

## Functional Characterization of Glycosylation-Deficient Human P-Glycoprotein Using A Vaccinia Virus Expression System

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**Abstract.** P-glycoprotein (P-gp), the product of human *MDR1* gene, which functions as an ATP-dependent drug efflux pump, is N-linked glycosylated at asparagine residues 91, 94, and 99 located within the first extracellular loop. We report here the biochemical characterization of glycosylation-deficient (Gly<sup>-</sup>) P-gp using a vaccinia virus based transient expression system. The staining of HeLa cells expressing Gly<sup>-</sup> P-gp (91, 94, and 99N→Q), with P-gp specific monoclonal antibodies, MRK-16, UIC2 and 4E3 revealed a 40 to 50% lower cell-surface expression of mutant P-gp compared to the wild-type protein. The transport function of Gly<sup>-</sup> P-gp, assessed using a variety of fluorescent compounds indicated that the substrate specificity of the pump was not affected by the lack of glycosylation. Additional mutants, Gly<sup>-</sup> D (91, 94, 99N→D) and Gly<sup>-</sup> Δ (91, 94, 99 N deleted) were generated to verify that the reduced cell surface expression, as well as total expression, were not a result of the glutamine substitutions. Gly<sup>-</sup> D and Gly<sup>-</sup> Δ Pgps were also expressed to the same level as the Gly<sup>-</sup> mutant protein. <sup>35</sup>S-Methionine/cysteine pulse-chase studies revealed a reduced incorporation of <sup>35</sup>S-methionine/cysteine in full length Gly<sup>-</sup> P-gp compared to wild-type protein, but the half-life (~3 hr) of mutant P-gp was essentially unaltered. Since treatment with proteasome inhibitors (MG-132, lactacystin) increased only the intracellular level of nascent, mutant P-gp, the decreased incorporation of <sup>35</sup>S-methionine/cysteine in Gly<sup>-</sup> P-gp appears to be due to degradation of improperly folded mutant protein by the proteasome and endoplasmic reticulum-associated proteases. These results demonstrate

that the unglycosylated protein, although expressed at lower levels at the cell surface, is functional and suitable for structural studies.

**Key words:** Multidrug resistance — P-glycoprotein — Drug transport — Glycosylation — Vaccinia virus

### Introduction

P-glycoprotein (P-gp)<sup>1</sup> mediated drug efflux is one of several ways in which cells develop the multidrug resistance (MDR) phenotype. MDR is characterized by cellular resistance to natural product and cytotoxic agents with diverse structures and unrelated mechanisms of action (Gottesman & Pastan, 1993; Gottesman, Pastan & Ambudkar, 1996; Ambudkar et al., 1999).

P-gp, a 170 kDa phosphoglycoprotein containing 1280 amino acids, is an energy dependent drug efflux pump. The topological model based on hydrophathy analysis of the primary sequence indicates that it contains two highly homologous halves, each containing six transmembrane helices and one nucleotide-binding domain. Both nucleotide binding domains contain highly conserved amino acid sequences, termed the Walker A and B motifs, which are commonly involved in ATP hydrolysis and also an additional dodecapeptide, the C or linker region (Gottesman & Pastan, 1993; Ambudkar, 1995). Due to the presence of these domains, P-gp belongs to a superfamily of transporters referred to as the ATP Binding Cassette (ABC) transporters (Higgins, 1992). Furthermore, the C region (dodecapeptide), the signature peptide of ABC transporters, may be involved in the coupling of ATP hydrolysis to drug transport. Both ATP sites can bind and hydrolyze ATP, and although both sites are required for P-gp function, only one site is utilized during single turnover (Ambudkar et al.,

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1999; Hrycyna et al., 1998; Senior, Al-Shawi & Urbatsch, 1995).

