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# Functional Characterization of a Glycine 185-to-Valine Substitution in Human P-Glycoprotein by Using a Vaccinia-based Transient Expression System

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Human P-glycoprotein (Pgp) is a 170-kDa plasma membrane protein that confers multidrug resistance to otherwise sensitive cells. A mutation in Pgp, G185→V, originally identified as a spontaneous mutation, was shown previously to alter the drug resistance profiles in cell lines that are stably transfected with the mutant *MDR*1 cDNA and selected with cytotoxic agents. To understand the mechanism by which the V185 mutation leads to an altered drug resistance profile, we used a transient expression system that eliminates the need for drug selection to attain high expression levels and allows for the rapid characterization of many aspects of Pgp function and biosynthesis. The mutant and wild-type proteins were expressed at similar levels after 24–48 h in human osteosarcoma (HOS) cells by infection with a recombinant vaccinia virus encoding T7 RNA polymerase and simultaneous transfection with a plasmid containing *MDR*1 cDNA controlled by the T7 promoter. For both mutant and wild-type proteins, photolabeling with [$^{3}$H]azidopine and [$^{125}$I]iodoarylazidoprazosin, drug-stimulated ATPase activity, efflux of rhodamine 123, and accumulation of radiolabeled vinblastine and colchicine were evaluated. In crude membrane preparations from HOS cells, a higher level of basal Pgp-ATPase activity was observed for the V185 variant than for the wild-type, suggesting partial uncoupling of drug-dependent ATP hydrolysis by the mutant. Several compounds, including verapamil, nicardipine, tetraphenylphosphonium, and prazosin, stimulated ATPase activities of both the wild-type and mutant similarly, whereas cyclosporin A inhibited the ATPase activity of the mutant more efficiently than that of the wild-type. This latter observation explains the enhanced potency of cyclosporin A as an inhibitor of the mutant Pgp. No differences were seen in verapamil-inhibited rhodamine 123 efflux, but the rate of accumulation was slower for colchicine and faster for vinblastine in cells expressing the mutant protein, as compared with those expressing wild-type Pgp. We conclude that the G185→V mutation confers pleiotropic alterations on Pgp, including an altered basal ATPase activity and altered interaction with substrates and the inhibitor cyclosporin A.

## INTRODUCTION

The successful treatment of cancer through chemotherapy is often hindered by the intrinsic and acquired resistance of cancer cells to drugs. This resistance is often associated with enhanced expression of the human multidrug resistance (*MDR*1) gene (Gottesman

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and Pastan, 1993; Gottesman *et al.*, 1995). The *MDR*1 gene encodes a 170-kDa protein, termed P-glycoprotein (Pgp) or the multidrug transporter, which is expressed on the plasma membranes of multidrug-resistant (MDR) cells. P-glycoprotein is a 1280-amino-acid protein organized as two homologous halves, each of which is thought to span the plasma membrane bilayer six times and each of which contains an ATP-binding/utilization domain (Chen *et al.*, 1986; Gottesman and Pastan, 1993). These structural elements identify the *MDR*1 gene product as a member of the superfamily of ATP-binding cassette (ABC) transporters (Higgins, 1992) that includes the cystic fibrosis transmembrane regulator (CFTR; Riordan *et al.*, 1989), the peptide transporters involved in antigen presentation (TAP1 and TAP2; Zhang *et al.*, 1995), and the yeast STE6 **a**-mating factor exporter (McGrath and Varshavsky, 1989).

Pgp is an energy-dependent transporter that extrudes a wide spectrum of compounds and drugs. Drugs that are known to be substrates of Pgp enter cells by passive diffusion because they are generally hydrophobic compounds that may have a positive charge at physiological pH (Gottesman and Pastan, 1993), but they share few other characteristics. Studies have shown that Pgp can be specifically photolabeled with 8-azido-ATP (Cornwell *et al.*, 1987; Azzaria *et al.*, 1989), and mutagenesis of some of the residues at the nucleotide-binding sites results in a failure of the protein to confer resistance (Azzaria *et al.*, 1989). Pgp also exhibits both basal and drug-stimulated ATPase activities, comparable to other transport ATPases, in both crude and purified/reconstituted systems (Ambudkar *et al.*, 1992; Doige *et al.*, 1992; Sarkadi *et al.*, 1992; Al-Shawi and Senior, 1993; Shapiro and Ling, 1994). In addition, ATP-dependent drug transport has been demonstrated in both membrane vesicles from multidrug-resistant cells (Horio *et al.*, 1988) and proteoliposomes containing purified Pgp (Sharom *et al.*, 1993; Ambudkar, 1995; Shapiro and Ling, 1995).

The exact mechanism by which the energy derived from ATP hydrolysis is transduced into transport of drugs out of the plasma membrane or cytoplasm remains to be elucidated. So that the mechanism of drug efflux can be understood, it will be necessary to identify precisely the domains and amino acid residues involved in drug binding and transport, determine the role of the two ATP-binding/utilization sites, and characterize the interactions between different domains. A mutation in Pgp leading to substitution of valine for glycine at position 185 has been the subject of great interest, because it has been identified as a spontaneous mutation that renders cells relatively more resistant to colchicine with reduced resistance to vinblastine (Choi *et al.*, 1989; Kioka *et al.*, 1989). An extensive biochemical characterization of the V185 mutation will help to shed light on the key elements that are involved in substrate specificity and harnessing of the energy of ATP to transport.

One of the most important approaches to understand the structure-function relationships of Pgp has been to analyze the effects of specific mutations on the ability of Pgp to confer resistance to various cytotoxic agents. Although this approach has proven to be valuable, it is time consuming, and the level of expression may still not be sufficient for extensive biochemical characterization. Additionally, the common and necessary practice of selection of cells on introduction of *MDR*1 cDNA with cytotoxic drugs in these experiments either to determine the relative resistance or to drive expression to high levels may lead to unknown pleiotropic cellular effects, including activation of alternate endogenous drug resistance mechanisms.

Eukaryotic transient protein expression systems such as the recombinant baculovirus (Miller, 1993) and vaccinia virus (Moss, 1991) systems offer the potential to achieve high levels of synthesis of a protein of interest. Pgp expressed by the baculovirus system (Germann *et al.*, 1990) has been valuable for large-scale synthesis for in vitro biochemical studies (Sarkadi *et al.*, 1992, 1994; Loo and Clarke, 1994; Rao, 1995). However, this system is not well-suited for functional studies in intact cells, because infection with baculovirus results in disruption of the plasma membrane, making the cells permeable and leaky (our unpublished results). Additionally, there is a concern that proteins expressed in insect cells are modified differently and hence behave differently from those synthesized in mammalian cells. As compared with the baculovirus expression system, the vaccinia virus system offers a number of advantages. The protein of interest can be expressed in a variety of human and other mammalian cells; with the use of a recombinant vaccinia virus–T7 RNA polymerase hybrid (vaccinia–T7) expression system, site-directed or random mutants can be expressed rapidly (Fuerst *et al.*, 1986; Moss, 1991) without the necessity of generating a recombinant vaccinia virus for each mutant and, importantly, without needing to impose drug selection. Previously, Pgp was expressed in HeLa cells by using the vaccinia–T7 system to study its potential chloride channel (Valverde *et al.*, 1992) or chloride channel-regulating activity (Hardy *et al.*, 1995). However, this expression system has not been used to study properties related to the drug transport function of Pgp.

In this paper, using the vaccinia–T7 system, we examined the effects of the V185 mutation on cell surface and total expression of Pgp, binding and energy-dependent transport of drugs in intact cells, and drug-stimulable ATPase activity in crude membrane preparations. We have learned that this mutation is more pleiotropic than previously believed, leading to partial uncoupling of ATPase activity from drug binding and an altered interaction with substrates and the inhibitor

cyclosporin A, suggesting that the mutation alters the structure of the transporter.

## MATERIALS AND METHODS

### Materials

Minimum essential medium with Earle's salts (EMEM), Opti-MEM, Iscove's modified Dulbecco's medium (IMEM), fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) without glucose and phenol red, trypsin, and lipofectin were obtained from Life Technologies (Grand Island, NY). DMEM was obtained from Bio-Whittaker (Walkersville, MD). [$^3$H] Colchicine and [$^{125}$I]iodoarylazidoprazosin (IAAP) were purchased from DuPont New England Nuclear (NEN; Boston, MA). [$^3$H]vinblastine and [$^3$H]azidopine were obtained from Amersham (Arlington Heights, IL). 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) was purchased from ICN (Irvine, CA). Cyclosporin A was obtained from Calbiochem (San Diego, CA).

