A Single Amino Acid Residue Contributes to Distinct Mechanisms of Inhibition of the Human Multidrug Transporter by Stereoisomers of the Dopamine Receptor Antagonist Flupentixol†

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ABSTRACT: Both cis and trans isomers of the dopamine receptor antagonist flupentixol inhibit drug transport and reverse drug resistance mediated by the human multidrug transporter P-glycoprotein (Pgp) with a stereoselective potency. The rate of ATP hydrolysis by Pgp and photoaffinity labeling of Pgp with the substrate analogue [^{125}I]iodoarylazidoprazosin ([^{125}I]IAAP) are modulated by each isomer in an opposite manner, suggesting different mechanisms for the inhibitory effect on drug transport. In this study we demonstrate that substitution of a single phenylalanine residue at position 983 (F983) with alanine (F983A) in putative transmembrane (TM) region 12 selectively affects inhibition of Pgp-mediated drug transport by both isomers of flupentixol. In F983A the stimulatory effect of *cis*(*Z*)-flupentixol and the inhibitory effect of *trans*(*E*)-flupentixol on ATP hydrolysis and [^{125}I]IAAP labeling were significantly altered. This indicates that F983 contributes to inhibition of drug transport by both isomers of flupentixol and plays an important role in stimulation and inhibition of ATP hydrolysis and [^{125}I]IAAP labeling by *cis*(*Z*)- and *trans*(*E*)-flupentixol, respectively. The near-wild-type level of drug transport by the F983A Pgp mutant dissociates susceptibility to inhibition by flupentixol from drug translocation, indicating the allosteric nature of the flupentixol interaction. The inhibitory effects of cyclosporin A on drug transport, drug-stimulated ATP hydrolysis, and [^{125}I]IAAP labeling as well as the stimulatory effect of verapamil on ATP hydrolysis by Pgp were minimally affected by substitution of F983, suggesting no global alteration in the structural and functional integrity of the mutant. Taken together, our data suggest that distinct mechanisms of inhibition of Pgp-mediated drug transport by the cis and trans isomers of flupentixol are mediated through a common site of interaction.

The human P-glycoprotein (Pgp)[1] is an ATP-dependent efflux pump for a variety of structurally unrelated anti-cancer drugs and other cytotoxic agents (*1*). The expression of functional Pgp contributes to resistance against multiple chemotherapeutic agents in cancer cells (*2, 3*). A number of chemical compounds have been identified that inhibit the transport function of Pgp and can chemosensitize certain multidrug resistant cancer cells (*2*). These compounds are collectively known as reversing agents or modulators (*2, 4, 5*). The effectiveness of some of these compounds in early phase clinical trials has encouraged efforts to design higher potency Pgp inhibitors. To develop reversing agents with the explicit goal of inhibiting drug transport by Pgp, a precise knowledge of the inhibitor interaction site(s) and the mechanisms involved in inhibition is required. However, the structural diversity among the reversing agents of Pgp presents a formidable challenge in defining their modes of action as well as their sites of interaction.

One approach to solve this problem is to analyze the inhibitory activity of various analogues of Pgp modulators in order to identify the structural features contributing to the inhibitory potential of a particular reversing agent (*6, 7*). Based on such studies with phenothiazines and thioxanthene derivatives, Ford et al. (*6, 8*) have outlined the structural components of these compounds important for inhibiting Pgp function. A tertiary amino group incorporated into a cyclic ring structure in a trans orientation at a distance of at least three carbons from the hydrophobic tricyclic ring nucleus was optimal for MDR (multidrug resistance) reversal by thioxanthenes (*6*). This study also suggested that thioxanthene derivatives locked into a trans configuration by an exocyclic double bond were more potent inhibitors than their cis counterparts. The reason behind this stereoselective potency was not clear.

cis and trans isomers of flupentixol have opposite effects on ATP hydrolysis and substrate recognition by Pgp. Both ATP hydrolysis and photoaffinity labeling with the substrate

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[1] Abbreviations: Pgp, P-glycoprotein; [^{125}I]IAAP, [^{125}I]iodoarylazidoprazosin; TM, transmembrane; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; FACS, fluorescence activated cell sorting.

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analogue [^{125}I]iodoarylazidoprazosin ([^{125}I]IAAP) were stimulated by *cis*(*Z*)-flupentixol, whereas both activities were effectively inhibited by *trans*(*E*)-flupentixol. Based on those results, distinct mechanisms of inhibition of Pgp-mediated drug transport by the two (cis and trans) stereoisomers can be proposed. To elucidate the underlying principle by which Pgp can functionally interact with a diverse array of structurally unrelated compounds and yet can distinguish between and respond differentially to a small structural variation such as stereoisomerism, the nature and sites of interaction of both the isomers of flupentixol need to be defined.

Structurally, Pgp is a 1280 amino acid cell surface phosphoglycoprotein encoded by the human *MDR*1 gene (*9*). It is composed of 12 putative transmembrane (TM) regions and 2 consensus nucleotide binding domains (NBD) that are arranged in 2 halves with considerable sequence homology between them (*9*). The putative transmembrane (TM) domains show a high degree of sequence homology among the members of the MDR family, which suggests an important role of these hydrophobic stretches in the function of the transporter. Genetic and biochemical studies have further emphasized the role of TM 5, 6 and TM 11, 12 on drug recognition and drug transport by Pgp (*10*−*13*), making them reasonable targets for mutagenesis in order to identify residues important for substrate as well as inhibitor interaction.

In this study we demonstrate that substitution of a single phenylalanine residue with alanine at position 983 in TM 12 of Pgp affects inhibition of Pgp-mediated drug transport by both *cis*(*Z*)- and *trans*(*E*)-flupentixol, and significantly alters their stereospecific effect on ATP hydrolysis and substrate recognition by Pgp, suggesting a common site of functional interaction for both isomers of flupentixol.

EXPERIMENTAL PROCEDURES

Chemicals. *cis*(*Z*)-Flupentixol and *trans*(*E*)-flupentixol were bought from Research Biochemicals International (Natick, MA). Bodipy FL-verapamil was obtained from Molecular Probes (Eugene, OR). [^{125}I]Iodoarylazidoprazosin ([^{125}I]IAAP) (2200 Ci/mmol) was supplied by DuPont New England Nuclear (NEN; Boston, MA). Cyclosporin A was purchased from Calbiochem (San Diego, CA). Restriction enzymes were purchased from Boehringer Mannheim (Indianapolis, IN) and New England Biolabs (Beverly, MA). Minimum essential medium with Earle's salts (EMEM), Opti-Eagle's minimal essential medium (Opti-MEM), Iscove's modified Dulbecco's medium (IMEM), trypsin-EDTA, Dulbecco's phosphate-buffered saline (PBS), and lipofectin were obtained from Gibco BRL Life Technologies Inc. (Grand Island, NY). Fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, UT). Dulbecco's modified Eagle's medium (DMEM) without glutamine was obtained from Quality Biological Inc. (Gaithersburg, MD).

Antibodies. The human Pgp-specific monoclonal antibody MRK-16 was a gift from Hoechst Japan Ltd. (*14*). Purified mouse IgG2a was obtained from Pharmingen (San Diego, CA). The Pgp-specific monoclonal antibody C219 (*15*) was a gift from Centocor, PA.

Cell Lines and Viruses. Human osteosarcoma (HOS, ATCC CRL1543) cells were grown as previously described (*16*). Briefly, cells were propagated as monolayer cultures at 37 °C in 5% CO_2 in EMEM supplemented with 4.5 g/L glucose, 5 mM L-glutamine, 50 units/mL penicillin, 50 μg/mL streptomycin, and 10% FBS. Human HeLa cells were grown as monolayers in DMEM supplemented with 4.5 g/L glucose, 5 mM L-glutamine, 50 units/mL penicillin, 50 μg/mL streptomycin, and 10% FBS. Recombinant vaccinia virus encoding bacteriophage T7 RNA polymerase (vTF7-3), used for expression of the wild-type or mutant *MDR*1 genes controlled by the T7 promoter in a transfected plasmid, was obtained from B. Moss (National Institutes of Health, Bethesda, MD). vTF7-3 was propagated and purified as described previously (*17, 18*).