Numerous eukaryotic secreted and membrane proteins, including P-gp, contain N-linked glycosylation, or the addition of oligosaccharide side chains to asparagine (Asn) residues. N-linked glycosylation occurs when the Asn residue resides within the consensus sequence, Asn-X-The/Ser, where X can be any amino acid except proline (Kornfeld & Kornfeld, 1985). Enormous diversity exists among N-linked oligosaccharides; however, they all share a common core arrangement of sugars that are attached to the protein in the rough endoplasmic reticulum. From studies using N-glycosylation deficient mutants, radiolabeled oligosaccharides, glycosylation inhibitors (e.g., tunicamycin) and glycosidases (e.g., neuraminidase, N-glycanase), P-gp appears to contain complex N-linked glycosylation (Richert et al., 1988; Schinkel et al., 1993). Although there are ten possible consensus sequences/sites for N-linked glycosylation, only three are believed to be exposed to the lumen of the endoplasmic reticulum and thus glycosylated (Schinkel et al., 1993). All three sites are located within the first extracellular loop, between transmembrane domains 1 and 2, at amino acid residues Asn 91, Asn 94 and Asn 99 (Fig. 1). This loop, which contains between two and four glycosylation sites for all mammalian P-gps, has otherwise little amino acid conservation (*see* Schinkel, 1993). Therefore, the conservation of these glycosylation sites suggests an evolutionary constraint for their maintenance in P-gp. The generation of drug resistant, N-glycosylation deficient clones demonstrate that the absence of glycosylation does not directly affect the protein's ability to confer drug resistance. It was thus postulated that these sites might play a role in the sorting and stability of P-gp in the plasma membrane (Schinkel et al., 1993).

To elucidate the biochemical role of glycosylation in the generation of the MDR phenotype, and to produce a large amount of unglycosylated protein for structural studies, glycosylation-deficient mutants were generated in which Asn residues at positions 91, 94 and 99 were either replaced with Gln (Gly<sup>-</sup>), Asp (Gly<sup>-</sup> D), or all three Asn were deleted (Gly<sup>-</sup> Δ). The biochemical properties of the mutant protein, Gly<sup>-</sup>, including drug transport, drug binding, ATP hydrolysis, biosynthesis, degradation and interaction with the conformation sensitive monoclonal antibody, UIC2, were assessed using a transient vaccinia virus based expression system. These biochemical characterizations demonstrate that N-linked glycosylation is not required for the function of P-gp. However, a lack of glycosylation appears to affect protein levels during early stages of biosynthesis by increasing protein degradation by the proteasome and endoplasmic reticulum-associated proteases. Thus, our data demonstrate that the nonglycosylated P-gp, which exhibits biochemical properties similar to WT, should facilitate

the generation of three-dimensional crystals for structural studies.

## Materials and Methods

Dulbecco's modified Eagle's medium (DMEM), Iscove's modified Dulbecco's medium (IMDM), trypsin, L-glutamine, penicillin/streptomycin, and lipofectin were obtained from Life Technologies (Grand Island, NY). FBS was supplied by Hyclone (Logan, UT). [<sup>125</sup>I]-iodoarylazidoprazosin (2200 Ci/mmol) was obtained from NEN Research Products (Boston, MA). [α-<sup>32</sup>P]-8-azido-ATP (10–16 Ci/mmol) was obtained from ICN (Costa Mesa, CA). Bodipy-FL-paclitaxel (taxol), bodipy-FL-verapamil, bodipy-FL-vinblastine, bodipy-FL-prazosin, bodipy-prazosin-558/568, and calcein-AM were obtained from Molecular Probes (Eugene, OR). Cyclosporin A, lactacystin and MG-132 were obtained from Calbiochem (San Diego, CA). MRK16 and C219 antibodies were generous gifts from Hoechst Japan, Japan and Centocor (Malvern, PA), respectively. UIC2 and 4E3 monoclonal antibodies were obtained from Coulter, (Miami, FL) and Signet (Dedham, MA), respectively. FITC conjugated antimouse IgG2a secondary antibody was obtained from PharMingen (San Diego, CA). ECL reagents were obtained from Amersham Pharmacia Biotech, (Piscataway, NJ). Human *MDR1* cDNA, pRc/CMV-neoMDR1 encoding the 91, 94 and 99 N→Q was provided generously by Dr. Alfred Schinkel (Netherlands Cancer Institute). Recombinant vaccinia virus encoding bacteriophage T7 RNA polymerase (vTF7-3), which is required for the expression of a gene under the control of a T7 promoter, was obtained from Dr. B. Moss (NIAID, NIH, Bethesda, MD).