### Cell Lines and Viruses

Human osteosarcoma (HOS; ATCC CRL1543) 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. Three previously characterized NIH3T3 cell lines were grown as monolayer cultures in DMEM with 10% FBS and an appropriate concentration of colchicine. The drug-sensitive NIH3T3 cells were grown in the absence of colchicine. NIH3T3-MDR1-G185 and NIH3T3-MDR1-V185 (Currier *et al.*, 1992) are clones of NIH3T3 cells transfected with the wild-type pHaMDR1/A (G185) and mutant pHaMDR1/A (V185) *MDR*1 retroviral vectors (Ueda *et al.*, 1987) by the calcium phosphate co-precipitation method. The transfectants were initially selected in colchicine at 60 ng/ml. The wild-type NIH3T3-MDR1-G185 was maintained in the presence of 60 ng/ml colchicine, whereas the mutant NIH3T3-MDR1-V185, which confers greater relative resistance to colchicine, was exposed to increasing concentrations of drug and maintained in 1 μg/ml colchicine, but it expressed approximately the same amount of Pgp on the cell surface as NIH3T3-MDR1-G185 cells. Recombinant vaccinia virus encoding bacteriophage T7 RNA polymerase (vTF 7-3), which is required for the expression of the gene controlled by the T7 promoter in a transfected plasmid, was obtained from Dr. B. Moss (National Institutes of Health, Bethesda, MD). vTF 7-3 was propagated and purified as previously described (Earl *et al.*, 1991).

### Construction of Expression Vectors

A DNA fragment (1183 bp) between the second codon and the *Eco*RI site in the coding sequence of human *MDR*1 was obtained by PCR with pMDRXS (WT; Y. Sugimoto, M. M. Gottesman, and I. Pastan, unpublished data) as a template. After phosphorylating the 5′-end with polynucleotide kinase and digesting with *Eco*RI, the PCR fragment was subcloned into the linearized vector pTM1 (Elroy-Stein *et al.*, 1989) that was prepared by digestion with *Nco*I, treatment with the Klenow fragment of *E. coli* DNA polymerase, and followed by digestion with *Eco*RI to obtain pTM1–MDR1 (NE). Subsequently, pTM1–MDR1 (NE) was double digested with *Nsi*1 and *Xho*I and ligated to the remaining coding sequence of *MDR*1 isolated from pMDRXS (WT) by digesting with the same enzymes to obtain the expression vector pTM1–MDR1. To introduce the V185 mutation, we replaced the *Bgl*II fragment in the *MDR*1 coding region of pTM1–MDR1 by the *Bgl*II fragment encoding the V185 mutation obtained from pMDR2000XS (Pastan *et al.*, 1988). The sequences in the constructs were verified by automated sequencing with Prism Ready Reaction DyeDeoxy Terminator Sequencing Kit (Perkin-Elmer Corporation, Norwalk, CT).

### Expression of Pgp by an Infection–Transfection Protocol

A 70-80% confluent monolayer of HOS cells grown in 75-cm$^2$ tissue culture flasks was infected with vTF 7-3 and transfected with pTM1–MDR1 constructs as described (Elroy-Stein and Moss, 1991) with minor modifications. After washing the cells with Opti-MEM medium, we performed transfection with 15 μg of pTM1–MDR1 by using 45 μg of lipofectin together with infection with vTF 7-3 at a multiplicity of infection of 10 in 3.25 ml Opti-MEM. After infection–transfection, cells were incubated at 32°C. Cells were fed with 12 ml of EMEM supplemented with 10% FBS after 4 h and incubated for desired periods of time. Unless stated otherwise, after infection–transfection, cells were harvested at 24 h for assays with intact cells and 48 h for isolating crude membranes.

### Preparation of Crude Membranes

Cells were harvested by scraping and were washed twice in phosphate-buffered saline (PBS) containing 1% aprotinin (catalog number A6279; Sigma Chemical, St. Louis, MO). All subsequent procedures were performed at 4°C. Cells were incubated on ice for 45 min in lysis buffer containing 10 mM Tris, pH 7.5, and (in mM) 10 NaCl, 1 $MgCl_2$, 1 DTT, 1 AEBSF, and 1% aprotinin and subsequently disrupted by the use of a Dounce homogenizer (30 strokes each with pestles A and B). The undisrupted cells and nuclear debris were removed by centrifugation at 500 × *g* for 10 min. The low-speed supernatant was diluted twofold in resuspension buffer containing (in mM): 10 Tris, pH 7.5, 50 NaCl, 250 sucrose, 1 DTT, 1 AEBSF, and 1% aprotinin. The membranes were collected by centrifugation for 60 min at 100,000 × *g* and resuspended in resuspension buffer containing 10% glycerol. The protein content was determined by a modified Lowry method (Bailey, 1967), using bovine serum albumin as a standard.

### SDS-PAGE and Immunoblot Analysis

Electrophoresis and immunoblot analysis were performed as described previously (Germann *et al.*, 1996).

### Photoaffinity Labeling

Photoaffinity labeling of Pgp in intact cells (1 × 10$^6$ cells) with 1 μCi of [$^3$H]azidopine (specific activity, 46 Ci/mmol) or [$^{125}$I]IAAP (specific activity, 2200 Ci/mmol) was performed as described previously (Bruggemann *et al.*, 1989). After UV cross-linking, cells were lysed by subjecting to three freeze–thaw cycles in a buffer containing 10 mM Tris, pH 8.0, 0.1% Triton X-100, 10 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mM DTT, and 20 μg/ml DNase. The resulting lysates were subjected to SDS-PAGE and autoradiography. The signals on the autoradiograms were quantitated with NIH Image software after capturing the images on a Macintosh computer with the Eagle Eye still video system (Stratagene, La Jolla, CA).

### Measurement of ATPase Activity

Pgp-associated ATPase activity was measured by determining the vanadate-sensitive release of inorganic phosphate from ATP with a colorimetric method as described (Sarkadi *et al.*, 1992) with some modifications. Membrane suspensions (15 μg) were first incubated at 37°C for 5 min in a reaction mixture containing (in mM): 50 Tris, pH 7.4, 2 dithiothreitol, 5 sodium azide, 0.5 ouabain, 0.5 EGTA, and 10 $MgCl_2$. The drugs were added from stock solutions prepared in dimethyl sulfoxide, and the assay mixtures were then incubated for 3 min at 37°C. The reactions were started by the addition of 5 mM ATP to the assay mixtures (a total volume of 100 μl) and incubated at 37°C for desired periods of time (usually 20 min). Reactions were stopped by the addition of 100 μl of 5% SDS solution; the amount of inorganic phosphate released was measured by a colorimetric reaction.

### *Fluorescence-activated Cell-sorting (FACS) Analysis*

FACS analysis was performed with a FACSort flow cytometer equipped with CellQuest software (Beckton-Dickinson FACS System, San Jose, CA).

### *Determination of Cell Surface Expression of Pgp by MRK-16 Staining*

MRK-16 staining was performed as described previously (Germann *et al.*, 1996) but in IMEM supplemented with 5% FBS.

### *Rhodamine 123 Efflux and Accumulation Assays*

Cells were harvested by trypsinization, washed, and resuspended in IMEM supplemented with 5% FBS. To determine energy-dependent rhodamine 123 efflux, 500,000 cells were incubated either in 25 mM glucose containing DMEM or glucose-free DMEM (prewarmed to 37°C) containing 5 mM 2-deoxyglucose and 10 mM sodium azide (to deplete energy) for 20 min at room temperature. Cells were then pelleted by centrifugation at 200 × *g* for 5 min and loaded with rhodamine 123 by incubating at 37°C in medium containing 0.5 μg/ml rhodamine 123 (added from a stock of 1 mg/ml in DMSO). After 40 min, cells were pelleted and incubated further in their respective rhodamine 123-free media for an additional 40 min. Cells were then pelleted and resuspended in 300 μl of PBS and immediately analyzed by FACS to determine rhodamine 123 efflux. For rhodamine 123 accumulation measurements, 500,000 cells were incubated in 5 ml of IMEM (prewarmed to 37°C) containing 5% FBS and 1 μg/ml rhodamine 123 with or without 30 μM verapamil for 40 min at 37°C. Cells were then centrifuged at 200 × *g* for 5 min, resuspended in 300 μl of ice-cold PBS containing 0.1% BSA, and analyzed by FACS.

