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Loss of Cyclosporin and Azidopine Binding Are Associated with Altered ATPase Activity by a Mutant P-glycoprotein with Deleted Phe^{335}

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ABSTRACT

In this study, we further characterize a mutant P-glycoprotein (P-gp) that has a deletion of Phe^{335} and is resistant to inhibition by cyclosporins. Photoaffinity labeling with [^{3}H]cyclosporine and [^{3}H]azidopine revealed markedly decreased binding to the mutant P-gp compared with wild-type P-gp. Expression of the mutant P-gp in multidrug-resistant variant cell line MES-SA/DxP (DxP) cells was associated with a 2-fold higher basal ATPase activity relative to multidrug-resistant cell line MES-SA/Dx5 (Dx5) cells with wild-type P-gp. Cyclosporine inhibited ATPase activity in both cell types, whereas the cyclosporin D analog valspodar (PSC 833), vinblastine, and dactinomycin stimulated ATPase activity in Dx5 but not in mutant DxP cells. Moreover, the cell lines differed in their responses to verapamil, which produced greater stimulation of ATPase in Dx5 than DxP cells. Verapamil significantly reversed the [^{3}H]daunorubicin accumulation defect in wild-type Dx5 cells, but it had no significant effect on [^{3}H]daunorubicin accumulation in the mutant DxP cells. Verapamil was not transported by cells expressing either mutant or wild-type P-gp. Vanadate trapping of azido-ATP was markedly impaired in mutant P-gp. In conclusion, our data demonstrate that Phe^{335} of transmembrane 6 is an important amino acid residue for the formation of cyclosporine and azidopine drug-binding site(s). Phe^{335} also plays a role in the coupling of verapamil binding and modulation of daunorubicin intracellular accumulation in wild-type P-gp. In addition, Phe^{335} in transmembrane 6 may play a role in coupling drug binding to ATPase activity. The deletion of Phe^{335} results in a significant increase in the basal ATPase activity with a concomitant decrease in its ability to trap ATP and transport some P-gp substrates.

The multidrug transporter P-glycoprotein (P-gp) is an ATP-dependent drug efflux pump that is expressed in both normal and malignant tissues and has been associated with clinical multidrug resistance (MDR) and poor prognosis in some cancers (for review, see Ling, 1992; Gottesman, 1993; Sikic, 1993). Clinical trials with anticancer drugs combined with inhibitors of P-gp, also called MDR modulators, such as the cyclosporin D analog valspodar (PSC 833; PSC), are ongoing in efforts to reverse resistance to chemotherapy (for review, see Sikic, 1999).

There is increasing evidence that domain-domain interactions of P-gp are critical for drug recognition, binding, and transport. These interactions involve the transmembrane (TM) regions and ATPase catalytic sites within the nucleotide-binding domains (Tamai and Safa, 1991; Bruggemann et al., 1992; Greenberger, 1993; Kajiji et al., 1993; Loo and Clarke, 1993, 1994; Senior et al., 1995; Chen et al., 1997; ., Dey et al., 1998; Hrycyna et al., 1998a). Analysis of P-gp mutants derived either from drug selection or site-directed mutagenesis has furthered our understanding of the structure-activity relationships between P-gp and its substrates or modulators (Loo and Clarke, 1993; Hanna et al., 1996; Chen et al., 1997; Ma et al., 1997).

We have recently described a mutation of the *MDR*1 gene that arose in the multidrug-resistant variant cell line MES-SA/DxP (DxP), by coselection of the MDR human sarcoma cell line MES-SA/Dx5 (Dx5) with doxorubicin and PSC. DxP had an altered MDR phenotype and was resistant to modulation by cyclosporins (Chen et al., 1997). Our previous study demonstrated that survival of cells exposed to doxorubicin and PSC in a multistep selection occurred as a result of a Phe^{335} deletion in TM 6 of P-gp (Chen et al., 1997).

To further characterize the phenotype conferred by deletion of Phe^{335} and to determine whether Phe^{335} is involved in forming the major binding site(s) for cyclosporins and azidopine, we performed photoaffinity labeling of P-gp with [^{3}H]cyclosporine, [^{3}H]azidopine, and 8-[α-^{32}P]azido-ATP.

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ABBREVIATIONS: P-gp, P-glycoprotein; MDR, multidrug resistance; PSC, cyclosporin D analog PSC 833 (valspodar); TM, transmembrane; DxP, multidrug-resistant variant cell line MES-SA/DxP; Dx5, multidrug-resistant cell line MES-SA/Dx5; Pi, inorganic phosphate.

These studies demonstrated substantial loss of binding of cyclosporine and azidopine by the mutant P-gp associated with both altered ATP binding and ATPase activity.

Materials and Methods

Drugs and Chemicals. [^{3}H]Cyclosporine (8.7 Ci/mmol, [^{3}H]azidopine (54 Ci/mmol), and [^{3}H]verapamil (84 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). PSC and photolabeled [^{3}H]dihydro-D-Ser^8 cyclosporine (an azidophenyl group was attached to D-Ser^8) (29.8 Ci/mmol) were provided by Novartis Pharmaceuticals Corp., East Hanover, NJ (formerly Sandoz Pharma Ltd., Basel, Switzerland). Doxorubicin was obtained from Adria Laboratories (Columbus, OH) and vinblastine from Eli Lilly and Co. (Indianapolis, IN). All other anticancer agents and chemicals were obtained from the National Cancer Institute and Sigma Chemical Co. (St. Louis, MO).

Cells and Tissue Culture. Details of the development and characterization of the human sarcoma cell line MES-SA and its MDR variant Dx5 have been described (Harker et al., 1983; Harker and Sikic, 1985). The DxP cell line was derived from coselection of Dx5 cells with doxorubicin plus PSC. The cell stocks were negative for mycoplasma infections by a polymerase chain reaction assay (Chen et al., 1997). The cultures of MES-SA and its variants were grown in McCoy's medium as detailed in previous reports (Chen et al., 1997).

Determination of P-Glycoprotein Expression. Flow cytometric analysis of P-gp expression with the UIC2 monoclonal antibody has been described previously (Chen et al., 1994, 1997). The light-enhanced chemiluminescence Western blot protocol (Amersham) was used for the detection of P-gp. Both total cell lysates and membrane fractions from the exponentially growing cells were used for P-gp immunoblotting with the monoclonal antibody C219 (Signet Inc., Dedham, MA) as described (Chen et al., 1994, 1997).