## CONSTRUCTION OF MUTANT CDNA

Using PCR, an *Xba* I site was introduced into pRc/CMV-neoMDR1 with primer P1, GAG GTG ATC ATG GAT CTA GAA GGG GAC C and primer, P2, GTG AAC ATT TCT GAA TTC C, which contains an *Eco* RI site. Using the convenient restriction sites, *Xba* I and *Eco* RI, the mutated fragment was inserted into the pBAC-MDR1(H6) transfer vector for the baculovirus expression system (Ramachandra et al., 1998). A *Bgl* II fragment from pBAC-MDR1(H6), containing the 91, 94 and 99 N→Q mutations, was cloned into pTM1-MDR1(H6) (Ramachandra et al., 1998) plasmid for the vaccinia virus expression system, which generated the glycosylation-deficient Gly<sup>-</sup> P-gp.

*Avr* II and *Nsi* I fragments were generated by PCR to introduce aspartate residue at position 91, 94 and 99 (N→D, Gly<sup>-</sup> D) or to delete residues 91N, 94N and 99N (Gly<sup>-</sup> Δ) in pTM1-MDR1 (H6). Oligonucleotides *Avr* II (+) (CAT TCC TAG GGG TCT TTC), Gly<sup>-</sup> D(-) (GAA CCC TGT ATC ATC GAT ATC ACT TCT ATC AGT AAT GTC TGA CAT CAG ATC TTC TAA), *Nsi* I(-) (TCC CAT ACC AGA AGG CCA GAG) and Gly<sup>-</sup> D (+) (TTA GAA GAT CTG ATG TCA GAC ATT ACT GAT AGA AGT GAT ATC GAT GAT ACA GGG TTC) were used to generate Gly<sup>-</sup> D. Gly<sup>-</sup> Δ was generated by using the primers *Avr* II (+), *Nsi* (-), Gly<sup>-</sup> Δ (+) (GAA GAT CTG ATG TCA ATT ACT AGA AGT GAT ATC GAT ACA GGG TTC TTC) and Gly<sup>-</sup> Δ (-) (GAA GAA CCC TGT ATC GAT ATC ACT TCT AGT AAT TGA CAT CAG ATC TTC). All the construct sequences were verified in both directions by automated sequencing with the PRISM Ready Reaction Dye Deoxy Terminator Sequencing Kit (Perkin-Elmer, Norwalk, CT).

## CELL LINES AND VIRUSES

HeLa cells were propagated as a monolayer at 37°C, 5% CO<sub>2</sub>, 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) was propagated and purified as previously described (Earl, Cooper & Moss, 1991).

#### VACCINIA VIRUS EXPRESSION AND PREPARATION OF CRUDE MEMBRANES

A 70–80% confluent monolayer of HeLa cells was co-infected/transfected with the vTF 7-3 vaccinia virus (10 pfu/cell) and 15 µg of the control plasmid, pTM1, the wild-type plasmid, pTM1-MDR1(H6), or the mutant pTM1-MDR1(H6) Gly<sup>-</sup> plasmid using 45 µg of lipofection (Hrycyna et al., 1998). The cells were fed at 4 hr postinfection with 12 mL DMEM supplemented with 10% FBS and incubated for 24–48 hr at 32°C, 5% CO<sub>2</sub>. In an attempt to increase P-gp expression at the cell surface (Loo & Clarke, 1997), cyclosporin A (5 µM) was added to the media at 4 hr postinfection.

At 48 hr postinfection, crude membranes were prepared by hypotonic lysis as described previously (Hrycyna et al., 1998). Briefly, cells were incubated on ice in a hypotonic lysis buffer and disrupted with a dounce homogenizer. The undisrupted cells were removed by centrifugation at 500 × g for 10 min. The low speed supernatant was incubated with micrococcal nuclease (50 units/mL) in the presence of 1 mM CaCl<sub>2</sub> for 20–30 min on ice. The membranes were collected by centrifugation for 1 hr at 100,000 × g and resuspended in 10 mM Tris pH 7.5, 50 mM NaCl, 250 mM sucrose, 1 mM DTT, 1% aprotinin, 2 mM AEBSF and 10% glycerol, using a bent, blunt ended 23 gauge needle. Aliquots of crude membranes were stored at -70°C. The protein content was determined using a modified Lowry method (Bailey, 1967), using bovine serum albumin as a standard.