### *Drug Accumulation Assays with Radiolabeled Compounds*

Drug accumulation assays with either [$^3$H]colchicine (100 nM, 0.5 μCi/ml) or [$^3$H]vinblastine (45.5 nM, 0.5 μCi/ml) in HOS cells one day after infection–transfection were performed as described by Stein *et al.* (1994).

## RESULTS

### *Construction of Plasmid Vectors*

To express the wild-type and mutant Pgp in human and other mammalian cells, we constructed the vector pTM1–MDR1 by inserting the human *MDR1* cDNA sequence at the 3′ end of the encephalomyocarditis virus internal ribosome binding site (IRES) sequence downstream of the T7 promoter in pTM1 (Figure 1A). The T7 promoter-regulated gene in the pTM1-based expression plasmid can be expressed in cells that are infected with vTF 7-3, a recombinant vaccinia virus encoding bacteriophage T7 RNA polymerase (Fuerst *et al.*, 1986). The presence of an IRES sequence downstream from the T7 promoter makes the translation cap-independent, significantly enhancing the expression level (Moss, 1991).

### *Expression of the Wild-Type (G185) and Mutant (V185) Pgp*

Initially, by using the infection–transfection protocol outlined in Figure 1B, several cell lines were evaluated

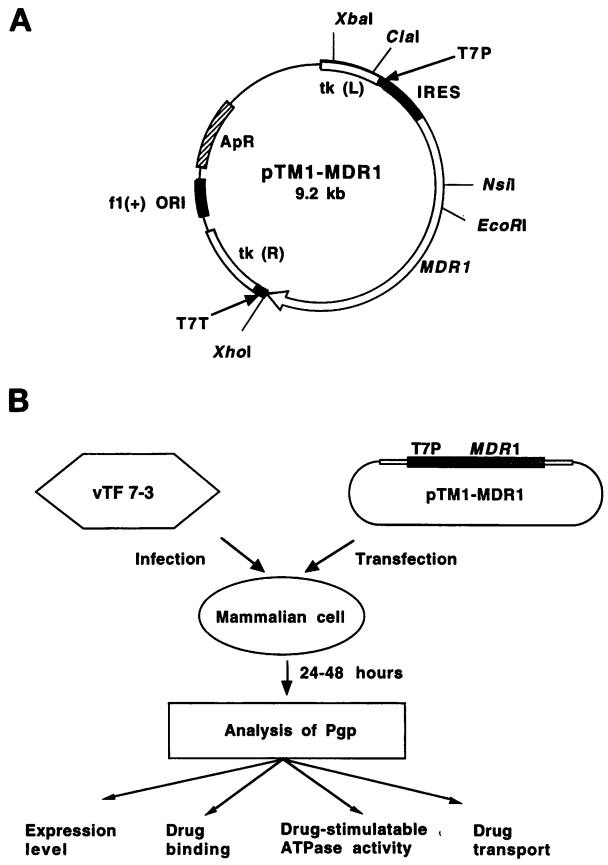


**Figure 1.** Pgp-expression plasmid and schematic diagram of the infection–transfection protocol and functional assays. The expression plasmid pTM1–MDR1 was constructed as described in MATERIALS AND METHODS. (A) The unique restriction sites and main features of the expression plasmid pTM1–MDR1. In this vector, *MDR1* is controlled by the T7 promoter (T7P), and the presence of an IRES sequence between the T7 promoter and *MDR1* sequence facilitates cap-independent translation on transfection of cells infected with vTF 7-3, a recombinant vaccinia virus encoding T7 RNA polymerase. T7 terminator (T7T) is present at the 3′-end of the *MDR1* cDNA. The expression cassette is flanked by segments of the vaccinia virus thymidine kinase (tk) gene, which enables use of this construct to generate and isolate recombinant vaccinia virus. (B) An outline of the infection–transfection protocol and functional assays that can be performed in the infected–transfected cells.

for their suitability in Pgp functional studies on the basis of their lack of detectable endogenous Pgp expression, ability to express functional Pgp, and low levels of endogenous membrane-associated ATPase activity. Human cells, including HeLa (cervical epidermoid carcinoma), MCF-7 (breast adenocarcinoma), HuTK$^-$ (thymidine kinase-deficient osteosarcoma), and HOS (osteosarcoma), were infected with vTF 7-3 and transfected with pTM1–MDR1. FACS analysis of the cells for cell surface expression by MRK-16 staining and rhodamine 123 accumulation revealed expression of functional Pgp in all these cell lines at the cell surface (our unpublished observations). HOS cells

were chosen for further study because they exhibited the lowest level of endogenous membrane-associated ATPase activity. Figure 2A shows the time course of Pgp expression in HOS cells as detected by immunoblot analysis with an anti-Pgp monoclonal antibody C219 (Georges *et al.*, 1990). A Pgp species of ~140 kDa was detected starting 8 h after infection–transfection, and the maximal expression was achieved at 48 h. Even when harvested at later time points (Figure 2A, lanes 4–7), the migration of Pgp on SDS-PAGE gels did not alter. However, when expressed in HeLa, both 140- and 170-kDa Pgp bands were visualized on the immunoblots (our unpublished observations).

The wild-type and a V185 variant were expressed with the infection–transfection protocol (Figure 2B, lanes 2–4), and compared with those expressed in NIH3T3 cells transfected with either the wild-type or V185 mutant cDNA, and selected for colchicine resistance (Figure 2B, lanes 5-7). The expression levels of the wild-type and V185 mutant were similar between infected–transfected HOS cells and transfected and drug-selected NIH3T3 cells. The expression level achieved in the vaccinia–T7 system was approximately threefold lower than the level in insect (Sf9) cells infected with recombinant *MDR*1 baculoviruses (compare Figure 2B, lane 1 showing 0.2 μg of crude membrane protein with lanes 3 and 4 showing 0.6 μg of crude membrane protein). Pgp expressed with the vaccinia–T7 and baculovirus systems migrated similarly (as 140-kDa bands) on SDS-PAGE gels, whereas Pgp synthesized in NIH3T3 cells migrated slower (at ~150 kDa and higher) on SDS-PAGE gels. It was concluded from several previous studies that the underglycosylated form of Pgp migrates as a 140-kDa band (Germann *et al.*, 1990), and complete glycosylation is not essential for the proper functioning of Pgp (Beck and Cirtain, 1982; Ling *et al.*, 1983; Germann *et al.*, 1990; Kuchler and Thorner, 1992; Schinkel *et al.*, 1993). Pgp from NIH3T3 cells, when treated with peptide-*N*-glycosidase F (Oxford GlycoSystems, Rosedale, NY), migrated similarly to treated and untreated Pgp from HOS cells (our unpublished observations), suggesting that Pgp synthesized in HOS cells is not N-glycosylated. It is possible that because vaccinia virus infection results in a shut-off of host protein synthesis (Moss, 1991), the cellular enzymes responsible for Pgp glycosylation may not be fully active in infected cells. It is also likely that, even if the endogenous machinery is active, it may not be able to properly modify such a large number of recombinant Pgp molecules synthesized in a relatively short time period. Intriguingly, V185-Pgp from NIH3T3 cells selected with 1 μg of colchicine/ml migrated slower than wild-type Pgp synthesized in NIH3T3 cells cultured in the presence of 60 ng of colchicine/ml.

### *Photoaffinity Labeling of Pgp*

Photoaffinity labeling experiments with [$^3$H]azidopine and [$^{125}$I] IAAP that used intact cells were performed to determine the drug-binding properties of Pgp. After labeling, cells were lysed, and the lysates were analyzed by SDS-PAGE and autoradiography. As shown in Figure 3, A and B, both the wild-type and V185 proteins expressed in the vaccinia–T7 system were labeled efficiently with both azidopine and IAAP. Vinblastine, a known substrate of P-glycoprotein, and cyclosporin A, a known inhibitor, competed for the photoaffinity labeling, demonstrating that the labeling is specific. As expected, no 140-kDa proteins were labeled in control cells infected with vTF 7-3 and transfected with the vector DNA.