Plasma Membrane Preparation. Both DxP and Dx5 cells were maintained in the same concentration used for drug selection (Chen et al., 1997). Dx5, DxP, and the wild-type MES-SA cells (1 to 2 $\times$ 10^8 cells) were scraped and washed twice with Dulbecco's-PBS (Life Technologies, Rockville, MD) at 4°C. The cells were sonicated in buffer A (10 mM Tris-HCl, pH 7.4; 10 mM NaCl; 1.5 mM $MgCl_2$; and 100 μg/ml phenylmethylsulfonyl fluoride). Cell disruption was confirmed by light microscopy. The sonicated solution (10 ml) was overlayed on 25 ml of freshly made sucrose (35%, w/v) and centrifuged at 18,000*g* in a swinging bucket rotor (SW27; Beckman Instruments, Palo Alto, CA) for 60 min at 4°C. The interface fractions (mem-

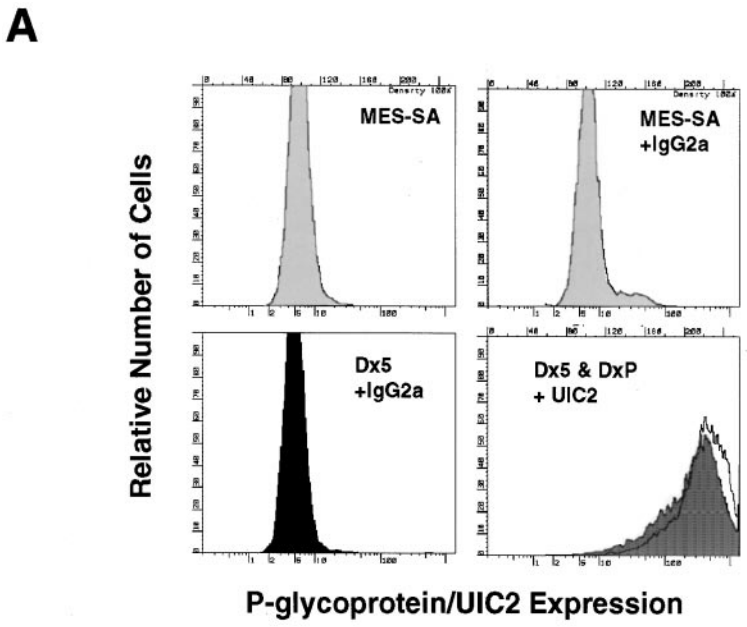

Fig. 1. Membrane P-gp expression. A, flow cytometric analysis of membrane P-gp expression with the Texas Red-conjugated UIC2 staining. MES-SA is the negative control for UIC2 staining. P-gp expression is depicted as Texas Red density (*x*-axis) of the histogram plot. One of at least three independent experiments is shown. B, Western blotting with the monoclonal antibody C219 was described previously (Chen et al., 1997). Purified plasma membranes were used for this experiment. One of the three experiments is shown.

branes) were collected and resuspended in buffer B (10 mM Tris-HCl, pH 7.4; 250 mM sucrose; and 100 μg/ml phenylmethylsulfonyl fluoride), and then ultracentrifuged at 100,000*g* for 1 h at 4°C. Finally, the pellets were resuspended and were freshly used for the photoaffinity labeling experiments or ATPase activity assays.

Photoaffinity Labeling with [^{3}H]Cyclosporine Derivative and [^{3}H]Azidopine. The isolated membranes of MES-SA, Dx5, and DxP cells were incubated with a serial concentration of photolabeled [^{3}H]dihydro-D-Ser^8 cyclosporine (50 to 500 nM) and [^{3}H]azidopine (50 to 500 nM) at 25°C for 1 h and irradiated for 15 min at 4°C with a UV lamp. The photolabeled membranes were analyzed by 7.5% SDS-polyacrylamide gel, dried, visualized by autoradiography, and quantitated with an Alpha Innotech image analyzer (San Leandro, CA) (Chen et al., 1997).

ATPase Activity Assay. P-gp-associated drug-stimulated ATPase activity was determined by measuring the vanadate-sensitive release of inorganic phosphate from ATP with a colorimetric method as previously described (Hrycyna et al., 1998b). Briefly, membrane preparations (20 μg) were initially incubated in the reaction mixture assay buffer (50 mM Tris-HCl, pH 7.5; 5 mM sodium azide; 2 mM EGTA, pH 7.0; 1 mM ouabain; 2 mM dithiothreitol; 50 mM KCl; and 10 mM $MgCl_2$) at 37°C for 5 min, substrates were added, and the reactions were started by adding 5 mM ATP into assay mixture in the presence of or absence of substrates. The reactions were incubated for 30 min at 37°C. Finally, the reactions were terminated by the addition of 5% (w/v) SDS solution and the amount of inorganic phosphate (Pi) released is measured at 880 nm relative to phosphate standard (Sigma Chemical Co.).

Vanadate-Induced Trapping of 8-[α-^{32}P]Azido-ATP to P-gp. Vanadate-induced trapping of 8-[α-^{32}P]azido-ATP was performed as described by Senior and coworkers (Urbatsch et al., 1995) with slight modifications. Briefly, orthovanadate solutions (10 mM) were prepared from Na_3VO_4 (Sigma Chemical Co.). 8-[α-^{32}P]Azido-ATP (17.5 Ci/mmol) was purchased from ICN Biomedicals, Inc. (Irvine, CA). The membrane preparations from MES-SA, Dx5, and DxP cells were preincubated with 300 μM vanadate in the presence of 100 μM verapamil at 37°C for 3 min and subsequently labeled by 8-[α-^{32}P]azido-ATP in the labeling buffer containing 3 mM $MgSO_4$, 2 mM ouabain, 0.1 mM EGTA, and 40 mM Tris-HCl (pH 7.4) at 37°C for 30 min. The reactions were cross-linked under a UV light, heated for 5 min at 100°C, and analyzed by a 4 to 20% Tris-glycine gradient gel (Fisher Scientific Co., Pittsburgh, PA). The gels were stained by Coomassie blue, dried, and exposed to films.

Na^+/K^+ ATPase Expression. The expression of Na^+/K^+ ATPase in membrane suspensions of MES-SA, Dx5, and DxP cells was determined by Western blotting (Chen et al., 1997). The anti- Na^+/K^+ ATPase monoclonal antibody (clone M7-PB-E9) (Affinity Bioreagents, Golden, CO) was diluted (1:250) in 1× TBST buffer (Tris-buffered saline, pH 7.6, 0.1% Tween 20), recognized by anti-mouse Ig-horseradish peroxidase at 1:2000 dilution (in 1× TBST buffer with 5% milk), and detected by enhanced chemiluminescence (Amersham, Arlington, IL). The results were quantified with an Alpha Innotech image analyzer.

Cellular Accumulation of ^{3}H-Labeled Drug. Intracellular drug accumulation of [^{3}H]daunorubicin, [^{3}H]vinblastine, and [^{3}H]cyclosporine was assessed in the presence or absence of either PSC or verapamil. Verapamil uptake in MES-SA, Dx5, and DxP cells was quantified with [^{3}H]verapamil. Approximately 1×10^6 cells/well (6-well Falcon tissue culture plates) were incubated with a series of verapamil concentrations (0.1, 1.0, 10, and 50 μM) in which each contains [^{3}H]verapamil over 10, 30, and 60 min. All values were normalized to protein content as previously described (Beketic-Oreskovic et al., 1995).

