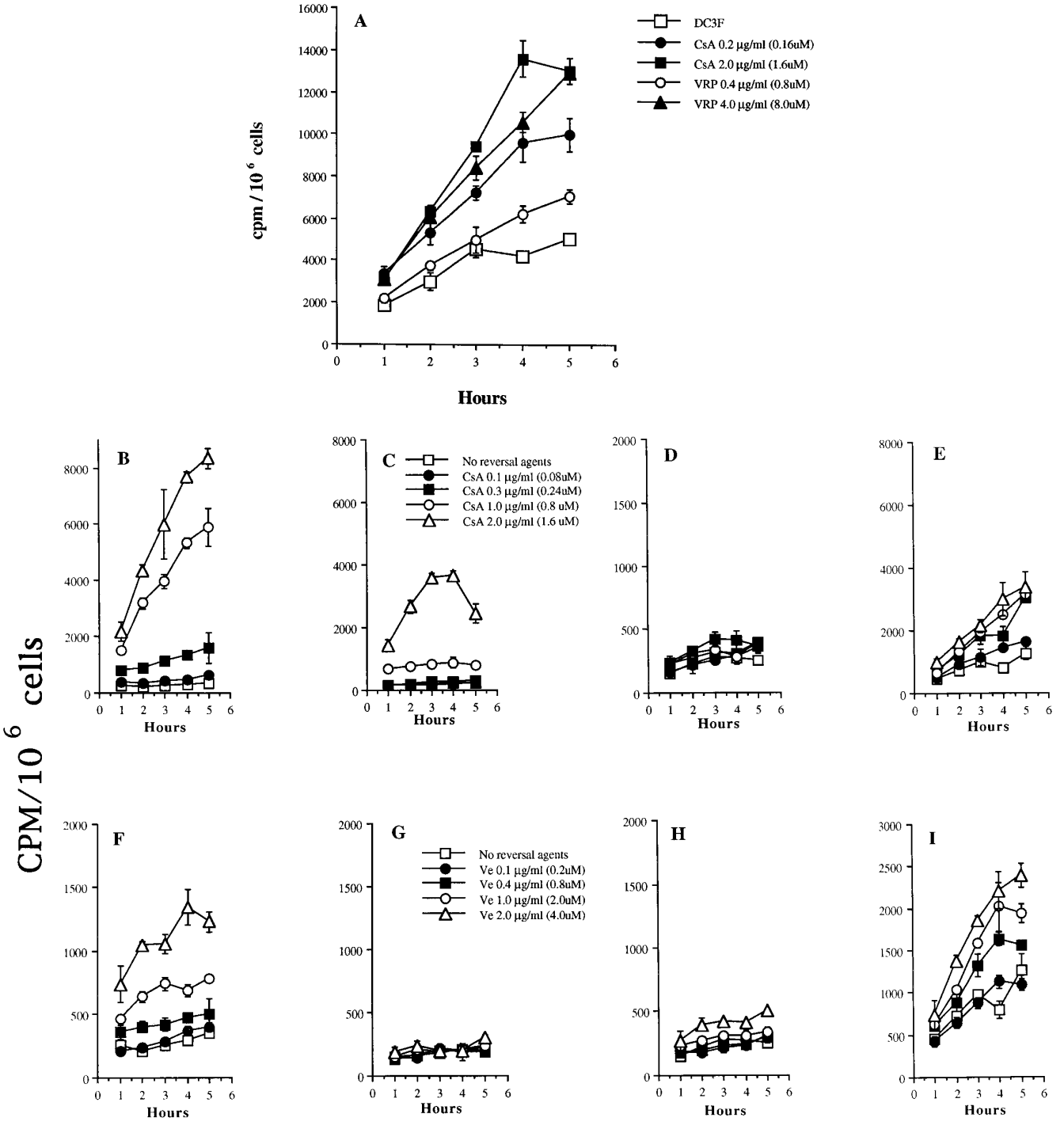

Fig. 4. Accumulation of [^{3}H]VCR by parental DC-3F cells and transfectants. All assays were performed in triplicate, and the values shown represent the mean of three determinations. A, [^{3}H]VCR accumulation by DC-3F cells in the presence of CsA or VRP. B–E, [^{3}H]VCR accumulation by transformants in the presence of CsA. F–I, [^{3}H]VCR accumulation by transfectants in the presence of VRP. B and F, DC-3F/G338A339. C and G, DC-3F/A338. D and H, DC-3F/P339. E and I, DC-3F/A338P339.