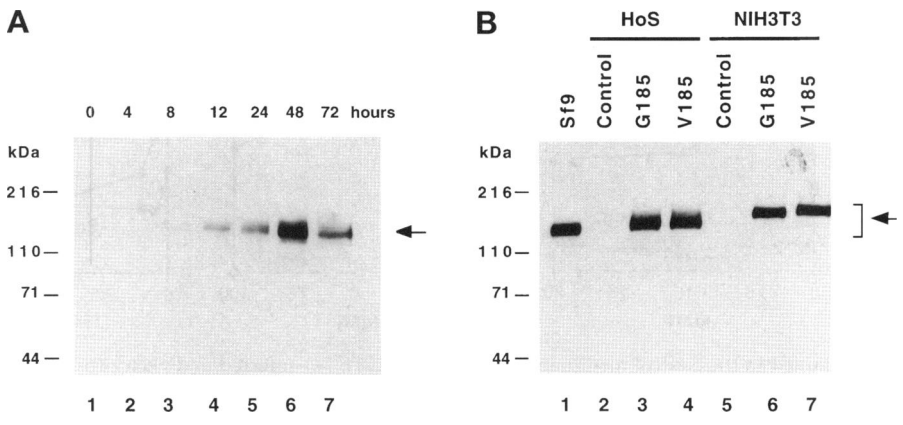


**Figure 2.** Expression of Pgp. (A) HOS cells were infected with vTF 7-3, transfected with pTM1–MDR1, and analyzed for protein expression at various time points (hours after transfection/infection are indicated on top). Cells were lysed, and total cell lysates (15 μg of protein/lane) were subjected to immunoblot analysis with anti-Pgp monoclonal antibody C219. (B) Immunoblot analysis of the membrane preparations from Sf9 cells infected with recombinant *MDR*1-baculoviruses (lane 1), HOS cells infected with vTF 7-3 and transfected with pTM1 (lane 2), HOS cells infected with vTF 7-3 and transfected with pTM1–MDR1 (wild-type/G185) (lane 3), HOS cells infected with vTF 7-3 and transfected with pTM1–MDR1 (V185; lane 4), drug-sensitive NIH3T3 cells (lane 5), NIH3T3-G185 cells (lane 6), and NIH3T3-MDR1-V185 cells (lane 7). In lane 1, 0.2 μg and in other lanes 0.6 μg of membrane proteins were loaded. The position of Pgp is shown by an arrow.

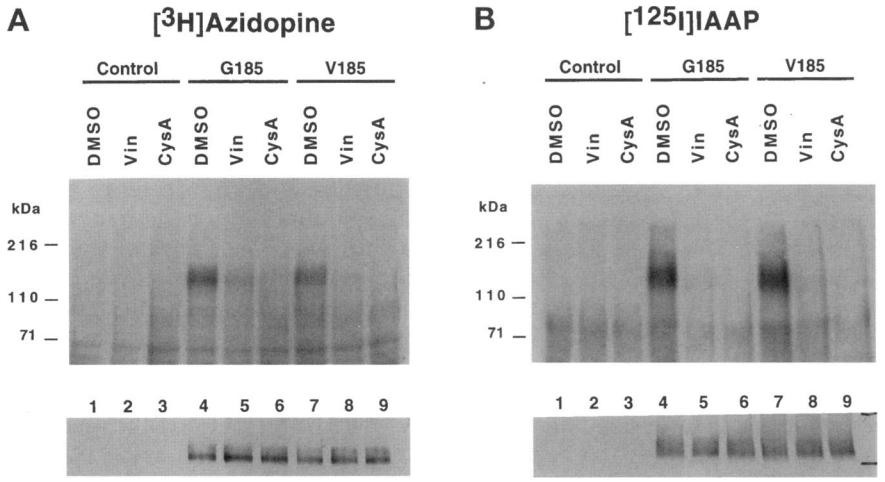


**Figure 3.** Photoaffinity labeling of Pgp. Intact HOS cells infected with vTF 7-3 and transfected with pTM1–MDR1 were photoaffinity labeled in the presence or the absence of competitors with [$^3$H] azidopine (A) and [$^{125}$] IAAP (B), as described in MATERIALS AND METHODS. After labeling, cells were lysed, and the lysates were analyzed by SDS-PAGE and autoradiography. Where indicated, vinblastine (Vin) and cyclosporin A (CsA) as competitors at 25 and 10 $\mu$M, respectively, and DMSO as control were added. To ensure equal loading of Pgp, aliquots from samples after photoaffinity labeling were analyzed by immunoblot analysis with anti-Pgp antibody C219 (shown in the bottom panel).

### *Stimulation of Pgp-ATPase Activity by Selected Compounds*

The ATPase activity of Pgp synthesized in the vaccinia–T7 system was measured as the vanadate-sensitive release of inorganic phosphate from $Mg^{2+}$ ATP. Known inhibitors of other membrane-associated ATPases such as sodium azide, ouabain, and EGTA were included in the assay mixtures to inhibit the activities of $F_0$–$F_1$ ATPase, $Na^+$, $K^+$ ATPase, and $Ca^{2+}$-dependent ATPase, respectively. Membrane preparations from vTF 7-3–infected HOS cells that were transfected with the vector DNA, pTM1, exhibited ATPase activity of ~4-5 nmols $\cdot$ min$^{-1}$ $\cdot$ mg$^{-1}$ of protein. The basal membrane-associated ATPase activity increased to 10-15 nmols $\cdot$ min$^{-1}$ $\cdot$ mg$^{-1}$ of protein in cells infected with vTF 7-3 and transfected with pTM1–MDR1. Compounds such as verapamil, nicardipine (Figure 4A), tetraphenylphosphonium (TPP), and prazosin (Figure 4B) stimulated the ATPase activities in crude membranes containing Pgp. Maximum stimulation of ~2.8-fold was observed with prazosin. Cyclosporin A, a potent inhibitor of Pgp, inhibited both the basal and verapamil-stimulated ATPase activities (Figure 4C). None of the above compounds had any effect on the ATPase activity of the membrane preparations from vTF 7-3–infected and vector-transfected HOS cells.

The ATPase activities of G185-Pgp were compared with those of V185-Pgp by using membrane preparations from infected–transfected HOS cells (Table 1 and Figure 5). Interestingly, the basal vanadate-sensitive ATPase activity of V185-Pgp was approximately twofold higher than that of G185-Pgp (Figure 5A), whereas the activities obtained in the presence of sodium orthovanadate were similar. Higher levels of basal ATPase activity for the V185 variant were also observed in assays performed with membrane preparations from NIH3T3 cells or *MDR*1 baculovirus-infected insect cells (our unpublished results). As for the wild-type Pgp ATPase activity, V185-Pgp ATPase ac-

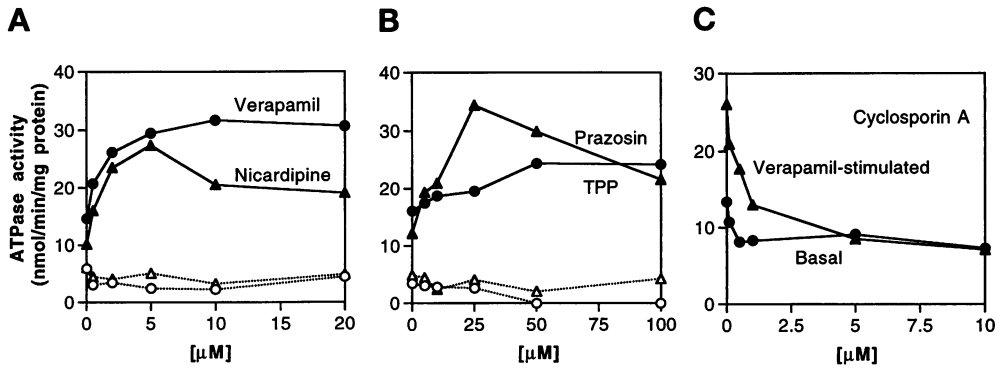


**Figure 4.** Pgp-ATPase activity in the isolated membranes from infected–transfected HOS cells. The vanadate-sensitive activities calculated as the differences between the ATPase activities measured in the absence and presence of vanadate (300 $\mu$M) are plotted. The representative data from at least two experiments are shown. Activities obtained in the membranes isolated from vTF 7-3–infected and pTM1–MDR1 (wild-type)-transfected cells are shown as filled symbols, and the activities obtained in the control membranes isolated from vTF 7-3–infected and vector (pTM1)-transfected cells are shown as open symbols. (A) Stimulation by verapamil (○, ●) and nicardipine (△, ▲). (B) Stimulation by TPP (○, ●) and prazosin (△, ▲). (C) Inhibition of basal ATPase activity (●) and activity stimulated in the presence of 5 $\mu$M verapamil (▲) by cyclosporin A.