Statistical Analysis. The unpaired *t* test was performed with the StatView software program (Version 4.51) from Abacus Concepts (Berkeley, CA) on a Macintosh Power G3 computer.

Results

P-gp Expression. Membrane P-gp expression was measured in conjunction with each photoaffinity labeling experiment. Dx5 and DxP cells always expressed a similar amount of membrane P-gp by both flow cytometric analysis with the UIC2 monoclonal antibody and immunoblotting with C219 in these experiments (Fig. 1).

Photoaffinity Labeling with [^{3}H]Cyclosporine. Photoaffinity-labeling experiments were performed to evaluate the binding of cyclosporine to P-gp. The plasma membrane-enriched fractions from drug-sensitive cells MES-SA and MDR cells Dx5 and DxP cells were incubated with a photolabeled [^{3}H]cyclosporine derivative ([^{3}H]dihydro-D-Ser^8 cyclosporine). The [^{3}H]cyclosporine derivative labeled P-gp in wild-type Dx5, but not in MES-SA cells. The DxP cells displayed a 3- to 10-fold decrease in [^{3}H]cyclosporine (250 nM) labeling compared with Dx5 cells (Fig. 2). At the higher [^{3}H]cyclosporine-labeling concentrations (250 and 500 nM), only the wild-type P-gp of Dx5 cells was saturated. In contrast, the mutant P-gp in DxP cells displayed a 3- to 5-fold decrease in [^{3}H]cyclosporine labeling without saturation. These results were normalized to cell membrane P-gp expression as shown in Fig. 1.

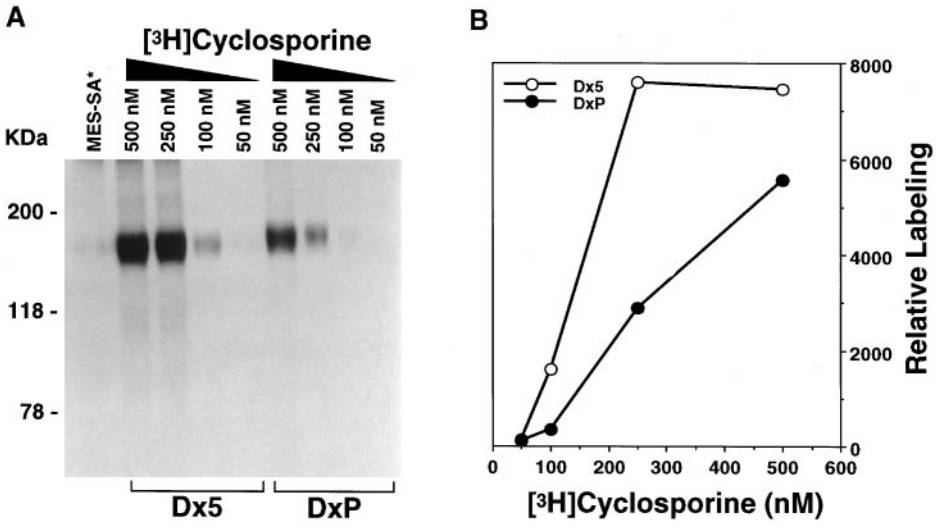

Fig. 2. Photoaffinity labeling of P-gp (P170) by [^{3}H]cyclosporine. Membrane-enriched fractions from MES-SA cells (negative control for P-gp expression), wild-type Dx5 MDR cells, and mutant DxP cells (deletion of Phe^{335}) were incubated with a [^{3}H]cyclosporine derivative at the indicated concentrations in the presence of dimethyl sulfoxide. The samples were fractionated on 7.5% SDS-polyacrylamide gel, visualized by autoradiography (A), and quantitated by digitized image analysis (B). *, MES-SA cells were incubated with 500 nM [^{3}H]cyclosporine.

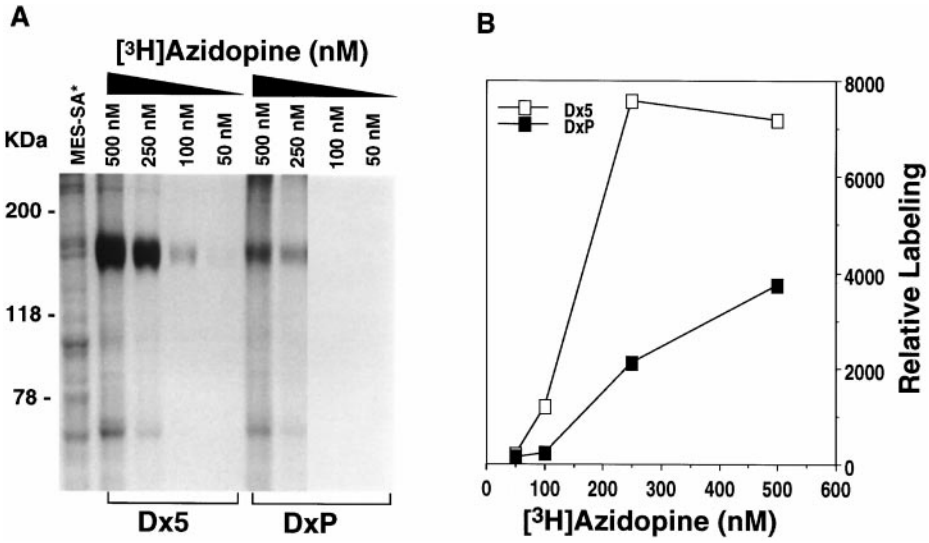

Fig. 3. Photoaffinity labeling of P-gp (P170) by [^{3}H]azidopine. Photoaffinity labeling of P-gp (P170) by [^{3}H]azidopine. Membrane enriched fractions containing equal amounts of P-gp were incubated with [^{3}H]azidopine at the indicated concentrations in the presence of dimethyl sulfoxide as described in Fig. 2. *, MES-SA cells were incubated with 500 nM [^{3}H]cyclosporine.