Mutagenesis and Vector Construction. In transmembrane domain 12 of human P-glycoprotein, alanine mutations were constructed using the pTM1-MDR1 vector system (*19*). In this system, the human *MDR*1 cDNA sequence is inserted at the 3′ end of the encephalomyocarditis virus internal ribosome entry site (IRES) sequence downstream from the T7 promoter in pTM1 (*20, 21*). Alanine mutations were introduced using a PCR mutagenesis method (*22*) at positions L975, V981, F983, M986, V988, Q990, and V991 (*23*). PCR fragments containing the respective mutations spanned the unique *Nde*I and *Pst*I sites in the human *MDR*1 cDNA and were subsequently cloned into these sites. All sequences (the *Nde*I−*Pst*I fragment in entirety) were verified by automated sequencing using PRISMReady Reaction DyeDeoxy Terminator Sequencing Kit from Perkin-Elmer Corp. (Norwalk, CT).

Expression of P-glycoprotein by a Recombinant Vaccinia Virus-Mediated Infection−*Transfection Protocol.* The transient expression system used was based on the method previously developed (*20, 24*). A 70−80% confluent monolayer of HOS or HeLa cells was infected with vTF7-3 and transfected with pTM1-MDR1 constructs as described previously (*19, 25*).

Fluorescence Activated Cell Sorting (FACS). To measure cell surface expression, 5×10^5 cells were incubated at 4 °C for 30 min with 5 μg of the monoclonal antibody MRK-16 (*14*) or 5 μg of purified mouse IgG2a (as control) in 200 μL of IMEM supplemented with 5% FBS. The cell suspension was then diluted to 5 mL in cold medium and centrifuged at 200*g* for 5 min. Washed cell pellets were resuspended in 200 μL of cold IMEM containing 3 μg of FITC-labeled anti-mouse IgG and incubated at 4 °C for 30−45 min. Cells were washed as above and resuspended in 350 μL of cold PBS and analyzed by fluorescent activated cell sorting (FACS) (Becton-Dickinson FACS System, San Jose, CA). The results are presented as cell counts versus cell-associated fluorescence intensity.

To measure the accumulation of fluorescent substrates, 5×10^5 cells were harvested and washed as described above for MRK-16 staining and incubated for 30 min at 37 °C in 5 mL of IMEM containing 5% FBS and 0.5 μM Bodipy FL-verapamil, with or without 5 μM cyclosporin A or other reversing agents (such as flupentixol). Cells were then pelleted by centrifugation at 200*g* for 5 min and resuspended in 350 μL of ice-cold PBS and analyzed by FACS. The results are presented as cell counts versus intracellular fluorescence intensity.

Preparation of Cell Lysate. Cells (5×10^5) were harvested, washed in PBS, and resuspended in 100 μL of 10 mM Tris-

HCl, pH 8.0, 0.1 Triton X-100, 10 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mM dithiothreitol (DTT), 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 1% aprotinin, and 10 units/μL micrococcal nuclease (TD buffer). Cells in TD buffer were frozen at −70 °C and subsequently thawed at 37 °C. The freeze−thaw cycle was repeated 3 times with intermittent vortexing of the cells. Following freeze−thaw, 25 μL of Laemmli SDS−PAGE sample loading buffer (*26*) was added and kept at room temperature for 30 min. Samples were vortexed firmly prior to SDS−PAGE and immunoblot analysis.

Preparation of Crude HeLa Membranes. Crude membranes were prepared as described previously by Ramachandra et al. (*19, 25*). Briefly, cells (from 10 × 75 cm^2 tissue culture flasks) were harvested, washed twice in ice-cold PBS containing 1% aprotinin, resuspended in an approximate volume (0.5 mL/75 cm^2 tissue culture flask) of hypotonic lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 1 mM AEBSF, and 1% aprotinin), and frozen at −80 °C. Frozen cells were thawed and incubated on ice for 45 min and disrupted by repeated (50 times) strokes of a Dounce homogenizer. Following lysis, undisrupted cells and nuclei were removed by centrifugation at 500*g* for 10 min. The low-speed supernatant was treated with micrococcal nuclease (50 units/mL) in the presence of 1 mM $CaCl_2$ for 30 min on ice. The membranes were collected by centrifugation at 100000*g* for 60 min and resuspended by passing through a hypodermic needle (gauge size 19 and then 23) in 400 μL of resuspension buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 250 mM sucrose, 1 mM DTT, 1% aprotinin, 1 mM AEBSF) containing 10% glycerol, and stored at −80 °C in aliquots. Protein content was determined by a modified Lowry method (*27*) using bovine serum albumin as a standard.

Measurement of ATPase Activity. Pgp-associated ATPase activity was measured by determining the level of sodium orthovanadate-sensitive release of inorganic phosphate from ATP using a colorimetric method as described (*19, 28*) with minor modifications. Membrane suspensions (20 μg of protein) were preincubated a 37 °C for 5 min in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 5 mM sodium azide, 2 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), 2 mM ouabain, 2 mM dithiothreitol (DTT), 50 mM KCl, 10 mM $MgCl_2$. Drugs were added to the assay and incubated for 3 min at 37 °C. For drugs that were not water-soluble, stock solutions were made in dimethyl sulfoxide (DMSO) in such a way that the final concentration of DMSO in the assay never exceeded 1%. This concentration of DMSO did not exhibit any effect on the Pgp ATPase activity. The assay was initiated by the addition of 5 mM ATP (pH 7.0) to a total volume of 100 μL, and incubated at 37 °C for 20 min. The rate of ATP hydrolysis remain linear up to 60 min. Reactions were stopped by addition of 100 μL of 5% SDS, and the amount of inorganic phosphate released was measured by a colorimetric detection method, previously described (*29*). The rates of ATP hydrolysis were expressed as nanomoles of ATP hydrolyzed per milligram of total membrane protein per minute (nmol mg^{-1} min^{-1}). The vanadate-sensitive activities were calculated as the differences between the ATPase activities obtained in the absence and presence of 300 μM sodium orthovanadate. To determine the concentrations of drug required to achieve maximal and half-maximal stimulation or inhibition of the basal ATPase activity, data were subjected to nonlinear regression analysis using graphic program GraphPad Prism (GraphPad Software Inc.). In all cases the basal ATPase activity (in absence of drug) was subtracted before curve-fitting. In the case of inhibition of the ATPase activity, all values were expressed as percent of the activity that was achieved in the absence of the inhibitor. To save space, actual curve fits are not shown. Each experiment was repeated 2−3 times, and similar results were obtained. Data for each figure represent the average of at least two identical experiments.

Photoaffinity Labeling with [^{125}I]IAAP. Photoaffinity labeling of crude membranes was carried out according to Dey et al. (*30*). Briefly, an aliquot of membranes (25 μg of protein) was incubated for 10 min under subdued light with 4 nM [^{125}I]IAAP at room temperature in 50 mM Tris-HCl, pH 7.5, and 1% aprotinin (labeling buffer), in a final volume of 100 μL. Following incubation, membranes were illuminated with a UV lamp (General Electric no. F15T8-BLB; 365 nm) for 10 min, with assay tubes sitting on water at room temperature. After cross-linking, 25 μL of 5× Laemmli SDS−PAGE sample buffer was added to the reaction mixture and kept at room temperature for another 30 min before analyzing by SDS−PAGE. Where appropriate, membranes were preincubated for 3−5 min with the indicated concentrations of Pgp modulators, cyclosporin A, *cis*(*Z*)-flupentixol, or *trans*(*E*)-flupentixol, prior to the addition of [^{125}I]IAAP.