#### FLUORESCENCE-ACTIVATED CELL-SORTING (FACS) ANALYSIS

A FACSort flow cytometer equipped with Cell Quest software (Becton-Dickinson, San Jose, CA) was utilized for FACS analysis.

#### DETERMINATION OF CELL SURFACE EXPRESSION

Cell surface expression of P-gp was detected in HeLa cells by using the MRK16, UIC2 and 4E3 monoclonal antibodies. Labeling was done using 1 µg of antibody per 100,000 cells at 4°C for 40 min. The cells were washed with IMDM supplemented with 5% FBS. Anti-mouse IgG<sub>2a</sub> FITC-conjugated secondary antibody was added at 1 µg per 250,000 cells and incubated at 4°C for 40 min. Cell surface expression of P-gp was measured by FACS, as described previously (Germann et al., 1996).

#### SDS-PAGE AND IMMUNOBLOT ANALYSIS

SDS-PAGE and immunoblotting were performed as previously described (Hrycyna et al., 1998). Immunoblot analysis, with the monoclonal antibody C219 (0.3 µg/ml), was used to probe the total cellular content of WT and mutant P-gp. SDS-PAGE samples were separated on a 1.5 mm thick, 8% Tris-glycine gel and transferred to a 0.44 µm nitrocellulose membrane at 400 mA for 1 hr using a Tris (25 mM)-glycine (192 mM) buffer with 15% (v/v) methanol. The membrane was blocked for 25 min with 20% dry milk. The blot was incubated with C219 (1:4000) in 5% dry milk overnight and was washed three times with 10 mL PBST (15 min each). HRP-conjugated anti-mouse secondary antibody was added at 1:15000 in 5% dry milk and the blot was incubated for 1 hr. The blot was washed three times (15 min each) with

PBST. After incubation with the ECL reagents for 1 min the blot was developed, as per the manufacturer's instructions.

#### DRUG ACCUMULATION ASSAYS

Cells were harvested after trypsinization by centrifugation at 500 × g and resuspended in 1 mL IMDM supplemented with 5% FBS. The fluorescent substrates, bodipy-FL-taxol (0.1 µM), bodipy-FL-verapamil (0.5 µM), daunorubicin (3 µM), bodipy-FL-vinblastine (0.5 µM), calcein-AM (0.5 µM), bodipy-FL-prazosin (0.5 µM), and bodipy-558/568-prazosin (0.5 µM) were added to 500,000 cells in 5 mL in the presence or absence of cyclosporin A (5 µM). The cells were incubated at 37°C for 40 min and pelleted at 500 × g. For daunorubicin accumulations, an additional incubation with only IMDM or IMDM with cyclosporin A was performed for 40 min at 37°C. The pellet was resuspended in 300 µL PBS prior to FACS analysis (Hrycyna et al., 1998).

#### PHOTOAFFINITY LABELING

Drug binding properties were analyzed by photoaffinity labeling with the prazosin analogue, [<sup>125</sup>I]-iodoarylazidoprazosin (IAAP). WT, Gly<sup>-</sup> and control, pTM1 infected-transfected HeLa crude membranes (30 µg) were photoaffinity labeled with IAAP (~2–4 nM) using a 365 nm UV source for 10 min. Labeling was done with P-gp alone and in the presence of cyclosporin A (20 µM). P-gp was immunoprecipitated with the polyclonal antibody, PEPG-13 (Hrycyna et al., 1998). The immunoprecipitated proteins were separated on an 8% Tris-glycine gel and autoradiography was performed.

#### ATPASE ACTIVITY AND VANADATE-INDUCED TRAPPING OF ADP

Drug-stimulated ATP hydrolysis was determined at 37°C, in the presence and absence of 0.3 mM ortho-vanadate, by measuring the release of inorganic phosphate from Mg-ATP as described previously (Ramachandra et al., 1996).