**Table 1.** Effects of selected compounds on vanadate-sensitive ATPase activities of the wild-type (G185)-Pgp and V185-Pgp in membranes from vTF 7-3–infected and *MDR*1-transfected HOS cells

| | Concentration required for half-maximal stimulation [μM] | | Maximal stimulation-fold | |
|---|---|---|---|---|
| Compound[a] | G185 | V185 | G185 | V185 |
| Verapamil | 1.1 | 1.3 | 2.2 | 1.7 |
| Nicardipine | 0.8 | 1.5 | 2.6 | 1.7 |
| TPP | 25.0 | 14.7 | 1.5 | 1.8 |
| Prazosin | 22.4 | 10.6 | 2.8 | 1.6 |

[a] For each drug at least five concentrations were used, as shown in Figure 4, to determine these values.

tivity was stimulated by verapamil, nicardipine, TPP, and prazosin, although the fold stimulation and the concentrations required for half-maximal stimulation differed within a factor of two (Table 1). Cyclosporin A inhibited the ATPase activities of both the wild-type and V185, but the inhibition was more effective in the case of V185-Pgp (Figure 5B). The concentrations required for 50% inhibition of verapamil-stimulated activities of G185-Pgp and V185-Pgp (determined in the presence of 5 μM verapamil) were 250 and 25 nM, respectively, indicating a significantly higher affinity for cyclosporin A in the case of the V185 mutant protein.

### *FACS Analysis for Cell Surface Expression of Pgp*

Expression of Pgp at the cell surface was examined by FACS after staining with MRK-16, a monoclonal antibody that recognizes an external epitope of human Pgp (Hamada and Tsuruo, 1986). A considerable amount of Pgp was detected 24 h after infection–transfection at the cell surface (Figure 6A), and trypan blue exclusion or staining with propidium iodide revealed that most of the cells were intact. Although total Pgp expression was higher 48 h after infection–transfection, cell viability was greatly reduced (our unpublished observations). Therefore, cells were routinely analyzed 24 h after infection–transfection for cell surface expression and functional properties. It can also be seen from Figure 6A that typically ~70-80% of the infected–transfected HOS cells express Pgp molecules at the surface, and the introduction of the V185 mutation has no effect on the amount of Pgp expressed at the cell surface. In NIH3T3 cells that were transfected with either the wild-type or V185 mutant *MDR*1 cDNA and selected in colchicine to induce high-level expression, the expression levels (Figure 6B) were comparable to the levels obtained in infected–transfected HOS cells. As expected, Pgp expression was detected in nearly all of the *MDR*1-transfected and colchicine-selected NIH3T3 cells.

### *Analysis of Rhodamine 123 Transport*

In efflux and accumulation assays, the abilities of the wild-type and mutant forms of Pgp-expressing HOS and NIH3T3 cells to transport rhodamine 123, a fluorescent dye, were determined. In efflux assays, cells were incubated in either 25 mM glucose-containing DMEM or glucose-free DMEM containing 5 mM 2-deoxyglucose and 10 mM sodium azide (to deplete ATP) for 20 min at room temperature; then the cells were loaded with rhodamine 123 in these respective media. After 40 min, cells were transferred to and incubated in rhodamine 123-free media for another 40 min. As shown in Figure 7, after 40 min incubation in rhodamine 123-free medium that followed loading with rhodamine 123, the accumulation was drastically reduced only in Pgp-expressing cells that were continuously incubated in glucose-containing medium, but not in glucose-free medium containing sodium azide and 2-deoxyglucose in both infected–transfected HOS

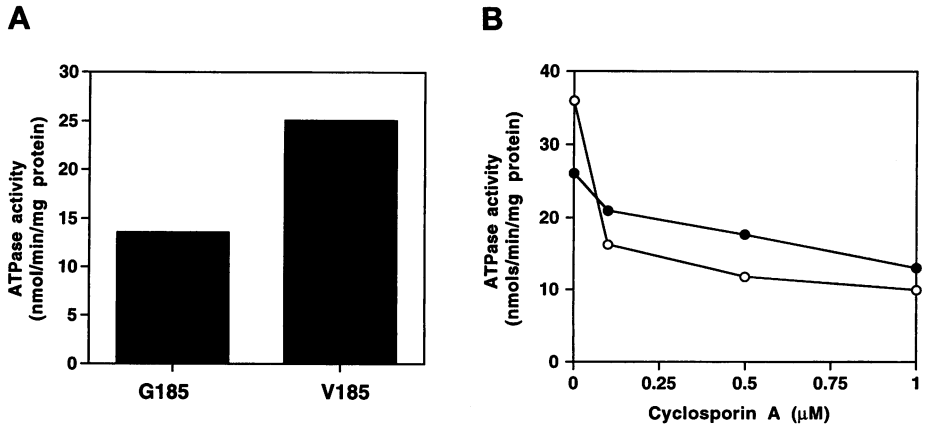


**Figure 5.** ATPase activities of the wild-type (G185)-Pgp and V185-Pgp expressed in infected–transfected HOS cells. (A) Basal activity (assayed in the absence of drugs) of G185-Pgp and V185-Pgp. (B) Effect of cyclosporin A on verapamil (5 μM)-stimulated ATPase activity of G185 (●) and V185 (○).

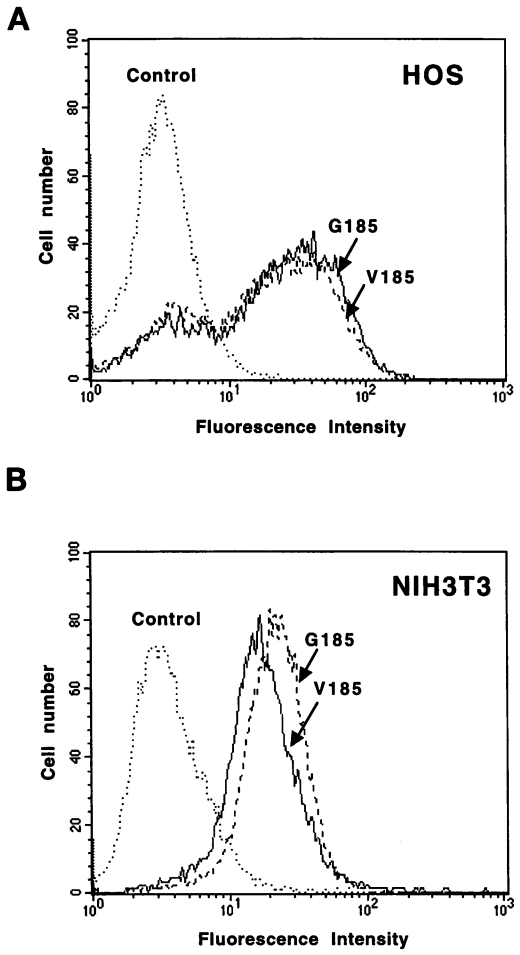


**Figure 6.** Analysis of cell surface expression of Pgp. Infected–transfected HOS (A) and stably transfected NIH3T3 (B) cells expressing either the wild-type (G185) or mutant (V185) Pgp were subjected to FACS analysis after staining with human Pgp surface epitope-specific monoclonal antibody MRK-16, as described in MATERIALS AND METHODS. vTF 7-3–infected and vector DNA-transfected HOS and drug-sensitive NIH3T3 cells were included as controls.

(Figure 7A) and transfected and selected NIH3T3 (Figure 7B) cells. In this assay, the V185 variant seemed to behave similarly to the wild-type transporter (our unpublished observations). In Pgp-negative control cells, incubation with or without glucose had no effect on intracellular rhodamine 123 levels, and the rhodamine levels were similar to that observed in Pgp-expressing cells incubated in glucose-free medium.