Quantification of Radioactivity in Protein Bands. Gels were presoaked in Novex gel drying solution for 10 min and dried overnight between two pieces of cellophane. For visualization of the radioactive bands, dried gels were exposed to Kodak Bio-Max MR X-ray film. Exposure times were adjusted such that the bands with higher intensity in adjacent lanes can be best resolved. To determine the [^{125}I]IAAP photo-cross-linked to Pgp, the radioactivity associated with each band was quantified from the dried gels by exposing to a phosphorimager screen and analyzed using a STORM 860 phosphorimaging system (Molecular Dynamics). For each set of experiments, the number of counts associated with the Pgp band in the absence of any modulator was considered as 100%, and values for others were expressed as percent control. To determine the concentrations of drug required to achieve maximal and half-maximal stimulation or inhibition of [^{125}I]IAAP labeling, data were subjected to nonlinear regression analysis using graphics program GraphPad Prism (GraphPad Software Inc.). Each experiment was repeated 2−3 times, and similar results were obtained. Data presented in each figure represent the average of at least two identical experiments.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE) and Immunoblot Analysis. Electrophoresis and immunoblot analysis were performed as described previously (*19, 25*). Pgp-specific monoclonal antibody C219 (*15*) was used at a dilution of 1:4000 for detection of the wild-type and mutant Pgp's. Goat anti-mouse IgG conjugated with horseradish peroxidase was used as secondary antibody at a dilution of 1:5000. HRP-conjugated secondary antibody bound to the nitrocellulose was detected by using the HRP-catalyzed luminol-based chemiluminescence reaction (ECL Western blotting system) from Amersham Life Science Products. The light emission signal was captured on Kodak Bio-Max MR film.

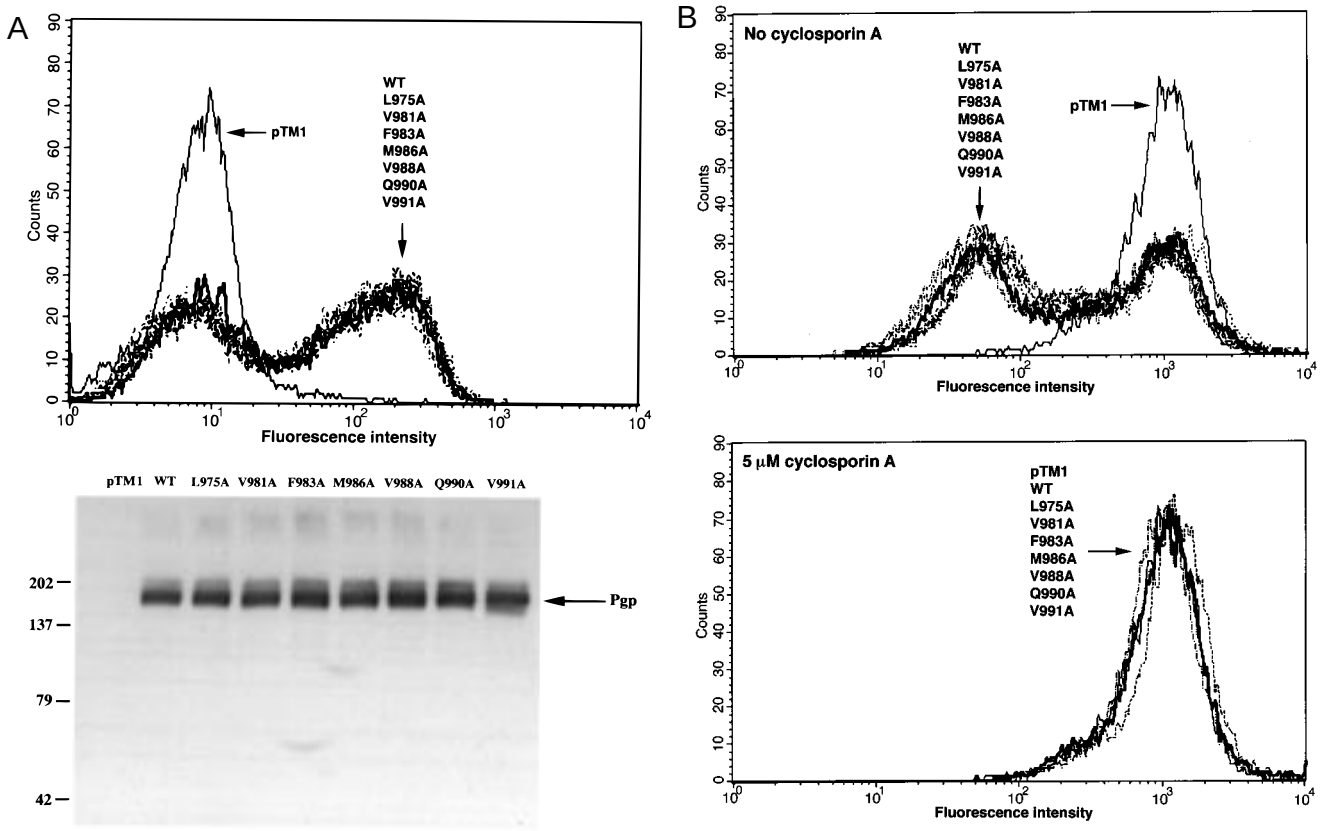

FIGURE 1: Expression and functional analysis of the wild-type and mutant Pgp's. Human osteosarcoma (HOS) cells infected with vTF7-3 were transfected with either pTM1 (control) (—), pTM1-MDR1 (wild type) (—), pTM1-MDR1-L975A (L975A) (•••), pTM1-MDR1-V981A (V981A) (- - -), pTM1-MDR1-F983A (F983A) (— —), pTM1-MDR1-M986A (M986A) (thick dashes), pTM1-MDR1-V988A (V988A) (—••—), pTM1-MDR1-Q990A (Q990A) (—•—), or pTM1-MDR1-V991A (V991A) (—•••—) plasmid DNA. At 24 h post-infection, cells were harvested and washed with PBS. (A, upper panel) Cells were subjected to FACS analysis after staining with human Pgp external epitope-specific monoclonal antibody MRK-16 (*14*), as described under Experimental Procedures. (A, lower panel) Total cell lysates were prepared from each cell type, and immunoblot analysis with Pgp-specific monoclonal antibody C219 was performed as described under Experimental Procedures. (B) Similar to section A; cells were infected with vTF7-3, and transfected with either pTM1 (control) (—), pTM1-MDR1 (wild type) (—), pTM1-MDR1-L975A (L975A) (•••), pTM1-MDR1-V981A (V981A) (- - -), pTM1-MDR1-F983A (F983A) (— —), pTM1-MDR1-M986A (M986A) (thick dashes) pTM1-MDR1-V988A (V988A) (—••—), pTM1-MDR1-Q990A (Q990A) (—•—), or pTM1-MDR1-V991A (V991A) (—•••—) plasmid DNAs. Bodipy FL-verapamil accumulation in infected-transfected HOS cells were determined by FACS in the presence (lower panel) and absence (upper panel) of 5 μM cyclosporin A.

RESULTS

Substitution of Phenylalanine Residue 983 with Alanine Selectively Affects Inhibition of Drug Transport by both cis(Z)- and trans(E)-Flupentixol. In a recent study, seven amino acid residues, L975, V981, F983, M986, V988, Q990, and V991, in the putative TM 12 of human Pgp were substituted individually by alanine (L975A, V981A, F983A, M986A, V988A, Q990A, and V991A). These residues are not strictly conserved among the members of the MDR family of transporters. None of these substitutions had any effect on Pgp-mediated drug transport or its inhibition by cyclosporin A (*23*). To determine whether these amino acid residues have any direct involvement in the interaction of Pgp with the reversing agent flupentixol, intracellular accumulation of the fluorescent substrate Bodipy FL-verapamil was measured in cells expressing any of the seven single mutants, in the presence and absence of *cis*(*Z*)- and *trans*(*E*)-flupentixol.