ATP hydrolysis was also confirmed by vanadate-induced trapping of [ $\alpha$ -<sup>32</sup>P]-8-azido-ADP. HeLa crude membranes expressing WT and Gly<sup>-</sup> P-gp (50 µg) were incubated at 37°C with [ $\alpha$ -<sup>32</sup>P]-8-azido-ATP (25 µM) in the presence and absence of vanadate (0.3 mM) (Urbatsch et al., 1995). Verapamil (30 µM) was added to the assay to ensure the stimulation of ATP hydrolysis. After 10 min incubation at 37°C, the reaction was terminated by the addition of ice-cold mixture of 10 mM ATP and 20 mM MgCl<sub>2</sub> and the trapped [ $\alpha$ -<sup>32</sup>P]-8-azido-ADP was cross-linked by exposure to UV light (365 nm) as described earlier (Ramachandra et al., 1998). P-gp was immunoprecipitated with the polyclonal antibody, PEPG-13 (Hrycyna et al., 1998). SDS-PAGE was done on 8% Tris-glycine gels and autoradiography was performed.

#### <sup>35</sup>S-LABELING OF VACCINIA VIRUS INFECTED-TRANSFECTED HELa CELLS

At 16–20 hr post-infection, HeLa cells expressing WT and Gly<sup>-</sup> P-gp were washed three times with DMEM without methionine and cysteine (DMEM [-Cys, Met]) and 2 mL DMEM/well [-Cys, Met] was added. The cells were incubated for 30 min at 32°C. The DMEM [-Cys, Met] was removed and 2 mL DMEM [Cys, Met] + Trans-<sup>35</sup>S label (final conc. 583–700 µCi/mL) was added and the cells were pulsed at 32°C for 5 min. The cells were washed with DMEM and chased at 32°C with the DMEM for the desired time intervals. Prior to harvesting, the cells were washed with ice-cold PBS and solubilized with

1–2 mL RIPA buffer at 4°C for 20 min. The wells were washed with 1–2 mL RIPA buffer. Cell extracts were clarified by spinning at 100,000 × *g* for 30 min. To quantitate the incorporation of radioactivity, proteins from cell lysates were precipitated with trichloroacetic acid. Cell extracts, in aliquots of 2–10 µL, were taken for precipitation and 0.1 mL 1% ovalbumin and 1.0 mL 20% trichloroacetic acid was added. The samples were incubated at 4°C for 5 min and spun in an Eppendorf centrifuge at 4°C for 10 min at 14,000 rpm. The supernatant was discarded. The pellet was solubilized in 0.1 mL 1N NaOH by vortexing and neutralized by the addition of 0.3 mL of 0.4 M acetic acid. Radioactivity in the protein sample was quantified in a liquid scintillation counter. For protein quantitation, a modified Lowry protocol was followed using bovine serum albumin as a standard (Bailey, 1967). P-gp was immunoprecipitated with the polyclonal antibody, PEPG-13 (Hrycyna et al., 1998). The samples were analyzed by SDS-PAGE and autoradiography. The radioactivity incorporated into P-gp was quantified by excising the radioactive band, solubilization and liquid scintillation counting (Dey et al., 1997).

#### TRYPsinIZATION OF P-GLYCOPROTEIN IN CRUDE MEMBRANES

HeLa crude membrane preparations (50 µg) were treated with various amounts of trypsin for 5 min at 37°C. The reaction was stopped with 5 × excess of trypsin inhibitor and immunoblotting was performed as described above with the monoclonal antibody, C219.

#### CONFORMATION-SENSITIVE UIC2 BINDING TO WT AND GLY<sup>-</sup> P-GLYCOPROTEIN

WT and Gly<sup>-</sup> P-gp expressing HeLa cells were incubated in the presence and absence of 5 µM cyclosporin A for 5 min at 37°C, prior to the addition of UIC2 antibody. UIC2 staining was carried out by using 1 µg antibody/100,000 cells at 37°C. Subsequent secondary antibody incubation and FACS analysis was performed as described above.

#### TREATMENT WITH PROTEASOME INHIBITORS

Vaccinia virus infected HeLa cells, transfected with pTM1, WT, or Gly<sup>-</sup> DNA, were treated with either 5 µM lactacystin or 10 µM MG-132 for 24 hr. Cell lysates were prepared and immunoblotting was performed as described above with the monoclonal antibody C219.