In accumulation experiments, after incubation in normal glucose-containing IMEM for 40 min, Pgp-expressing HOS (Figure 8, left panels) and NIH3T3 (Figure 8, right panels) cells accumulated considerably less rhodamine 123 than drug-sensitive NIH3T3 cells and control HOS cells transfected with vector DNA. In the presence of 30 $\mu$M verapamil, a known reversing agent (Gottesman and Pastan, 1993), Pgp-expressing cells accumulated rhodamine 123 similarly to control cells because of inhibition of efflux of rhodamine 123 from cells. These data indicate that the reduced accumulation is due to Pgp. Taken together, these results demonstrate energy-dependent Pgp-mediated rhodamine 123 efflux that can be inhibited by verapamil. In accumulation experiments, the wild-type and V185-mutant Pgp-expressing cells behaved similarly, indicating that the V185 mutation does not alter the ability of Pgp to transport rhodamine 123 (Figure 8).

### *Analysis of Accumulation of Colchicine and Vinblastine*

The wild-type and V185 mutant transporters were examined in accumulation experiments for their abilities to transport radiolabeled colchicine and vinblastine. Figure 9 shows time course analyses of the accumulation of colchicine in HOS cells (Figure 9A) infected with vTF 7-3 and transfected with pTM1–MDR1 constructs and NIH3T3 cells transfected with *MDR*1 and selected with colchicine (Figure 9C). When wild-type Pgp was expressed by the vaccinia–T7 system (Figure 9, A and B) or by *MDR*1 cDNA transfection and colchicine selection (Figure 9, C and D), the accumulation of colchicine was reduced, as compared with control HOS cells that were infected with vTF 7-3 and transfected with vector DNA or drug-sensitive NIH3T3 cells. In both HOS (Figure 9, A and B) and NIH3T3 cells (Figure 9, C and D), MDR1-V185 transfectants accumulated less colchicine than did the MDR1-G185 (wild-type) transfectants. Sixty minutes after cyclosporin A was added at 10 $\mu$M to inhibit the transport activity of Pgp, the levels of colchicine accumulation in both wild-type and V185 mutant *MDR*1-transfected HOS and NIH3T3 cells were comparable to the levels observed in Pgp-negative control cells (Figure 9, B and D). These results demonstrate that the reduced level of accumulation of colchicine is restored by a known inhibitor of the efflux activity of Pgp.

After determining the Pgp-mediated transport of colchicine in cells expressing Pgp with the vaccinia–T7 system, we compared the ability of the wild-type and V185 transporters to efflux vinblastine. The results presented in Figure 10 show that both the wild-type and V185 Pgp-expressing cells accumulated less vinblastine than the control Pgp-negative cells. In contrast to the colchicine accumulation experiments, accumulation of vinblastine was higher in the MDR1-V185 infected–transfected HOS cells (1.87 pmol/million cells/60 min) than in the MDR1-G185 infected–transfected cells (1.38 pmol/million cells/60 min). Immunoblot analysis of the cells used in colchicine and vinblastine uptake studies showed similar levels of the wild-type and V185 proteins in both HOS and NIH3T3 cells, indicating that the observed difference in their

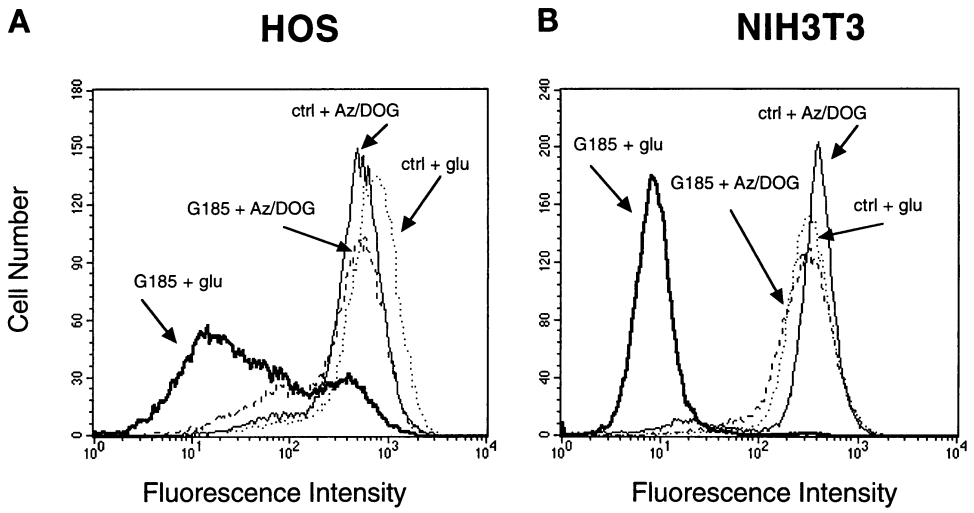


**Figure 7.** Energy-dependent efflux of rhodamine 123. Rhodamine 123 efflux was measured in HOS cells infected with vTF 7-3 and transfected with either pTM1–MDR1 (wild-type/G185) or vector DNA (control; A) and in drug-sensitive (ctrl) and MDR1(wild-type/G185)-transfected NIH3T3 (B) cells. After incubating in either glucose-free DMEM containing 5 mM 2-deoxyglucose and 10 mM sodium azide (Az/DOG; to deplete energy) or 25 mM glucose-containing DMEM (glu) for 20 min, cells were incubated in media containing rhodamine 123 (0.5 μg/ml). After loading for 40 min, cells were further incubated in their respective rhodamine 123-free medium for an additional 40 min and subsequently analyzed for rhodamine 123 efflux by FACS. Similar results were obtained with the MDR1(V185) mutant, but the data were not shown for clarity of presentation.

transport properties is not due to a difference in protein levels (our unpublished observations).

## DISCUSSION

### *Characterization of the V185 Mutation in Pgp with the Vaccinia–T7 Transient Protein Expression System*

The unusually broad substrate specificity of the multidrug transporter has been a subject of considerable speculation (Gottesman *et al.*, 1995). Mutations such as G185V, which change substrate specificity, should provide critical information about drug-transporter interactions. Transient expression of the V185 mutant and wild-type Pgp with a vaccinia–T7 system has facilitated the analysis of this important mutation without the confounding effects of drug selection of cultured cells. In this work we show that transiently expressed mutant and wild-type Pgp share many similar features but differ in substrate and inhibitor specificity and in basal ATPase activity, suggesting an uncoupling of substrate and ATPase sites.

When expressed in NIH3T3 cells on *MDR*1 transfection and colchicine selection (at 60 ng/ml), wild-type Pgp migrated as a 150-kDa band on SDS-gels (Figure 2). Surprisingly, the V185-Pgp synthesized in NIH3T3 cells that were selected for higher resistance to colchicine (1 μg/ml) migrated much more slowly than the wild-type protein, indicating that the mutant protein is modified differently. The observation that V185-Pgp synthesized in HOS cells with a vaccinia–T7 system migrated similarly to its wild-type counterpart indicates that the differential mobility of the V185 protein synthesized in NIH3T3 cells is not due to the mutation per se but likely is the result of either expression in mouse cells and/or selection with a higher concentration of colchicine. Selection of NIH3T3 cells with the higher level of colchicine may have selected or induced mechanisms responsible for this differential modification. Attempts to express the V185 mutant in NIH3T3 by using the recombinant vaccinia–T7 system to rule out the possibility that the differential modification is due to the expression of the mutant protein in mouse cells were not successful. The exact nature of the modification that results in the aberrant mobility of the mutant protein remains to be determined.