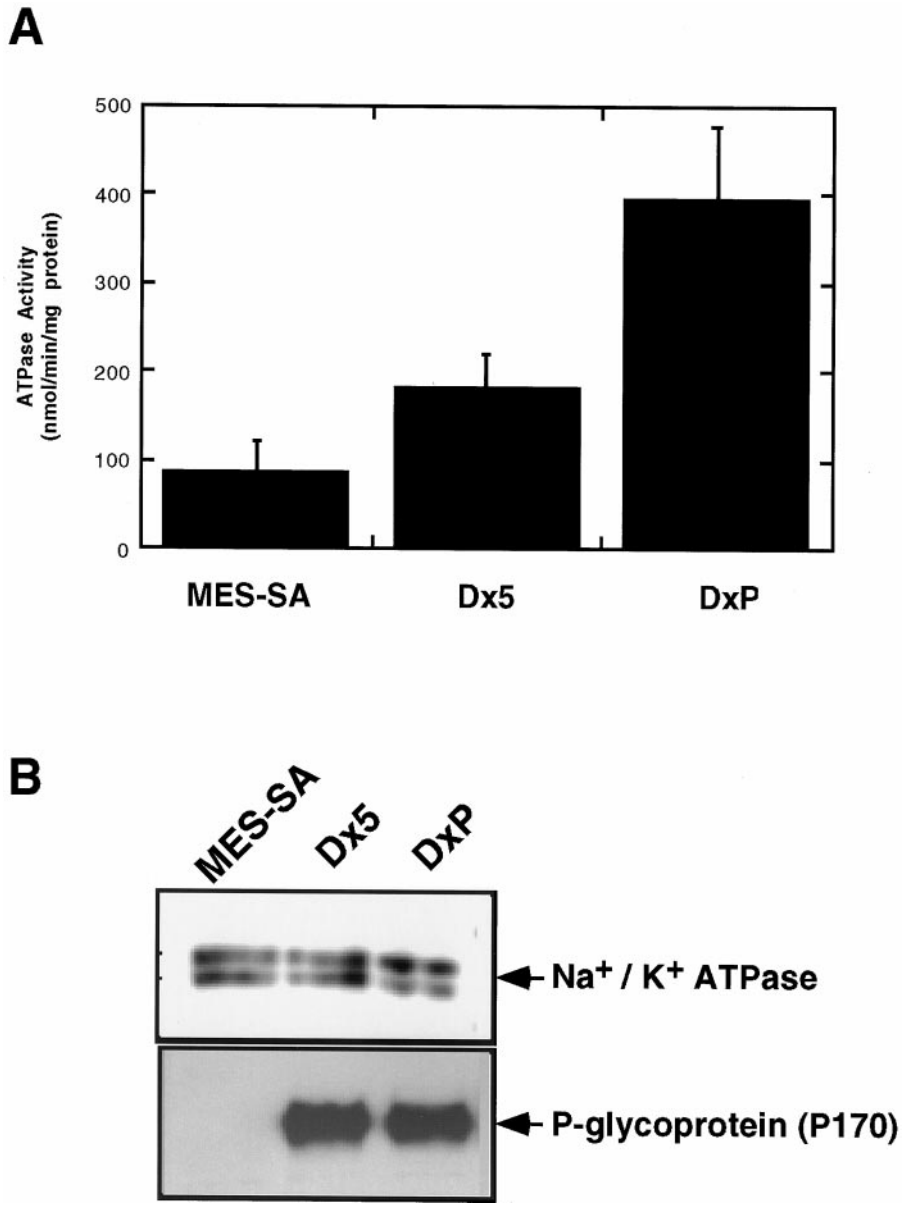

Fig. 4. The basal ATPase activity in MES-SA, Dx5, and DxP cells. A, ATPase activity was determined by measuring the vanadate-sensitive release of Pi from ATP as described in *Materials and Methods* with membrane preparations containing 20 μg of protein. The mean of five independent experiments with standard deviations has been depicted. B, Na^+/K^+ ATPase and P-gp expression: Equal amounts of membrane preparations from MES-SA, Dx5, and DxP were used to quantitate the relative expression of Na^+/K^+ ATPase and P-gp by Western blotting with the anti-Na^+/K^+ ATPase monoclonal antibody and C219. The detailed procedures have been published previously (Chen et al., 1997).

Photoaffinity Labeling with [^{3}H]Azidopine. Photoaffinity-labeling experiments also were performed to evaluate the binding of azidopine to P-gp with the procedures described above. The cells expressing mutant P-gp manifested decreased [^{3}H]azidopine binding to P-gp (Fig. 3). This decreased binding of azidopine in DxP cells was 2-, 4-, and 5-fold at 500, 250, and 100 nM [^{3}H]azidopine concentrations, respectively (Fig. 3, A and B).

Basal ATPase Activity. The basal ATPase activity was determined by measuring vanadate-sensitive release of Pi from ATP. Basal ATPase activity was elevated 2-fold in Dx5 cells (181 nmol/min/mg protein, $P = .08$) and 4-fold elevated in DxP cells (394 nmol/min/mg protein, $P = .004$) compared with the wild-type MES-SA cells (86 nmol/min/mg protein). Of note, DxP cells displayed 2-fold higher ATPase activity than wild-type Dx5 cells ($P = .03$) (Fig. 4A). However, the level of Na^+/K^+ ATPase (an integral ATP-dependent transporter of Na^+ and K^+ across cell membranes) was identical among MES-SA, Dx5, and DxP cells (Fig. 4B).

Drug-Stimulated ATPase Activity. Equal amounts of P-gp-containing membranes from Dx5 and DxP cells were assayed for the stimulation of ATPase activity by P-gp substrates. Dx5 cells demonstrated 100, 68, and 82% stimulation of ATPase activity after exposure to verapamil, vinblastine, and dactinomycin, respectively. PSC only slightly increased basal ATPase activity in these cells, whereas cyclosporine inhibited the ATPase by 35% (Fig. 5A). In contrast to Dx5 cells, DxP cells showed a 20% elevation of ATPase activity by verapamil, approximately a 50% inhibition by PSC, and a 25% inhibition by cyclosporine, vinblastine, and dactinomycin (Fig. 5A). The differences in drug-stimulated ATPase activities in Dx5 versus DxP cells were assessed by unpaired *t*-tests, and significant differences were found for verapamil ($P = .04$), PSC ($P = .05$), vinblastine ($P = .04$), and dactinomycin ($P = .05$). Drug concentration-dependent ATPase stimulation by verapamil was observed in both Dx5 and DxP cells, but there was no stimulation in parental MES-SA cells, which do not express P-gp (Figs. 1, 4B, and 5B).

Vanadate-Induced Trapping of 8-[α-^{32}P]Azido-ATP to P-gp. Vanadate-induced trapping of 8-[α-^{32}P]azido-ATP specifically at a single catalytic site of P-gp has been well established (Urbatsch et al., 1995). With the same approach, we found that 8-[α-^{32}P]azido-ATP specifically labels wild-type P-gp (expressed in Dx5 cells) only in the presence of vanadate (Fig. 6A). However, the mutant P-gp (expressed in DxP cells) showed a marked loss (40% by densitometry) of 8-[α-^{32}P]azido-ATP labeling relative to that of wild-type P-gp under the same labeling conditions (Fig. 6, A and B).

Verapamil Cellular Accumulation and Effects on Drug Accumulation. Intracellular accumulation of [^{3}H]verapamil in MES-SA, Dx5, and DxP cells reached a steady state at 30 min, and did not differ among the three cell lines (Fig. 7).