The wild-type and mutant Pgp's were transiently expressed in human osteosarcoma (HOS) cells using a vaccinia virus-mediated infection−transfection system. Cell surface expression of the wild-type and mutant Pgp's was determined by staining with monoclonal antibody MRK-16, specific for a human Pgp external epitope. Consistent with the previous report, all the mutant Pgp's (L975A, V981A, F983A, M986A, V988A, Q990A, and V991A) were expressed on the cell surface at a comparable level to that of wild-type (WT) Pgp (Figure 1A, upper panel). This was evident from the enhanced fluorescence intensity associated with the cells compared to that of the control cells, infected with the vector DNA (pTM1) only (Figure 1A, upper panel). Since only 50% of the cells were transfected by this protocol, the low fluorescence intensity peak in Figure 1A represents cells that were not transfected. MRK-16 staining exclusively detects Pgp molecules that are present on the cell surface. Therefore, to ensure that the same levels of Pgp were expressed in the mutants, immunoblot analyses of the total cell lysates of the same cells were performed, using Pgp-specific monoclonal antibody C219. All the mutants show similar levels of Pgp expression to that of the wild-type Pgp (Figure 1A, lower panel). No Pgp was detected in the control cells transfected with the vector plasmid (pTM1) only (Figure 1A, lower panel).

To measure transport function, cells were incubated for 30 min at 37 °C with 0.5 μM Bodipy FL-verapamil in the presence and absence of either 5 μM cyclosporin A, 10 μM

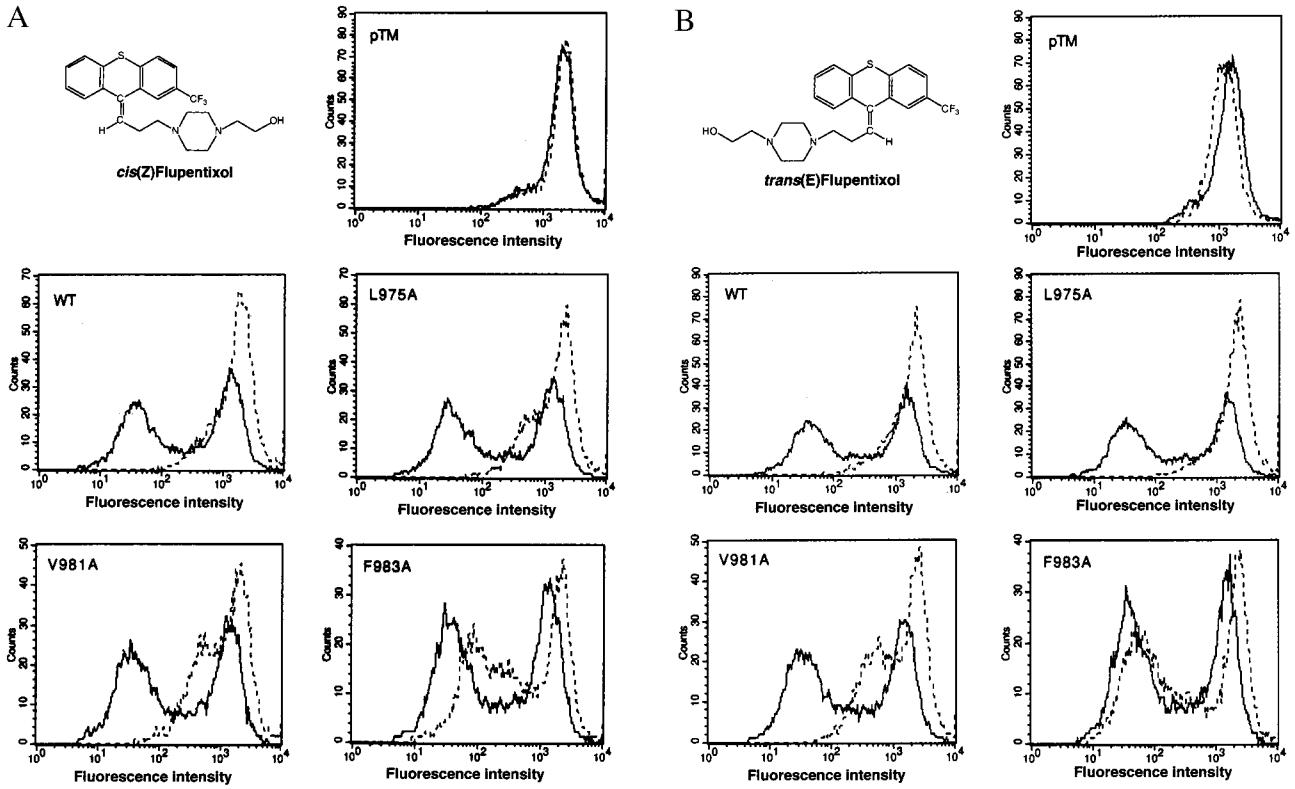

FIGURE 2: Effect of *cis*(*Z*)-flupentixol and *trans*(*E*)-flupentixol on Bodipy FL-verapamil accumulation. Steady-state accumulation of Bodipy FL-verapamil was measured by FACS in the presence (- - -) and absence (—) of 10 μM *cis*(*Z*)-flupentixol (A) or 5 μM *trans*(*E*)-flupentixol (B) in HOS cells infected with vTF7-3 and transfected with either pTM1 (pTM1), pTM1-MDR1 (WT), pTM1-MDR1-L975A (L975A), pTM1-MDR1-V981A (V981A), or pTM1-MDR1-F983A (F983A). For details, see Experimental Procedures.

cis(*Z*)-flupentixol or 5 μM *trans*(*E*)-flupentixol. The concentrations of reversing agents chosen were the minimum required to achieve complete inhibition of drug transport in cells expressing wild-type Pgp. After incubation, cells were washed in ice-cold PBS and analyzed by FACS. Cells transfected with the control plasmid DNA (pTM1) showed enhanced fluorescence intensity, indicating intracellular accumulation of the fluorescent drug (Figure 1B, upper panel). Cells expressing any of the seven mutants or the wild-type Pgp showed reduced accumulation of Bodipy FL-verapamil (Figure 1B upper panel), indicating active extrusion of the fluorescent drug analogue from the cells. Consistent with our results with cell surface expression of Pgp (Figure 1A, upper panel), only 50% of the cell population showed decreased accumulation of the drug. In the presence of 5 μM cyclosporin A, the steady-state accumulation of Bodipy FL-verapamil in all the cells expressing either wild-type or mutant Pgp's was increased to the level of that of the control cells (Figure 1B, lower panel) with no detectable expression of Pgp (Figure 1A). This reflects inhibition of Pgp-mediated efflux by cyclosporin A, resulting in increased intracellular accumulation. Similar results were obtained with other Pgp substrates, such as daunomycin, calcein AM, rhodamine, and Bodipy FL-taxol (data not shown). This suggested that none of the single amino acid substitutions have grossly affected the structural and functional integrity of Pgp.

In the presence of 10 μM *cis*(*Z*)-flupentixol or 5 μM *trans*(*E*)-flupentixol, the intracellular accumulation of Bodipy FL-verapamil in HOS cells expressing either wild-type Pgp or L975A was increased to approximately the same level as that of the control cells (Figure 2A,B), which had no detectable expression of Pgp, suggesting effective inhibition of Pgp-mediated efflux from the cells. The effects of both *cis*(*Z*)- and *trans*(*E*)-flupentixol on accumulation of Bodipy FL-verapamil in cells expressing V988A, Q990A, or V991A were identical to that observed in L975A (data not shown), suggesting that none of these substitutions affects inhibition of transport by flupentixol. However, the steady-state accumulation of the verapamil derivative in cells expressing F983A was minimally altered in the presence of 10 μM *cis*(*Z*)- or 5 μM *trans*(*E*)-flupentixol (Figure 2A,B), suggesting an impaired ability of this mutant to respond to inhibition of drug transport by both isomers of flupentixol. In cells expressing mutants V981A (Figure 2A,B) and M986A (data not shown), the level of intracellular accumulation of Bodipy Fl-verapamil was effectively increased by both isomers of flupentixol, but not to the same extent as that of the cells expressing wild-type Pgp. This suggested that inhibition of drug transport by *cis*(*Z*)- and *trans*(*E*)-flupentixol in V981A and M986A was not complete, indicating a moderate contribution of these residues to the interaction with flupentixol. Since substitution of F983 had the most profound effect on the inhibitory potential of flupentixol, the mutant F983A was our obvious choice for further characterization.