It was shown previously that the MDR1-V185 mutant multidrug transporter renders cells relatively more resistant to colchicine with reduced resistance to vinblastine (Choi *et al.*, 1989; Currier *et al.*, 1992; Cardarelli *et al.*, 1995). In photoaffinity labeling experiments, vinblastine competed for the labeling of both the wild-type and mutant proteins (Figure 3). Previously, it has been shown that vinblastine competes for the photoaffinity labeling of V185-Pgp with a greater affinity (Safa *et al.*, 1990; Bruggemann *et al.*, 1992); on the basis of observations in cell proliferation assays showing that vinblastine is a poorer substrate for V185-Pgp (Choi *et al.*, 1989; Currier *et al.*, 1992; Cardarelli *et al.*, 1995), it has been concluded that the V185 mutation reduces the rate of release of vinblastine from the transporter (Safa *et al.*, 1990). We consistently observed a higher basal ATPase activity for V185-Pgp when expressed either in vaccinia–T7/infected–transfected HOS cells, baculovirus-infected insect cells, or stably transfected NIH3T3 cells. This higher basal V185-Pgp ATPase activity was stimulated by several Pgp substrates and modulators (Table 1). These results suggest that the V185 mutation leads to a partial uncoupling of drug binding to ATP hydrolysis. Interest-

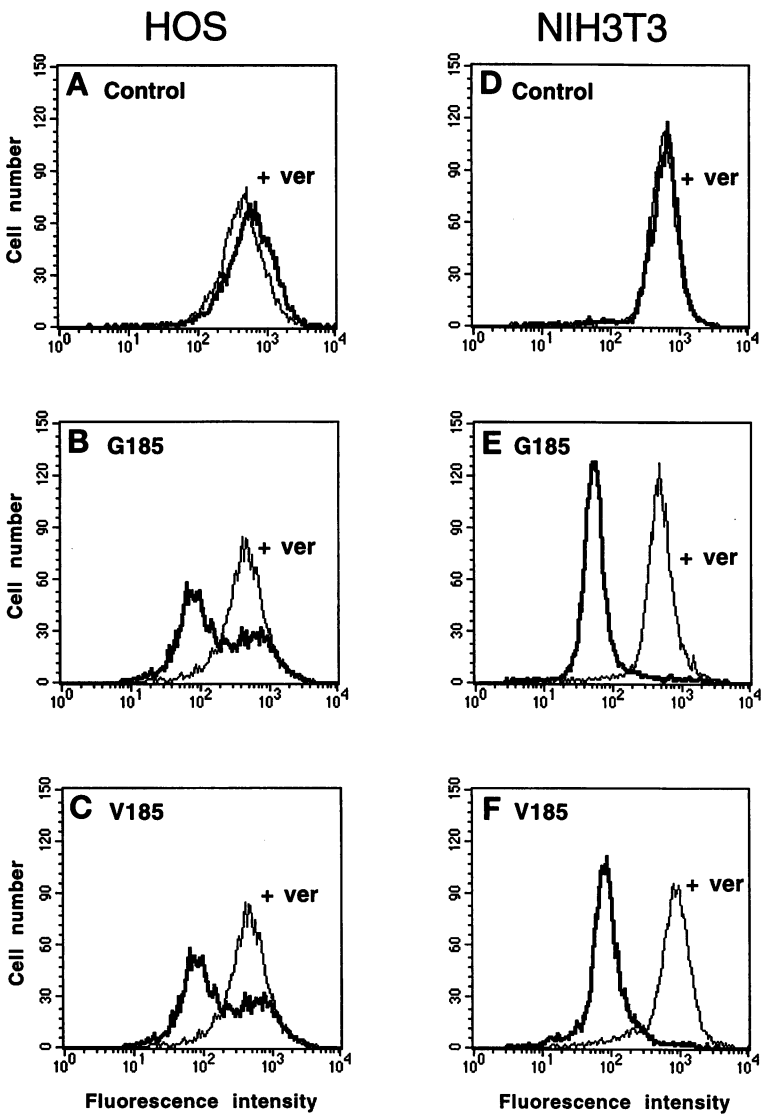


**Figure 8.** Rhodamine 123 accumulation and the effect of verapamil on rhodamine 123 efflux. Rhodamine 123 accumulation in infected–transfected HOS and stably transfected NIH3T3 cells was determined by FACS in the presence or absence of 30 $\mu$M verapamil (ver). Rhodamine 123 accumulation in HOS cells infected with vTF 7-3 and transfected with either vector DNA (control), pTM1–MDR1 (wild-type/G185), or pTM1–MDR1 (V185) is shown in left panels (A–C). Rhodamine 123 accumulation in drug-sensitive (control), *MDR*1-wild-type (G185), or *MDR*1-V185–transfected and colchicine-selected NIH3T3 cells is shown in the right panels (D–F).

ingly, Cyclosporin A, one of the more potent chemosensitizers, inhibited the drug-stimulated ATPase activity of V185-Pgp much more efficiently than that of the wild-type (Figure 5B), and the cyclosporin A concentration required for 50% inhibition of verapamil-stimulated activity of V185-Pgp was 10-fold lower than that required for the wild-type protein. An increased affinity for cyclosporin A in the case of V185-Pgp was also reported previously (Rao, 1995). The finding that cyclosporin A is a better inhibitor of V185-Pgp–ATPase activity is consistent with the results of a previous study that demonstrated that cyclosporin A is a powerful reversing agent of taxol and colchicine resistance for the mutant transporter, but it is a much less effective reversing agent for the wild-type transporter (Cardarelli *et al.*, 1995). In efflux assays that used the fluorescent substrate rhodamine 123, both the wild-type and mutant transporter behaved similarly. However, in accumulation experiments that used radioactive compounds, the mutant transporter is more efficient at reducing colchicine accumulation (Figure 9) and less efficient at reducing vinblastine accumulation (Figure 10) than the wild-type transporter. Similar results were obtained in the transport experiments that used recombinant vaccinia virus-infected and *MDR*1-transfected HOS cells and *MDR*1-transfected and colchicine-selected NIH3T3 cells (results of this study; Stein *et al.*, 1994), further validating the utility of this transient expression system for mutational analysis.

Taken together, the results from this study and previous work indicate that the G185V substitution affects the specificity of the substrate/inhibitor site on Pgp and its coupling to the ATP site. Because residue 185 is

**Table 2.** Comparison of human Pgp expression systems[a]

| | Expression system | | | | | |
|---|---|---|---|---|---|---|
| | Vaccinia virus | Baculovirus | Yeast | Transient transfectants | Stable transfectants | Transient viral transduction (pHaMDR1) |
| Mammalian cell expression | Yes | No | No | Yes | Yes | Yes |
| Amount of Pgp expressed[b] | +++ | ++++ | ++ | + | +++[c] | ++ |
| Analysis of cell surface expression[d] | Yes | Possible, not yet demonstrated | No | Possible, not yet demonstrated | Yes | Yes |
| Need to impose drug selection for expression | No | No | No | No | Yes | No |
| Time of optimal expression | Very rapid (12–48 hs) | Rapid (72 hs) | Rapid (24 hs) | Slow (d/wk) | Slow (wk/mo) | Rapid (24–48 hs) |
| Intact cell transport/efflux assays | Yes | Yes, but not well-suited | No | Not detectable | Yes | Yes |
| Photoaffinity labeling | Yes | Yes | Yes | Yes | Yes | Yes |
| ATPase activity[e] | Yes | Yes | No | Yes, only after partial purification (Loo and Clarke, 1995) | Yes | Possible, not yet demonstrated |
| Simplicity/versatility of mutational analysis[f] | ++++ No need to generate a new virus for each construct | + New virus usually necessary | ++ N/A | ++ N/A | ++ N/A | + Viral production necessary for each construct |

[a] This table does not include systems involving expression of Pgp from other mammalian species. Expression of human Pgp in *E. coli* has met with limited success (Evans *et al.*, 1995).

[b] The amount of Pgp expressed in baculovirus-infected insect cells comprises ~3% of the total membrane protein. This value is taken as the maximum.

[c] Expression level of the cell lines used in this study, NIH3T3–MDR1-G185 and NIH-3T3–MDR1-V185 (Currier *et al.*, 1992).

[d] FACS analysis using human Pgp-specific monoclonal antibodies.

[e] ATPase activity as measured in crude membrane preparations.

[f] Takes into account both the time and ease of constructing vectors and in evaluating expression and function.

not close to the primary domains known to be photoaffinity-labeled by Pgp substrates (Currier *et al.*, 1992), it seems likely that the G185V mutation at this site has pleiotropic effects on Pgp function, resulting from an altered higher order structure of the transporter.

### *Advantages of the Vaccinia–T7 System for the Functional Characterization and Mutational Analysis of Human Pgp*

To determine the functional consequences of the V185 mutation in Pgp, we used the vaccinia–T7 system in this study. Our results show that the transient expression of human Pgp in HOS cells with the recombinant vaccinia–T7 system is a useful tool to rapidly analyze cell surface and total expression, to analyze binding and energy-dependent transport of drugs in intact cells, and to measure drug-stimulable ATPase activity in crude membrane preparations. Because the transport of fluorescent and radiolabeled substrates was blocked by chemosensitizers such as verapamil or cyclosporin A, this system offers a unique opportunity to study the effects of many mutations on the action of different reversing agents. Because of the presence of a large amount of recombinant Pgp at the cell surface, the infected–transfected HOS cells behaved similarly in transport assays with either fluorescent compounds or radiolabeled drugs to NIH3T3 cells that had been transfected with *MDR*1 cDNA and selected for long periods of time for resistance to colchicine.