We previously reported that DxP cells with the mutant P-gp have a decreased ability to efflux [^{3}H]vinblastine or [^{3}H]cyclosporine, and are also resistant to modulation of drug transport by PSC (Chen et al., 1997). These cells differ also in their responses to verapamil. Verapamil significantly enhanced the accumulation of [^{3}H]daunorubicin in Dx5 cells, but not in DxP cells. There was no significant effect on the accumulation of cyclosporine and vinblastine by verapamil in either DxP or Dx5 cells (Fig. 8).

Discussion

Cellular expression of the efflux transporter P-gp confers a MDR phenotype, and is a major factor associated with clinical response to cancer chemotherapy. The cyclosporins and verapamil inhibit P-gp function and reverse the resistant phenotype (Sikic, 1993). We have recently reported that combined selection of the MDR cell line Dx5 with doxorubicin and PSC resulted in the DxP variant cell line that had an altered phenotype compared with its parental Dx5 cells, which express wild-type P-gp (Chen et al., 1997). This altered phenotype included loss of cross-resistance to dactinomycin, decreased cross-resistance to *Vinca* alkaloids, impaired transport of cyclosporine, and inability to modulate MDR by cyclosporins. The altered MDR phenotype of DxP cells was associated with the overexpression of a mutant P-gp with a deletion of Phe^{335} in TM 6, and was reproduced by transferring the mutant *MDR*1 gene into drug-sensitive cells (Chen et al., 1997).

It has been suggested that the various MDR substrates

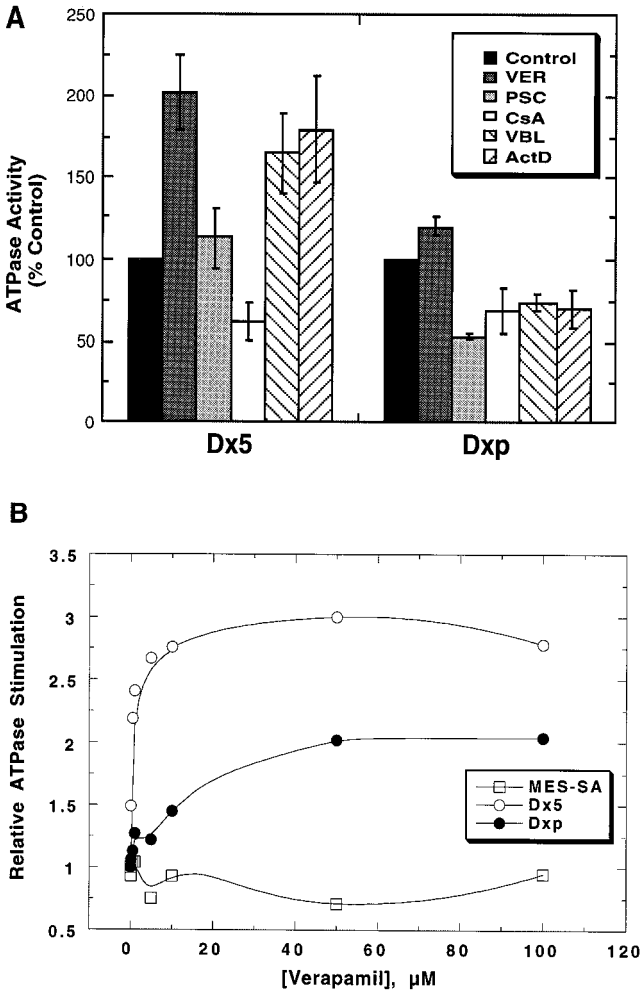

Fig. 5. Drug-stimulated ATPase activity. Equal amounts of P-gp-containing plasma membranes (20 μg of protein) from Dx5 and DxP cells were assayed. A, substrate concentrations used in this assay are 10 μM each. The means of three independent experiments with standard deviations are depicted. VER, verapamil; CsA, cyclosporine A; VBL, vinblastine, and ActD, dactinomycin. B, concentration-dependent stimulation of ATPase activity by verapamil in MES-SA, Dx5, and DxP cells. The ATPase activities are expressed relative to nonstimulated controls (100%).

and chemosensitizers compete for a common drug-binding site present in P-gp, which involves TM 5 and 6 and TM 11 and 12 (Bruggemann et al., 1992; Greenberger, 1993; Loo and Clarke, 1993). The Phe[335] deletion mutant provides insight into the relationship between P-gp structure and MDR modulation by cyclosporins. Labeling experiments with a photolabeled [^{3}H]cyclosporine derivative in this study demonstrated decreased binding of [^{3}H]cyclosporine to mutant