#### ABBREVIATIONS

FACS, fluorescence-activated cell sorter; P-gp, P-glycoprotein; Gly<sup>-</sup>, glycosylation-deficient P-glycoprotein (91, 94, 99 Asn→Gln); WT, wild-type; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; IMDM, Iscove's modified Dulbecco's medium; and IAAP, [<sup>125</sup>I]-iodoarylazidoprazosin.

### Results

#### CONSTRUCTION OF PLASMIDS TO EXPRESS GLYCOSYLATION-DEFICIENT P-GLYCOPROTEIN

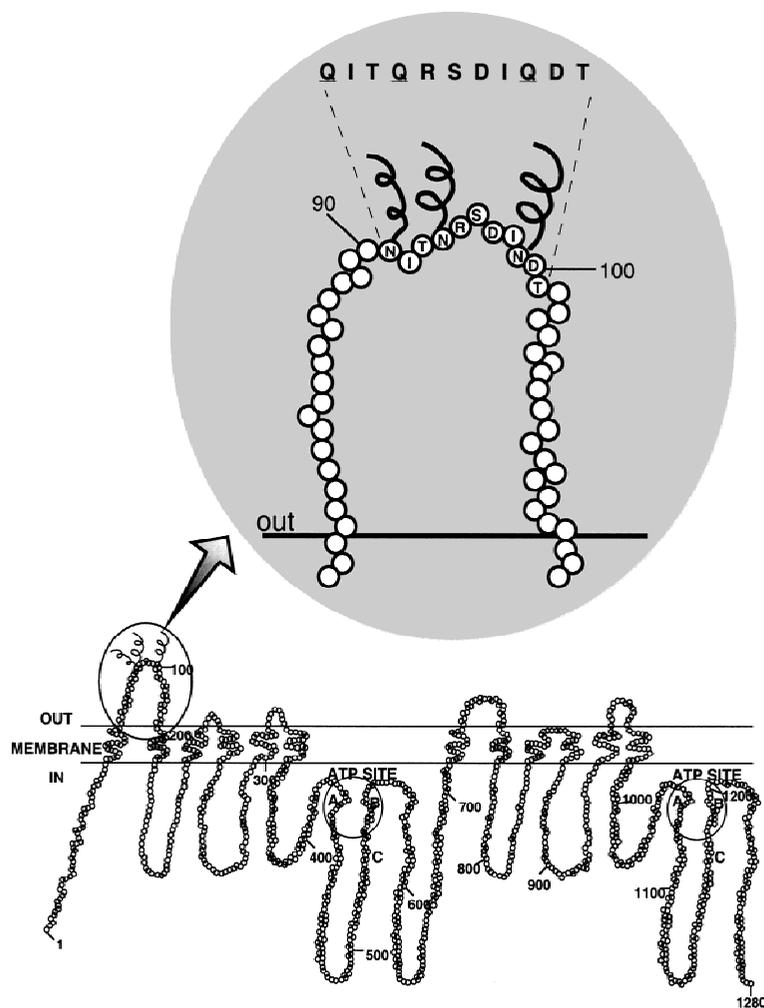
To express glycosylation-deficient P-gp in HeLa cells, *MDR1* cDNAs in the pTM1 vector were constructed in

which asparagine at positions 91, 94, and 99 were either mutated to glutamine (Gly<sup>-</sup>, N→Q), or aspartate (Gly<sup>-</sup> D, N→D) or were deleted all together (Gly<sup>-</sup> Δ, N Δ; *see* Fig. 1). The WT and mutated *MDR1* cDNA, containing a six-histidine tag at the carboxy terminus, was placed under the control of a T7 promoter and was down stream of an internal ribosome entry site in the pTM1 vector. As described previously, high levels of expression of WT and mutant *MDR1* could be obtained in mammalian cells upon infection with the vTF7-3 recombinant vaccinia virus encoding bacteriophage T7 RNA polymerase followed by transfection with the pTM1-*MDR1* vector (Ramachandra et al., 1996).