On transfection of vTF 7-3–infected HOS cells, typically 70-80% of the cells expressed functional Pgp at the cell surface (Figure 6A). Such high transfection efficiency in the vaccinia–T7 system is believed to be caused by simultaneous infection with vaccinia viruses, apparently involving fusion of the viral envelope with the plasma membrane during cationic liposome-mediated transfection (Moss, 1991). Additionally, as transfected plasmids are transcribed in the cytoplasm of vaccinia virus-infected cells, the require-

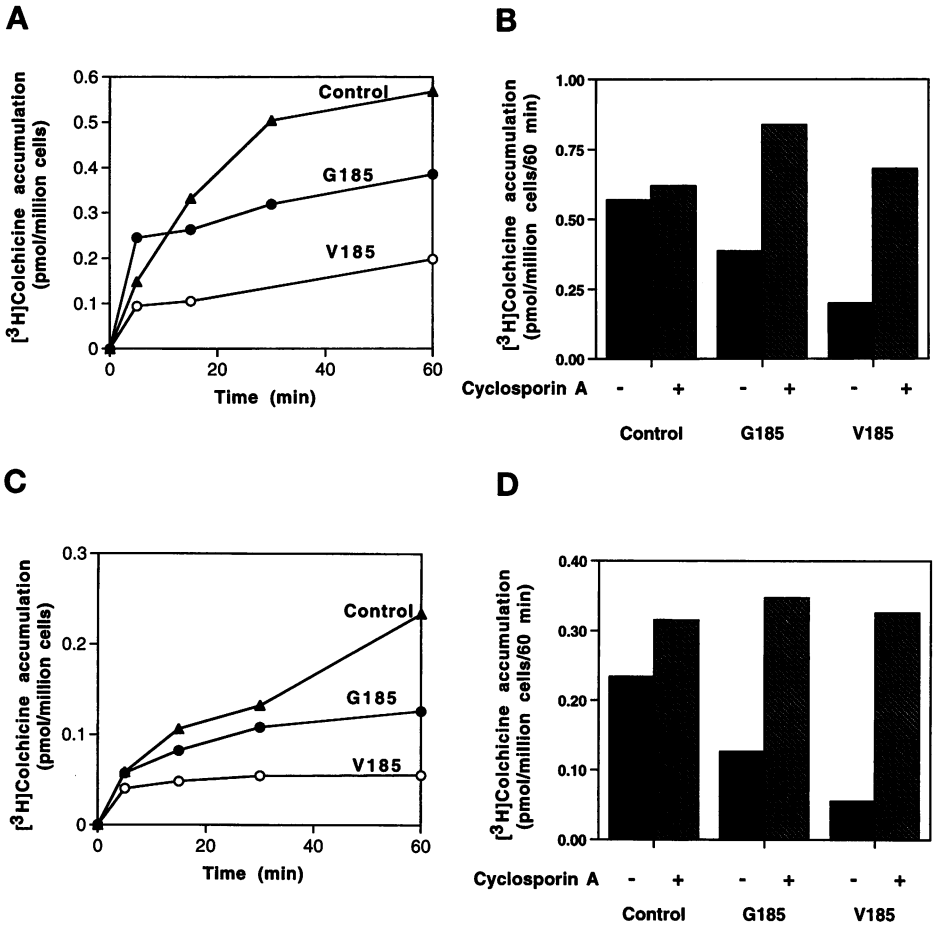


**Figure 9.** Colchicine accumulation in infected–transfected HOS and stably transfected NIH3T3 cells. Drug accumulation assays were performed as described in MATERIALS AND METHODS, and the representative data from at least two experiments are shown. (A) Time course of uptake of [$^3$H]colchicine in vTF 7-3–infected HOS cells that were transfected with either pTM1 (▲), pTM1–MDR1 (wild-type/G185) (●), or pTM1–MDR1 (V185) (○). (B) Effect of cyclosporin A at 10 $\mu$M on colchicine accumulation in infected–transfected HOS cells (60-min time point). (C) Time course of uptake of [$^3$H]colchicine in NIH3T3 drug-sensitive cells (▲) and *MDR*1(G185) (●) and *MDR*1(V185) (○) transfectants. (D) Effect of cyclosporin A at 10 $\mu$M on colchicine accumulation in NIH3T3 cells (60-min time point).

ment of plasmid transit to the nucleus is avoided, allowing virtually all transfected cells to express Pgp (Moss, 1991).

The vaccinia–T7 expression system has proven to be ideal for the study of almost all aspects of Pgp function in a single mammalian cell line. Previous attempts at heterologous expression of human Pgp have been successful, although none have been completely satisfying (Table 2; Evans *et al.*, 1995; Hrycyna *et al.*, 1996). Most of the early mutational studies of Pgp have used stable transfectants with *MDR*1 constructs. These transfectants are capable of expressing large amounts of protein but necessarily require stepwise MDR drug selections that can take periods of time ranging from weeks to months as a means of driving Pgp expression to high levels. Additionally, such selection schemes have always been the subject of debate because of the unknown effects of drug selection on cellular functions. The possible activation of endogenous drug resistance mechanisms when selected with cytotoxic agents may complicate interpretation of the data. The use of the vaccinia–T7 transient system that does not involve drug selection for Pgp expression eliminates the need to consider any possible pleiotropic cellular effects in the interpretation of the observed phenotypes.

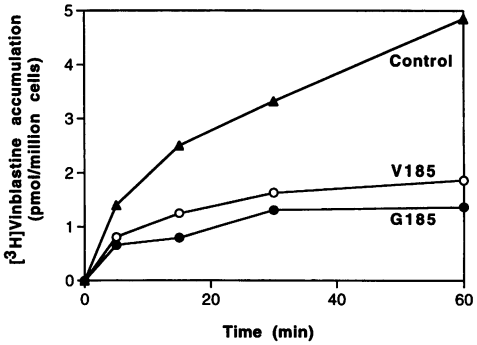


**Figure 10.** Vinblastine accumulation in infected–transfected HOS cells. Time course of [$^3$H]vinblastine accumulation was measured in vTF 7-3–infected HOS cells that were transfected with either pTM1 (▲), pTM1–MDR1 (wild-type/G185) (●), or pTM1–MDR1 (V185) (○), as described in MATERIALS AND METHODS. The representative data from at least two experiments are shown.

The ability of vaccinia viruses to infect most mammalian cells permitted us to choose an appropriate host system on the basis of low endogenous Pgp expression and high transfection efficiency. The vaccinia–T7 system will be useful in studies aimed at evaluating the nature of interactions of newly developed chemotherapeutic agents with the multidrug transporter in a particular cell type. Pgp can be expressed in a desired cell line by using the vaccinia–T7 system, and functional interactions of these compounds with Pgp can be determined for their ability to compete for photoaffinity labeling, to block the transport of fluorescent or radiolabeled compounds, and to modulate ATPase activity (Figure 1). Similarly, this system could be used to determine transport of a particular substrate in a specific cell type. Generation of recombinant vaccinia viruses encoding *MDR*1 by homologous recombination with the flanking vaccinia virus thymidine kinase gene in the vector pTM1–MDR1 would further increase the choice of host cells. For mutational studies, however, there is no need to generate a new virus for each construct, because almost all functional aspects of Pgp can be studied on protein expression in the infection–transfection procedure.

One of the drawbacks of the vaccinia–T7 system is that the infected–transfected cells cannot be used to measure relative resistance to MDR drugs in cell proliferation assays, because the infected cells are committed to virus-induced lysis. In the future, it may be possible to make use of a noncytopathic virus that will allow use in these types of assays. However, changes in substrate specificity resulting from mutations presently can be examined in transport assays by using fluorescent/radiolabeled compounds with infected–transfected cells. Additionally, because host protein synthesis is shut off later in the vaccinia virus infection, one should be cautious in using this system for studies in which host factors are critical for the expected function of the recombinant protein.

## ACKNOWLEDGMENTS

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