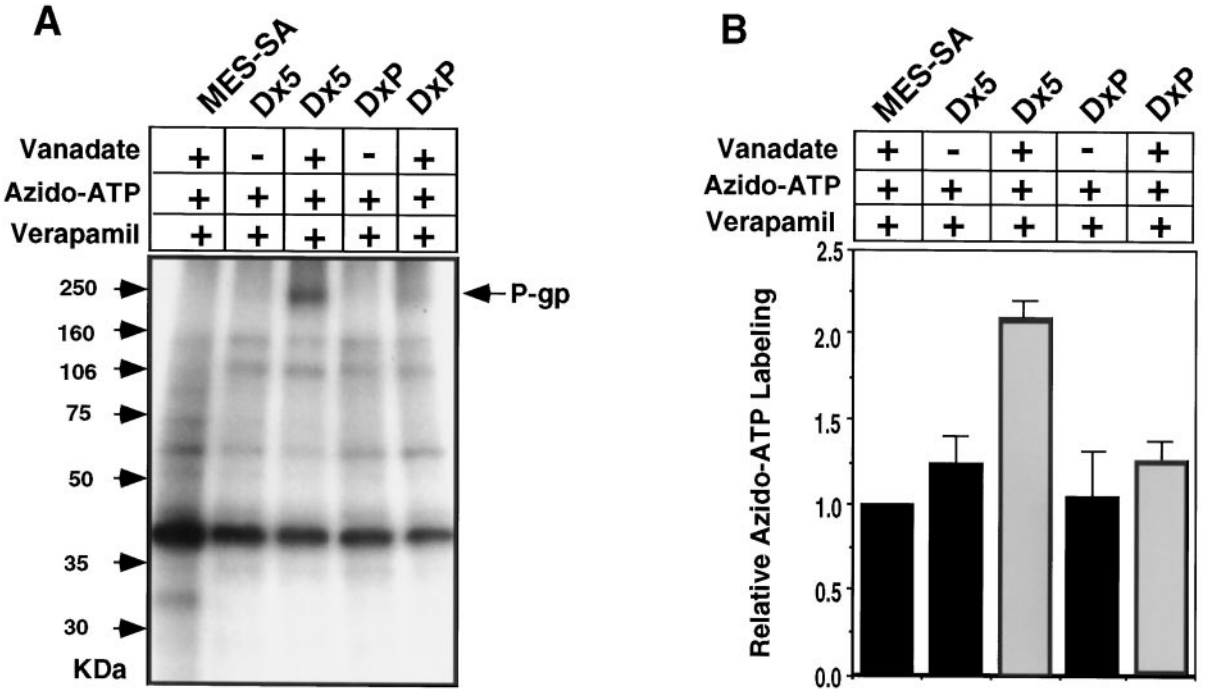

Fig. 6. 8-[α-^{32}P]Azido-ATP labeling of P-gp mediated by vanadate trapping. A, membrane preparations (~100 μg) from MES-SA, Dx5, and DxP cells were preincubated with (+) or without (−) 300 μM vanadate in the presence of 100 μM verapamil at 37°C for 3 min and were subsequently labeled by 5 μM 8-[α-^{32}P]azido-ATP (Azido-ATP) in the labeling buffer for 30 min. The reactions were placed on ice, irradiated under a UV light (wavelength = 254 nm) for 20 min, and then subjected to a 4 to 20% Tris-glycine gradient gel analysis. One of three experiments with similar results is shown. B, quantitative analysis of [α-^{32}P]azido-ATP labeling. The labeled P-gp bands were cut and 8-[α-^{32}P]8-azido-ATP counted. The results were normalized to equivalent membrane protein content, as determined by both densitometric analyses of the Coomassie blue stained gels and Western blotting of the membrane with a C219 monoclonal antibody. The means, relative to the MES-SA labeling control (100%), of three experiments with standard deviations are depicted.

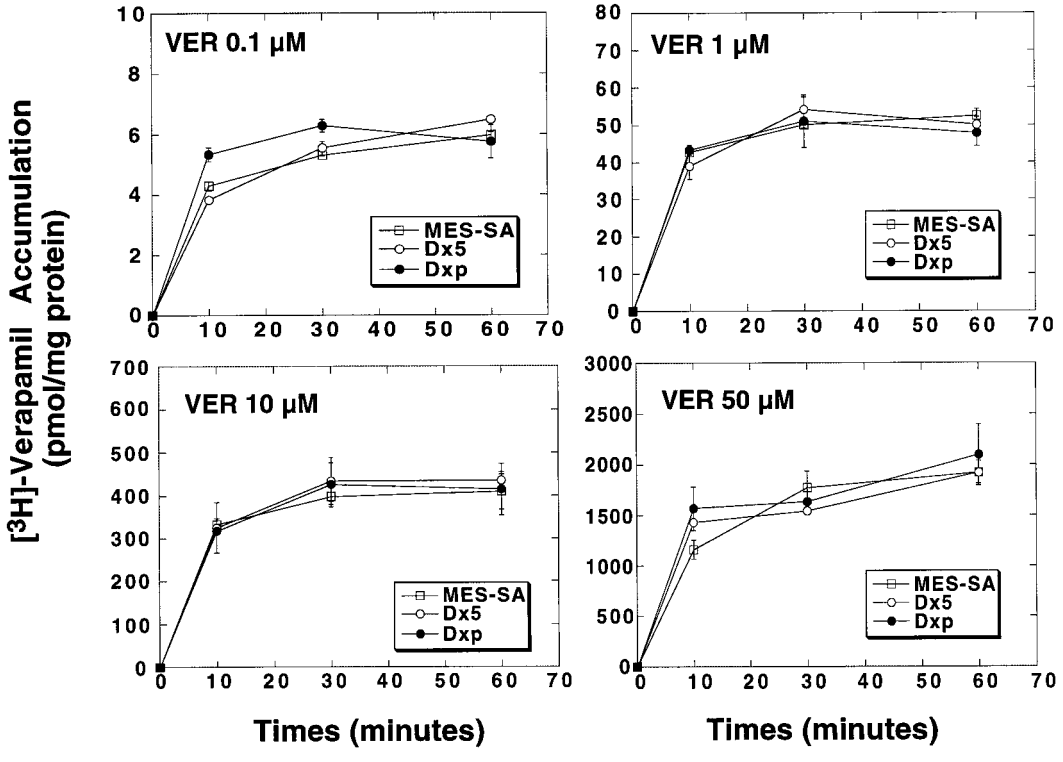

Fig. 7. [^{3}H]Verapamil transport. Verapamil transport was assessed by measured intracellular [^{3}H]verapamil accumulation overvarious time points. Approximately 1 × 10^6 cells/well were incubated with a range of verapamil concentrations (0.1, 1.0, 10, and 50 μM). All values were normalized to protein content.

P-gp, whereas wild-type P-gp of Dx5 cells maintained a high-affinity labeling (Fig. 2). Similar results also were found in our [^{3}H]azidopine-labeling experiment (Fig. 3). Therefore, the drug-binding domain for cyclosporins and azidopine includes or is significantly affected by the Phe^{335} residue of TM 6.

It is likely that multiple sites in P-gp mediate broad substrate specificity. Previous experiments have demonstrated that azidopine binds to P-gp at a binding site(s) different from vinblastine and cyclosporine binding site(s) in MDR cells (Tamai and Safa, 1991). In general, TM 5 and 6 and TM 11 and 12 may participate in binding of vinblastine, azidopine, rhodamine 123, and cyclosporins (Tamai and Safa, 1991; Bruggemann et al., 1992; Greenberger, 1993; Kajiji et al. 1993; Loo and Clarke, 1993; Chen et al. 1997; Ma et al., 1997; Demeule et al., 1998). However, the other TMs such as TM 4 and TM 10 also may be involved in the recognition of colchicine and doxorubicin, respectively (Loo and Clarke, 1993). Ferry et al. (1995) showed that P-gp on cell membranes possesses more than one drug acceptor site that could be allosterically coupled. As shown in Fig. 8, the modulation mediated by verapamil in wild-type P-gp cells seems to be more specific for a substrate such as daunorubicin rather than vinblastine or cyclosporine. Verapamil, like cyclosporine and PSC, is less effective in modulating daunorubicin accumulation in DxP cells (Fig. 8), indicating that the ΔPhe^{335} mutation is likely to affect both verapamil and cyclosporin binding to P-gp. However, verapamil is less effective than the cyclosporin analog PSC in modulating the accumulation of cyclosporine and vinblastine in Dx5 cells (Fig. 8). Potential changes in the interactions of verapamil with cyclosporine and vinblastine accumulation in the mutant cells are not assessable because of the decreased transport of these substrates by the mutant cells.