Stimulation of ATP Hydrolysis by cis(Z)-Flupentixol Is Considerably Altered in F983A. Reversing agents such as verapamil and cyclosporin A have been shown to modulate Pgp-mediated ATP hydrolysis. Since drug transport by F983A was considerably less sensitive to inhibition by *cis*(*Z*)-flupentixol, we studied the ability of *cis*(*Z*)-flupentixol to modulate the rate of ATP hydrolysis by this mutant. Crude membranes were isolated from HeLa cells expressing either

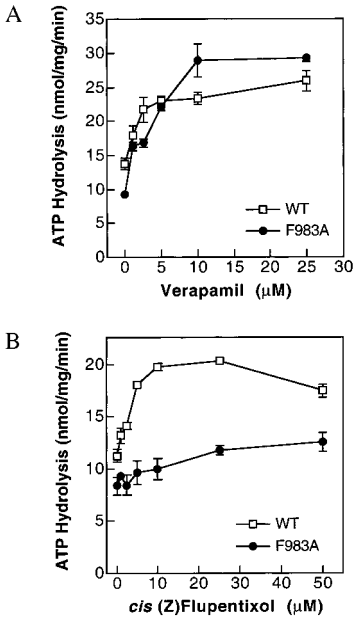

FIGURE 3: Effect of verapamil and *cis*(*Z*)-flupentixol on ATP hydrolysis by the wild-type and F983A Pgp's. Membranes were isolated from HeLa cells infected with vTF7-3 and transfected with either pTM1, pTM1-MDR1 (WT), or pTM1-MDR1-F983A (F983A) plasmid DNAs. HeLa cell membranes (20 μg of protein) containing wild-type Pgp (□) or F983A (●) were incubated either with the indicated concentrations of verapamil (A) or with *cis*(*Z*)-flupentixol (B) prior to measuring the rate of ATP hydrolysis. Vanadate-sensitive release of phosphate from MgATP was measured in the presence of 50 mM Tris-HCl (pH 7.5) (A) or 0.1 M MES[2-(*N*-morpholino)ethanesulfonic acid) (pH 7.0) (B), 5 mM sodium azide, 2 mM EGTA, 2 mM ouabain, 2 mM DTT, 50 mM KCl, 5 mM ATP, and 10 mM $MgCl_2$, as described under Experimental Procedures. Reactions were stopped after 20 min of incubation at 37 °C, and the rates of ATP hydrolysis were plotted as nmol mg^{-1} min^{-1} against the concentration of the modulators used. The figure represents the average (±standard error) of data from three different experiments.

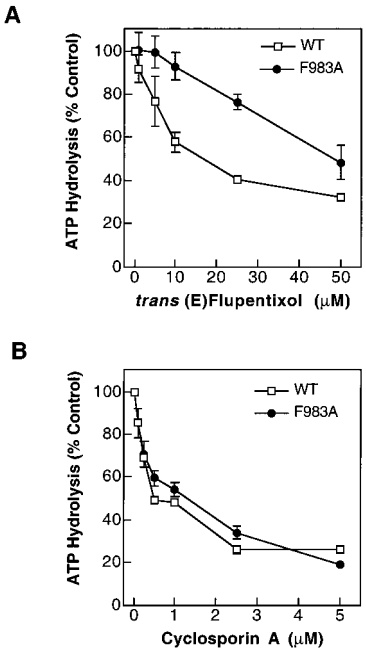

FIGURE 4: Effect of *trans*(*E*)-flupentixol and cyclosporin A on verapamil-stimulated ATP hydrolysis by the wild-type and F983A Pgp's. Vanadate-sensitive ATP hydrolysis by wild-type Pgp (□) and F983A (●) (20 μg of membrane protein) in HeLa cell membranes was measured as described in Figure 3, in the presence of 5 mM ATP, 10 mM $MgCl_2$, 25 μM verapamil, and the indicated concentrations of either *trans*(*E*)-flupentixol (A) or cyclosporin A (B). Values are expressed as percent control, considering the rate of ATP hydrolysis in the absence of any inhibitors as 100%. The figure represents the average (±standard error) of data from two different experiments.

the wild-type Pgp or F983A, and Pgp-ATPase activity was measured in the presence of varying concentrations of either verapamil or *cis*(*Z*)-flupentixol.

Consistent with their ability to transport Bodipy FL-verapamil, ATP hydrolysis by both wild-type Pgp and F983A in isolated membranes was stimulated by verapamil in a concentration-dependent manner (Figure 3A). Although the rate of ATP hydrolysis by F983A in the absence of verapamil was about 70% of that of the wild-type Pgp, the verapamil-stimulated activity was about the same in both Pgp's. A maximum rate of ATP hydrolysis between 25 and 28 nmol mg^{-1} min^{-1} was achieved by both wild-type and F983A at a verapamil concentration of 25 μM. Under the same experimental conditions, the basal as well as drug-stimulated ATPase activity in membranes isolated from cells transfected with pTM1 (vector only) never exceeded 5 nmol mg^{-1} min^{-1} (data not shown) (*23*).

cis(*Z*)-Flupentixol also stimulated the rate of ATP hydrolysis by wild-type Pgp in a concentration-dependent manner. A maximal activity of 20 nmol mg^{-1} min^{-1} was reported at 10−25 μM *cis*(*Z*)-flupentixol (Figure 3B). In contrast, stimulation by *cis*(*Z*)-flupentixol of ATP hydrolysis by F983A was significantly impaired, with a maximum of only 12−14 nmol mg^{-1} min^{-1} of ATP hydrolyzed at *cis*(*Z*)-flupentixol concentrations of 50 μM (Figure 3B). Due to a low basal rate of ATP hydrolysis by F983A, the fold stimulation by *cis*(*Z*)-flupentixol (2-fold) was about the same for both wild-type Pgp and F983A; however, in F983A this was achieved at a considerably higher concentration (Figure 3B).

The Ability of trans(E)-Flupentixol To Inhibit Verapamil-Stimulated ATP Hydrolysis Is Markedly Reduced in F983A. Since Bodipy FL-verapamil transport by F983A was less sensitive to inhibition by *trans*(*E*)-flupentixol, we studied its effect on verapamil-stimulated ATP hydrolysis by F983A. Isolated cell membranes from HeLa cells, expressing either wild-type Pgp or F983A, were preincubated with varying concentrations of *trans*(*E*)-flupentixol prior to the measurement of vanadate-sensitive ATP hydrolysis in the presence of 25 μM verapamil. Consistent with the previous experiment, in the absence of *trans*(*E*)-flupentixol the rate of verapamil-stimulated ATP hydrolysis by the wild-type and the mutant (F983A) was around 26 nmol mg^{-1} min^{-1}. This verapamil-stimulated ATP hydrolysis by wild-type Pgp was effectively inhibited by *trans*(*E*)-flupentixol with a concentration required for half-maximal inhibition (IC_{50}) of about 8 μM (Figure 4A). In contrast, the ability of *trans*(*E*)-flupentixol to inhibit verapamil-stimulated ATP hydrolysis by F983A was significantly reduced (Figure 4A). The concentration required for half-maximal inhibition was around 35 μM, which was at least 4-fold higher than was required for wild-type Pgp. This indicated that residue F983A also plays a crucial role in the inhibition of ATP hydrolysis by *trans*(*E*)-flupentixol.