tance was affected by each of the three mutant forms of Pgp1 (Table 2) suggests that VRP also interacts with TM6 and that this region may contain, or be part of, a COLC binding site as well (1). Studies to determine the affinity and kinetics of drug binding with both wild-type and mutant forms of Pgp1 are in progress.

In the absence of reliable secondary or tertiary structural data, any attempt to interpret these results in terms of the mechanism of action of Pgp1 is speculative, but some potentially interesting points can be considered. Although both of the mutations G338 to A338 and A339 to P339 replace amino acids that are nonpolar and aliphatic, with amino acids that display similar characteristics, alanine is more hydrophobic than glycine, whereas proline is much less hydrophobic than

alanine (27). Hence, the overall hydrophobic nature of the putative TM6 helix would be altered by either or both of these mutations (1). However, the replacement of glycine with alanine within an α-helix would also be expected to somewhat reduce conformational flexibility because the potential for the alanine side chain to form hydrogen bonds would be predicted to be greater than the single hydrogen of glycine. Replacement of alanine with proline, on the other hand, would be expected to disrupt the helix by not permitting hydrogen bonding and, because the secondary amino (imino) group is fixed in a rigid conformation, limit flexibility. The number of conformations available for the TM6 helix in the double mutant therefore might be expected to be less than those available for the wild-type. This suggests that as the helix becomes more structured (i.e., has less conformational flexibility), the ability to interact favorably with many different substrates becomes increasingly limited. Hence, selection for mutants that provide enhanced resistance to one drug might be expected to limit or reduce the ability of Pgp1 to recognize and transport others. This is consistent with the results presented here and would be particularly true if the mechanism of action of Pgp relies on a single complex binding site.

It is generally believed that Pgp mediates drug resistance by decreasing the intracellular concentration of cytotoxic drugs to nontoxic levels via an efflux pump-like activity. Reversal agents, particularly those such as CsA and VRP, which are also substrates for the pump, have been reported to act competitively with vinca alkaloids for common binding sites on Pgp (15). Moreover, mutations that alter resistance have also been shown to alter the potency of reversal agents (28). However, correlation of drug accumulation data with such observations is not straightforward. For example, in the presence of a reversal agent, drug accumulation may increase but may or may not reach that observed in drug-sensitive cells (16). In the work presented here, CsA is able to reverse resistance in transfectants expressing wild-type Pgp1 and, at 1.0 μg/ml, restores the accumulation of [^{3}H]VCR to that seen in drug-sensitive DC-3F cells (Fig. 4A and B–E). However, the amount of CsA required to achieve the RD_{50} for VCR in these same transfectants is ~0.06 μg/ml, ~17-fold less (Table 2). Hence, in this system, and in several others as well (16), a large discrepancy exists between the dose levels required for reversal and those required to affect accumulation of drug. Indeed, in the case of VRP, we were able to demonstrate only modest effects on drug accumulation (Fig. 4, F–I) at any dose, including those concentrations that were clearly able to reverse resistance (Table 2). However, accumulation of drug did occur in all cases, albeit much slower than in the presence of CsA. It has been pointed out (16) that attempts to correlate the results of relatively short drug accumulation assays with those from necessarily longer cell growth assays are difficult. Moreover, it is quite clear from the data in Fig. 3 and Table 2 that the amount of drug that does accumulate in the presence of VRP over the 72-hr growth assay used here is sufficient to inhibit cell growth. It is also clear, however, that the immediate effects of CsA and VRP on drug accumulation are very different and in transfectants are at least partially mediated by the nature of the Pgp1 transgene. Attempts to further understand these differences are in progress.

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