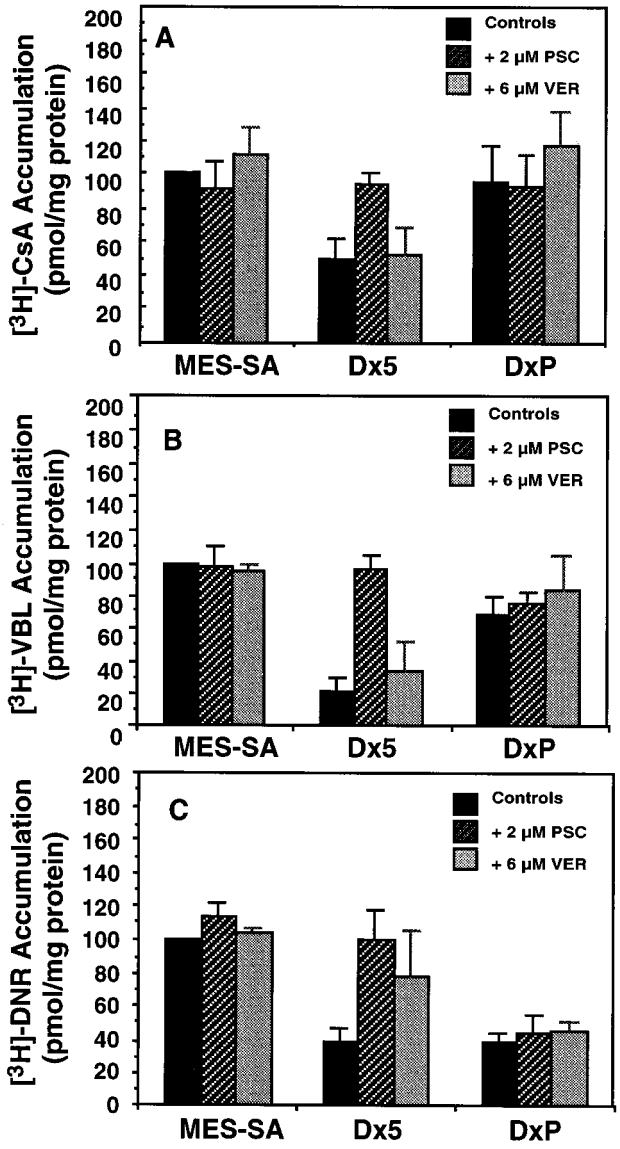

Fig. 8. The effects of verapamil on ^{3}H-labeled drug in both Dx5 and DxP cells. The intracellular accumulation of [^{3}H]cyclosporine (CsA) (250 nM) (A), [^{3}H]vinblastine (VBL) (50 nM) (B), and [^{3}H]daunorubicin (DNR) (0.5 μM) (C) in the presence or absence of either 2 μM PSC or 6 μM verapamil (VER) was measured at 37°C for 60 min. Each point is the mean plus S.D. of three independent experiments. MES-SA cells were used as a control (100%) in these experiments.

It is possible that allosteric changes in this mutant P-gp may enhance binding to specific sites by substrates because we have previously shown that DxP cells have enhanced iodoarylazidoprazosin labeling in the presence of both PSC and vinblastine (Chen et al., 1997). The newly identified diazirine-cyclosporine labeling sites on hamster P-gp (Demeule et al., 1998) indicate that the photoaffinity-labeling groups of cyclosporins have their preferential physical contact on the domain that is formed by TM 5 and 6 and TM 11 and 12 (Fig. 9). Thus, our data together with that of other studies support a model for P-gp with multiple drug-binding sites that accounts for variations in substrate binding and transport specificities (Fig. 9B; Dey et al., 1998).

The topological structure of P-gp in the native membrane may be present in different orientations and this feature may be important for its function (Zhang et al., 1996). When mutations are introduced into TM regions, the local and global topology of P-gp and the distribution of hydrophobic or hydrophilic residues that form the putative amphipathic helix might be altered (Fig. 9B). The hydrophobic residues (e.g., aromatic amino residues) in TM regions of P-gp form a helix configuration that may undergo diverse drug-dependent dynamic conformational changes, which in turn determine P-gp substrate specificity. Nonetheless, recent data also suggest that the more hydrophilic face of the TM helix may play an important functional role in drug recognition and transport by P-gp (Hanna et al., 1996).

Studies of P-gp suggest that it transports diverse substrates via an ATP-dependent pathway. The mechanisms that link substrate recognition, drug binding and ATPase activity are not well understood. Data from the mutagenesis work by Loo and Clarke (1997) suggest that TM 6 and TM 12 undergo conformational changes that bring these TM segments into contact. These changes are associated with drug binding and ATPase hydrolysis (Loo and Clarke, 1997). Our data indicate that inhibited ATPase activities by PSC, vinblastine, and dactinomycin in the mutant cells are associated with loss of cyclosporin or azidopine binding, suggesting that the Phe^{335} residue on TM 6 may participate in coupling drug binding to ATPase activity. Altered coupling to ATPase by ΔPhe^{335} P-gp may contribute to its reduced capacity to confer resistance to drugs such as vincristine, vinblastine, and dactinomycin (Chen et al., 1997). Stimulation or suppression

of ATPase activity may depend on the TM 6 helix conformation that involves Phe^{335} (Figs. 4 and 5).

The basal ATPase activity was significantly elevated by 2-fold (200%) in DxP cells relative to Dx5 cells, without a change in expression of Na^+/K^+ ATPase. A previous report showed that a mutant P-gp at the same site in TM 6 (Phe^{335}→Ala^{335}, F333A) displayed markedly increased (3-fold) basal ATPase activity relative to that of wild-type P-gp (Loo and Clarke, 1995). However, drug-stimulated ATPase activity in this mutant also was elevated, in contrast to the deleted Phe^{335}. Expenditure of ATPase activity, as demonstrated by the increase in basal ATPase activity by the mutant P-gp, also suggests that ATPase activity may depend on the helix conformation defined by Phe^{335}. In contrast to cyclosporine, which inhibited ATPase activity of both wild-type and ΔPhe^{335} P-gp, PSC retained its ability to stimulate ATPase activity in wild-type P-gp (Fig. 5A; Watanabe et al., 1997), but not in the ΔPhe^{335} P-gp (Fig. 5A).