To determine whether the loss of sensitivity to *trans*(*E*)-flupentixol in F983A reflected a general defect of the mutant

in its ability to respond to inhibitors of Pgp- ATPase activity, we studied the effect of cyclosporin A on verapamil-stimulated ATP hydrolysis by wild-type Pgp and F983A. Cyclosporin A inhibited verapamil-stimulated ATPase activity with equal potency in both wild type and F983A (Figure 4B). The concentrations for half-maximal inhibition were about 0.4 μM for both wild type and F983A. This suggested a selective effect of substitution of F983 on the inhibitory potential of *trans*(*E*)-flupentixol on ATP hydrolysis.

Stimulation by cis(Z)-Flupentixol of Photoaffinity Labeling with the Substrate Analogue [^{125}I]IAAP Is Considerably Affected in F983A. Unlike other reversing agents, such as cyclosporin A, *cis*(*Z*)-flupentixol stimulates [^{125}I]IAAP labeling of Pgp (*30, 31*). Since *cis*(*Z*)-flupentixol was unable to reverse drug transport by F983A, we examined the effect of *cis*(*Z*)-flupentixol on [^{125}I]IAAP labeling of F983A.

Isolated membranes from HeLa cells expressing wild-type Pgp or F983A were photoaffinity labeled with 4 nM [^{125}I]IAAP in the presence of varying (0−100 μM) concentrations of *cis*(*Z*)-flupentixol. *cis*(*Z*)-Flupentixol stimulated [^{125}I]IAAP labeling of wild-type Pgp in a concentration-dependent manner (Figure 5A) with a maximum of 9-fold stimulation observed at concentrations of 10−25 μM (Figure 5C). In contrast, stimulation of [^{125}I]IAAP labeling was significantly reduced in F983A (Figure 5B) with a maximal stimulation of only 2-fold (Figure 5C). However, stimulation reached its maximum at the same concentration of *cis*(*Z*)-flupentixol (10 μM) for both wild-type Pgp and F983A (Figure 5C). In both cases a progressive decrease in the stimulation of [^{125}I]IAAP labeling was found at concentrations of *cis*(*Z*)-flupentixol higher than 25 μM. Isolated membranes from HeLa cells transfected with the control plasmid DNA (pTM1) showed no detectable Pgp expression or photoaffinity labeling with [^{125}I]IAAP (data not shown), demonstrating the specificity of [^{125}I]IAAP labeling for Pgp. These results indicate that F983 also plays a crucial role in the stimulation of [^{125}I]IAAP labeling by *cis*(*Z*)-flupentixol.

Photoaffinity Labeling of F983A with [^{125}I]IAAP Is Relatively Resistant to Inhibition by trans(E)-Flupentixol. Photoaffinity labeling of Pgp with the substrate analogue [^{125}I]IAAP is effectively inhibited by most Pgp reversing agents and substrates (*32*). When HeLa cell membranes were photoaffinity labeled with [^{125}I]IAAP in the presence of varying concentrations of *trans*(*E*)-flupentixol, a concentration-dependent inhibition in [^{125}I]IAAP labeling of the wild-type Pgp (Figure 6A) was observed. The concentration of *trans*(*E*)-flupentixol required for half-maximal inhibition of [^{125}I]IAAP labeling was about 10 μM in wild-type Pgp. Inhibition of [^{125}I]IAAP labeling by *trans*(*E*)-flupentixol was considerably altered in F983A, in which case the concentration of *trans*(*E*)-flupentixol required for half-maximal inhibition was about 4-fold higher (about 40 μM) than that for the wild-type Pgp.

Photoaffinity labeling of the wild-type Pgp and F983A with [^{125}I]IAAP was also carried out in the presence of varying concentrations of cyclosporin A. In both Pgp's, cyclosporin A inhibited [^{125}I]IAAP labeling with similar potency (Figure 6B). Concentrations of cyclosporin A required for half-maximal and maximal inhibitions were around 0.035 μM for both wild-type Pgp and F983A. This excluded the possibility of a general loss of sensitivity to inhibitors of substrate recognition in F983A.

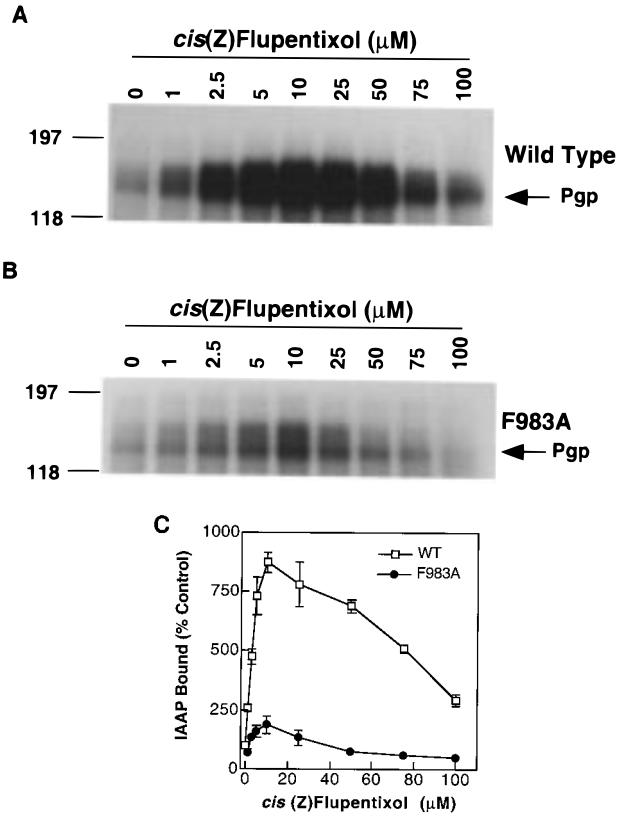

FIGURE 5: Effect of *cis*(*Z*)-flupentixol on [^{125}I]IAAP labeling of the wild-type Pgp and F983A. Membranes were isolated from HeLa cells infected with vTF7-3 and transfected with either (A) pTM1-MDR1 (WT) or (B) pTM1-MDR1-F983A (F983A) plasmid DNAs. 25 μg of HeLa membranes was preincubated at room temperature in the presence of the indicated concentrations of *cis*(*Z*)-flupentixol prior to incubation with 4 nM [^{125}I]IAAP for 10 min under subdued light and photo-cross-linked as described under Experimental Procedures. Following photo-cross-linking, 10 μg of membrane proteins was analyzed by SDS−PAGE and subjected to autoradiography. (C) Quantification of radioactivity associated with the wild-type (□) and F983A (●) Pgp's bands was done by a STORM 860 phosphorimaging system as described under Experimental Procedures. Values were expressed as percent control with labeling in the absence of *cis*(*Z*)-flupentixol as 100% for wild-type and mutant Pgp's respectively. The figure represents the average (±standard error) of data from two different experiments.