The mechanism of the coupling of ATP hydrolysis to drug transport is being actively investigated. Senior et al. (1995) have proposed a catalytic cycle for ATP hydrolysis by P-gp, in which P-gp hydrolysis was coupled to drug transport mediated by a transition of drug-binding status between a high-affinity inner-face site to a low-affinity outer-face site. This model also was supported by experiments demonstrating that human P-gp exhibits reduced affinity for substrates such as [^{125}I]iodoarylazidoprazosin or [^{3}H]azidopine during a catalytic transition state (Ramachandra et al. 1998). The

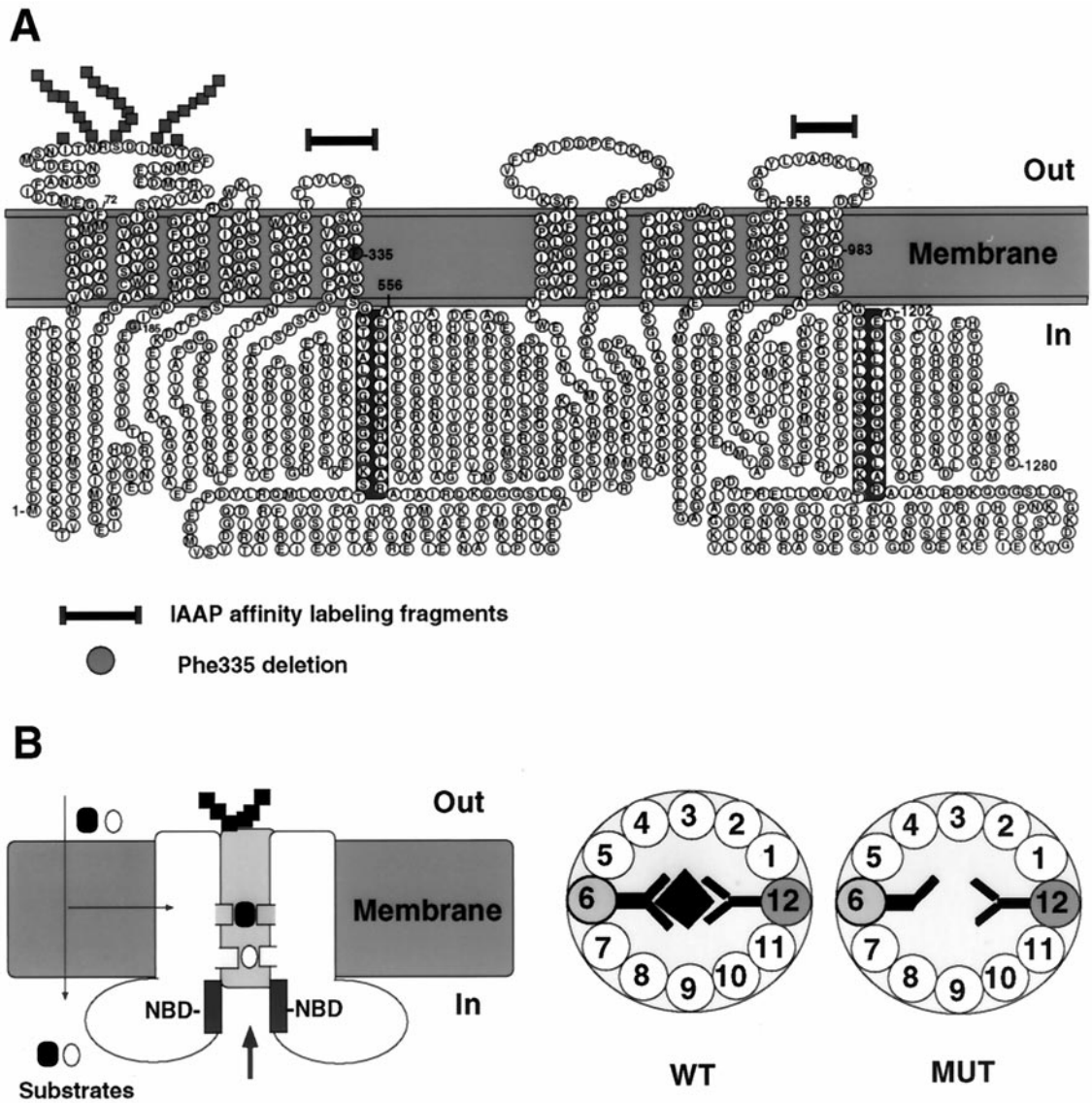

Fig. 9. A schematic representation of the TM orientation of P-gp. A, two-dimensional representation of P-gp, illustrating the orientation of the putative TM domains (TMDs), nucleotide-binding domains (NBDs), cytoplasmic loops, and the Phe^{335} deletion (●) (modified from Gottesman, 1993). The black chains denote glycosylation sites on P-gp. The ATP-binding sites were highlighted and the major iodoarylazidoprazosin (IAAP) photoaffinity-labeling regions were depicted based on previous evidence (Greenberger, 1993). B, hypothetical models for drug binding and transport by P-gp. The arrows indicate potential pathways of drug influx and efflux, both from within the membrane lipid bilayer and from the intracellular cytoplasmic compartment, as suggested by a low-resolution electron microscopic study and image analysis (Rosenberg et al., 1997). The different drug-binding sites formed by TMDs are depicted (left). The small circles labeled from 1 to 12 represent the first to the 12th TMDs on P-gp (the α-helix packing in the lipid bilayer, a top view) (right). The Y-bar fragments protruding from small circles represent the side chains of the TM α-helix. The drug-binding site for cyclosporins is formed by interaction of the side chains of both TM 6 and TM 12. WT, wild-type P-gp; MUT, mutant P-gp with Phe^{335} deletion; and (♦), cyclosporins.

results of our present study further suggest that the coupling of ATP hydrolysis to drug-binding domains involves Phe^{335} of TM 6 (Figs. 2–6, and 9). The higher basal ATP activity in these DxP cells may be a consequence of a conformation change in TM 6 of the mutant P-gp (manifested by decreased binding of substrates such as cyclosporins and azidopine), which also results in a conformational change of the ATP binding site at the first half of P-gp (Fig. 9A). Such a linkage of TM 6 and the ATP-binding site also might explain the reduced trapping of ATP by vanadate in the mutant P-gp.

The cyclosporins and verapamil illustrate the sometimes divergent properties of drug binding, transport, and ATPase stimulation in P-gp. Verapamil is not itself transported by either the wild-type or the mutant P-gp, although it binds to P-gp with a moderate affinity (as defined by inhibition of substrate transport and photoaffinity labeling), and stimulates ATPase activity (Figs. 5 and 7). Cyclosporine, by contrast, variably binds to and is transported by the wild-type and mutant P-gp, and inhibits rather than stimulates the basal ATPase activity (Chen et al., 1997; Figs. 2, 5, and 8). Thus, an association between drug binding and transport or between binding and ATPase stimulation is not an absolute requirement for MDR modulators to inhibit transport by P-gp and sensitize cells to MDR-related cytotoxic drugs.

In summary, the consequences of the deletion of Phe^{335} in DxP cells include an altered MDR phenotype, loss of cyclosporine and azidopine binding, altered basal and stimulated ATPase activity, decreased ATP binding, and altered drug transport. Our data demonstrate that Phe^{335} of TM 6 is an important amino acid residue that is involved in forming the drug-binding site. P-gp substrates such as cyclosporine, PSC, azidopine, dactinomycin, vinblastine, and rhodamine 123 share at least one binding domain involving Phe^{335} of TM 6 on P-gp. The examples of verapamil and cyclosporins reveal dissociations among drug transport, drug binding, and stimulation or inhibition of basal ATPase activities. Finally, our data suggest that Phe^{335} of TM 6 may play an important role in coupling drug binding to ATPase activity.

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