DISCUSSION

cis(*Z*) and *trans*(*E*)- isomers of the dopamine receptor antagonist flupentixol both inhibit Pgp-mediated drug transport and reverse drug resistance (*6, 8*). These two isomers of flupentixol have opposite effects on the rate of ATP hydrolysis by Pgp and photoaffinity labeling of Pgp with the substrate analogue [^{125}I]IAAP. Since both ATP hydrolysis and substrate recognition are essential for drug translocation, different effects of *cis*(*Z*)- and *trans*(*E*)-flupentixol on these functional aspects of Pgp suggest distinct mechanisms of inhibition of drug transport by the two isomers. To understand the mechanism by which Pgp, a transporter that interacts with so many structurally unrelated agents, can respond differentially to stereoisomers of the same compound, identification of the sites of flupentixol interaction with Pgp is essential. In this study we demonstrate that a single substitution of alanine for phenylalanine at position 983 in the putative TM 12 of Pgp markedly alters the ability of both cis and trans isomers of flupentixol to modulate ATPase activity and [^{125}I]IAAP labeling of Pgp, and also

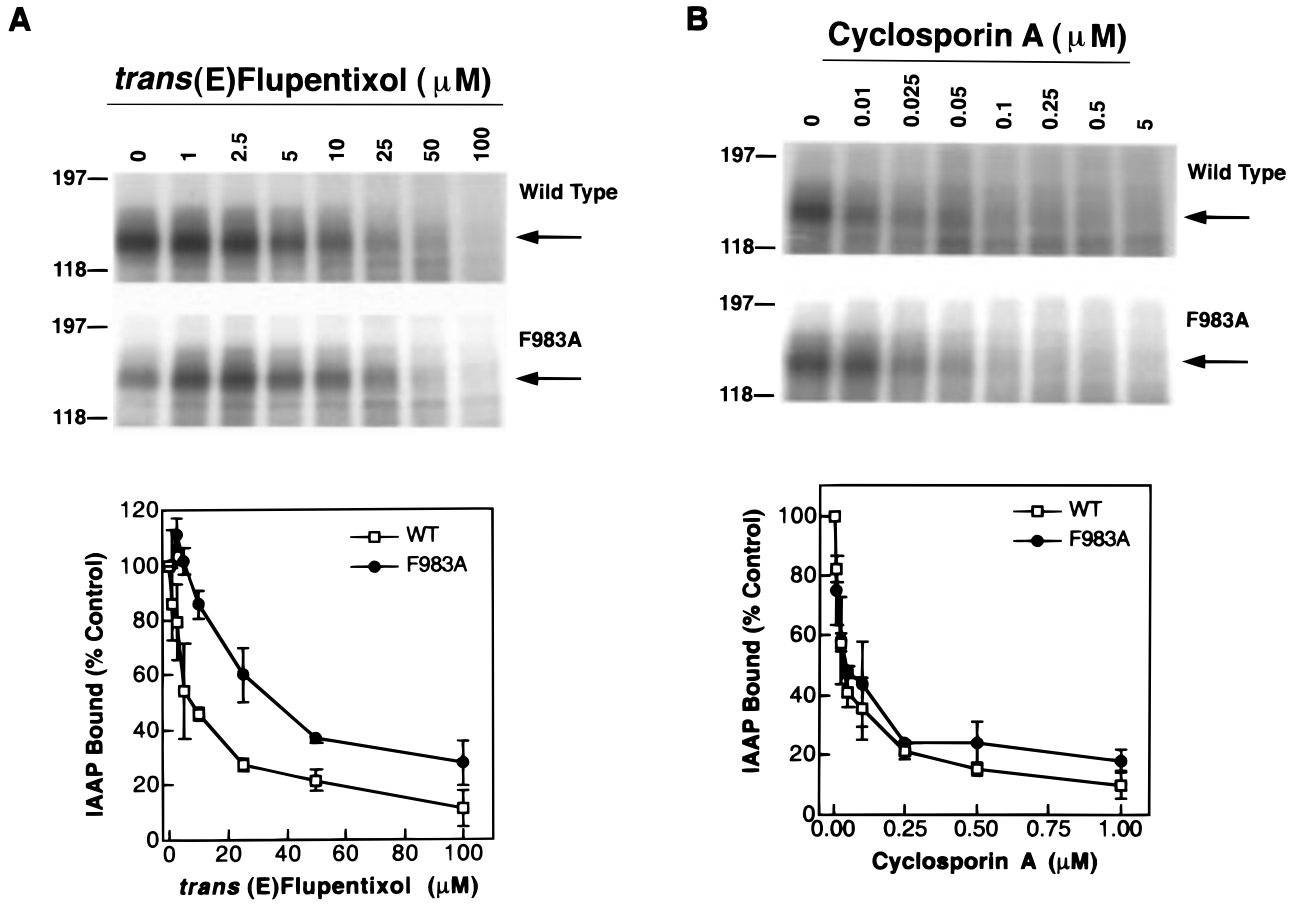

FIGURE 6: Effect of *trans*(*E*)-flupentixol and cyclosporin A on [^{125}I]IAAP labeling of the wild-type Pgp and F983A. HeLa membranes (25 μg of protein) containing either wild-type Pgp (top) or F983A (bottom) were preincubated at room temperature in the presence of the indicated concentrations of either *trans*(*E*)-flupentixol (A) or cyclosporin A (B) prior to incubation with 4 nM [^{125}I]IAAP, for 10 min, as described under Experimental Procedures. Following incubation, membranes were photo-cross-linked, and 10 μg of membrane proteins was analyzed by SDS−PAGE and subjected to autoradiography. Relevant portions of the autoradiograms are shown in the upper panels. Graphs show quantification of the radioactive bands by a STORM 860 phosphorimaging system as described. Radioactivity associated with the wild-type (□) and F983A (●) Pgp bands was expressed as percent control using the same method as in Figure 5. The figure represents the average (±standard error) of data from three different experiments.

affects inhibition of Pgp-mediated drug transport by both isomers of flupentixol.

Stereospecific Modulation by Flupentixol Involves Direct Interaction with Pgp. Both isomers of flupentixol also interact with other cellular proteins such as calmodulin (*33*, *34*), the dopamine D_2 receptor (*35*−*37*), and protein kinase C (*38*). Based on these observations, it has been suggested that the stereoselective potency of the two isomers on Pgp function could be mediated through other cellular targets (*8*). The fact that a single amino acid substitution in Pgp significantly alters the modulatory effects of both isomers of flupentixol on Pgp strongly suggests a direct interaction of both isomers with Pgp. Since *cis*(*Z*)- and *trans*(*E*)-flupentixol have opposite effects on ATP hydrolysis and [^{125}I]IAAP labeling, the evidence for modulation through direct interaction indicates a considerable degree of precision involved in the mechanism by which substrates or modulators are recognized by Pgp. Considering the structural diversity among the substrates and reversing agents of Pgp, the ability to differentiate a small structural difference like stereoisomerism reveals a novel mechanistic aspect of Pgp function.

Phenylalanine 983 Plays an Important Role in the Interaction of Flupentixol with Pgp. Of the seven amino acid residues in TM 12 that were individually substituted with alanine, replacement of F983 has the most dramatic effect on the ability of flupentixol to inhibit drug transport by Pgp. This loss of inhibition of drug transport by *cis*(*Z*)- and *trans*(*E*)-flupentixol suggests a crucial role for F983 in the interaction of flupentixol with Pgp. An active role of phenylalanine residues in the interaction of calmodulin with phenothiazines, compounds structurally related to flupentixol, has been proposed (*39*). Based on those analyses, a similar contribution of phenylalanine residues in the interaction of flupentixol with Pgp had been predicted by Hait and Aftab (*40*). Since substitution of F983 with alanine selectively alters the inhibitory potential of flupentixol with minimal effect on the functional interaction of Pgp with other reversing agents, such as verapamil and cyclosporin A (Figure 1B), our data are suggestive of a specific interaction of flupentixol with F983. Any global structural alteration impairing the general ability of Pgp to be inhibited by reversing agents seems unlikely in this mutant. Although it is premature to predict which structural feature of flupentixol may be directly engaged in the interaction that involves F983, the hydrophobic tricyclic ring nucleus could be a potential candidate.

Among the other residues that were substituted with alanine, V981 and M986 were the closest to F983 in the primary sequence of Pgp. Although drug transport was inhibited by both *cis*(*Z*)- and *trans*(*E*)-flupentixol in V981A and M986A (data not shown), inhibition was not complete,

suggesting a small but clear contribution of these two amino acid residues in the inhibitory effect of flupentixol on drug transport. It is to be noted that in all three mutants (F983A, V981A, and M986A) the inhibitory effect of both isomers (cis and trans) of flupentixol was affected. Therefore, it is possible that F983 and its neighboring residues constitute a site of interaction for flupentixol to which V981 and M986 contribute to a lesser extent.

Alanine is a structurally neutral amino acid that does not introduce constraint in the polypeptide backbone. Therefore, it is more probable that replacement of amino acid side chains such as valine, methionine, or phenylalanine with that of alanine would affect the geometric precision of the interaction site rather than the secondary structure. At this point it is difficult to assess whether F983 contributes directly to binding of flupentixol or is responsible for transmitting the signal after the modulator is bound. The precise role of F983 in the interaction of Pgp with flupentixol can be determined conclusively only if a photoaffinity analogue of the compound becomes available. It is also apparent from the quantitative effect of F983 substitution on the interaction with flupentixol that other residues are also involved in the process. The importance of phenylalanine residues in the TM regions of Pgp, in recognition and transport of Pgp modulators and substrates, has been reported earlier by other groups (*41*, *42*).

Separation of Modulatory Effects of Flupentixol from Drug Recognition and Transport Indicates the Allosteric Nature of Modulation. In F983A the inhibitory effect of *cis*(*Z*)- and *trans*(*E*)-flupentixol on Pgp-mediated drug transport was considerably reduced without much alteration in the ability of the protein to transport substrates (Figures 1B and 2A,B). This is consistent with a previous finding by Loo and Clarke (*41*) which demonstrated that replacement of F983 of human Pgp with alanine had no effect on the ability of the mutant to confer resistance to MDR drugs such as vinblastine, colchicine, actinomycin D, and adriamycin. Therefore, this dissociation of susceptibility to inhibition by flupentixol from drug transport in F983A strongly indicates the allosteric nature of the flupentixol interaction site with Pgp. Consistent with this, in F983A the modulatory potency of *cis*(*Z*)- and *trans*(*E*)-flupentixol on ATP hydrolysis and [^{125}I]IAAP labeling were significantly altered with only a minimal effect on the basal level of ATPase activity and [^{125}I]IAAP labeling.

Stimulation and Inhibition of ATP Hydrolysis and [^{125}I]IAAP Labeling Can Be Mediated through a Common Site of Interaction. The stimulatory and inhibitory effects of Pgp substrates and reversing agents on ATP hydrolysis have been studied extensively. Based on the kinetic analyses, the existence of two major sites of modulation with distinct stimulatory (active site) and inhibitory (inhibitory site) functions has been postulated (*43*). According to the proposed model, the differential affinity of the regulatory sites for a particular agent results in selective interaction of that compound with either of these two sites, which in turn determines the nature of the modulatory effect. Since *cis*(*Z*)-flupentixol stimulates ATP hydrolysis and *trans*(*E*)-flupentixol inhibits it, one would expect that the former interacts with the stimulatory site while the latter binds to the inhibitory site. The fact that substitution of a single amino acid residue (F983) alters both stimulation by *cis*(*Z*)-flupentixol and inhibition by *trans*(*E*)-flupentixol suggests

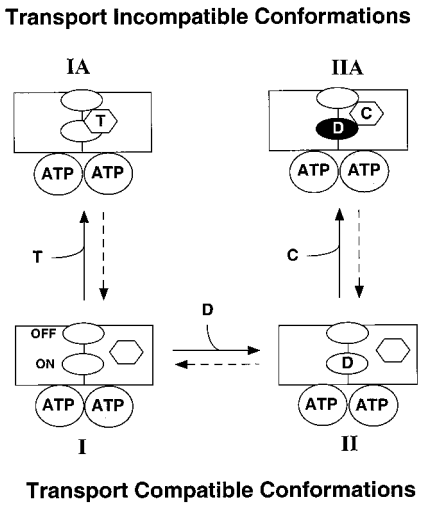

FIGURE 7: Hypothetical model explaining the mechanisms of action of *cis*(*Z*)- and *trans*(*E*)-flupentixol on Pgp function. TM domains and the ATP binding sites of Pgp are represented by squares and ovals, respectively. The ON and OFF sites are drug interacting sites along the translocating pathway and are designated by shaded (high-affinity site) and open ellipses (low-affinity site), respectively. The black ellipse represents a stabilized conformation with drug molecule bound to it. The flupentixol interaction site is depicted by the hexagon. The substrate molecule, *cis*(*Z*)-flupentixol, and *trans*(*E*)-flupentixol are shown as "D", "C", and "T", respectively. The solid arrows represent the favored reactions. Conformations I and II are transport-active while IA and IIA are not.

that the two sites either are spatially close to each other or are physically overlapping. The other possibility is that a common site of interaction for *cis*(*Z*)- and *trans*(*E*)-flupentixol exists that primarily recognizes the tricyclic ring nucleus of these compounds, whereas the relative orientation of the side chain piperazinyl group contacts distinct regions of the protein near the recognition site which eventually determines the nature of the modulatory effect.

Consistent with the idea of a single site of interaction, both *cis*(*Z*)-flupentixol-mediated stimulation (Figure 5C) and *trans*(*E*)-flupentixol-mediated inhibition (Figure 6A) of [^{125}I]IAAP labeling were also significantly reduced in F983A. *cis*(*Z*)-flupentixol-mediated stimulation of [^{125}I]IAAP labeling also shows a progressive decrease at concentrations higher than 25 μM. Based on this effect, a possible second site of interaction in Pgp for *cis*(*Z*)-flupentixol can be proposed, binding to which either inhibits [^{125}I]IAAP labeling directly or alters interaction of *cis*(*Z*)-flupentixol with the site that stimulates [^{125}I]IAAP labeling. In F983A, stimulation of [^{125}I]IAAP labeling was significantly altered with a maximal stimulation of only 2-fold compared to 9-fold stimulation in the wild-type Pgp (Figure 5A−C). However, the decrease in stimulation of [^{125}I]IAAP labeling was evident at the same concentration of *cis*(*Z*)-flupentixol for both F983A and wild-type Pgp. This suggest that substitution of F983 does not affect the interaction of *cis*(*Z*)-flupentixol with the putative second site within Pgp.

Selective effects of single amino acid replacements or deletions on substrate or modulator interaction have been reported by other groups (*41*, *42*). The unique ability of residue F983 to contribute to both stimulation and inhibition of ATP hydrolysis and substrate interaction ([^{125}I]IAAP labeling) singles it out from the others. Since modulatory effects on ATP hydrolysis and [^{125}I]IAAP labeling reflect

the mechanism by which drug transport is inhibited, our results suggest that a single amino acid residue can contribute to distinct mechanisms of inhibition of Pgp-mediated drug transport by the two isomers of flupentixol.

Recently, we proposed a scheme for the catalytic cycle of Pgp (*30*). A portion of the scheme that is relevant to our present study is depicted in Figure 7. In that scheme, the two nonidentical [^{125}I]IAAP interaction sites of Pgp were predicted as two regions of contact within the drug translocating pathway, one representing the initial recognition site (ON site) and the other from which the substrate molecule dissociates (OFF site). Our data suggest that *cis*(*Z*) and *trans*(*E*) isomers of flupentixol modulate Pgp function through a common site of interaction. This interaction site seems to be functionally, if not structurally, distinct from the site of substrate recognition. *cis*(*Z*)-Flupentixol stabilizes a conformation of Pgp that favors recognition of substrate (at the ON site) but prevents its translocation (Figure 7, IIA), whereas *trans*(*E*)-flupentixol affects the step of initial recognition of the substrate molecule by Pgp (Figure 7, IA). In both instances, drug transport is inhibited. We propose that the phenylalanine residue at position 983 constitutes an essential part of this modulatory site for flupentixol. Therefore, substitution of F983 with alanine results in reduced modulatory potency of both isomers of flupentixol on Pgp function. The model presented here provides the simplest explanation for our data, but other more complex possibilities cannot be completely ruled out.

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