#### EXPRESSION OF WILD-TYPE AND GLYCOSYLATION-DEFICIENT P-gp USING A VACCINIA VIRUS

By transiently expressing Gly<sup>-</sup> P-gp with the vaccinia virus expression system, the long-term pleiotropic effects of drug selection on stable transfectants and/or tunicamycin treatment could be avoided in the expression of Gly<sup>-</sup> P-gp. Furthermore, the loss of P-gp activity resulting from the use of glycosidases would be eliminated. Both WT and Gly<sup>-</sup> P-gp were expressed in HeLa cells using the vaccinia virus expression system. The HeLa cell line (cervical epidermoid carcinoma) was chosen due to its low level expression of endogenous P-gp, ability to express a high level of P-gp, and relative ease of transfection (Hrycyna et al., 1998; Ramachandra et al., 1998).

P-gp expression at the cell surface was detected at 24 hr post-infection-transfection using the monoclonal antibodies MRK16, UIC2 and 4E3, which recognize external epitopes on human P-gp as shown in Fig. 2. HeLa cells infected with virus and the control plasmid containing no cDNA, pTM1, show very low levels of endogenous P-gp expression. As shown in Fig. 2, the Gly<sup>-</sup> P-gp is expressed approximately 40–50% less than the WT P-gp based on median fluorescence intensity. An immunoblot analysis with the monoclonal antibody C219 also shows this difference in expression levels for Gly<sup>-</sup> P-gp and WT P-gp, as shown in Fig. 3. Both by FACS and immunoblot analysis, it was found that the Gly<sup>-</sup> D (91, 94, 99 N→D) mutant is expressed at a similar level as Gly<sup>-</sup> P-gp (91, 94, 99 N→Q). The mobility of the Gly<sup>-</sup> D protein was retarded compared to WT and N→Q mutant P-gp (Fig. 3), which might be due to change in the net charge on the protein as a result of the addition of three negatively charged amino acids (91, 94, 99 N→D). Comparable results were also obtained with Gly<sup>-</sup> Δ P-gp (91, 94, 99 N deleted; *data not shown*). Therefore, for further biochemical analyses, only the Gly<sup>-</sup> (91, 94, 99 N→Q) mutant P-gp was used. The



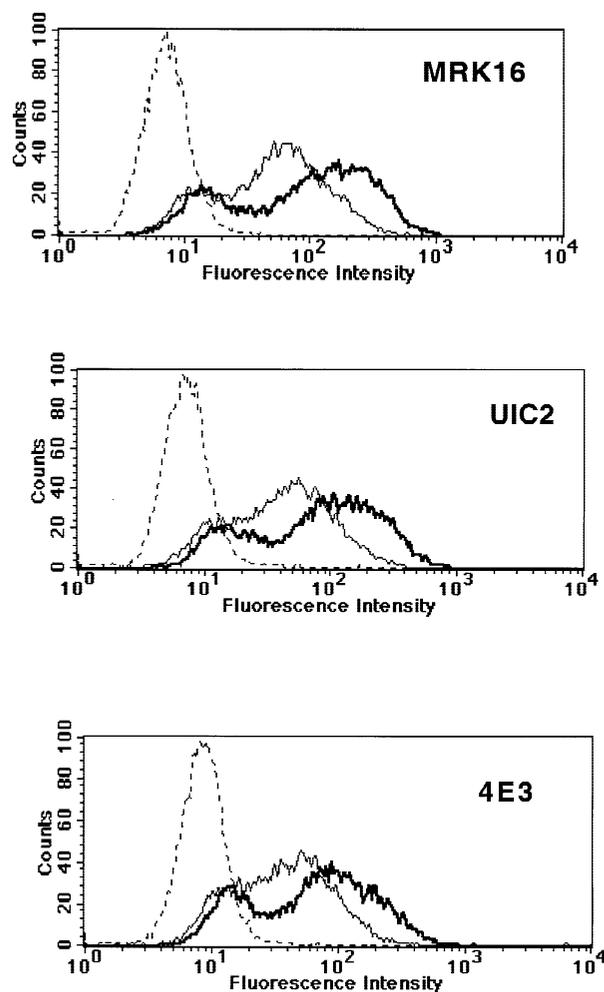
**Fig. 1.** Two-dimensional hypothetical model of P-glycoprotein structure. A hypothetical two-dimensional model of human P-gp based on hydropathy analysis of the amino acid sequence [adapted from Gottesman & Pastan (1988)]. The glycosylation sites in the first extracellular loop are indicated by the helical lines. Glycosylation occurs in the first extracellular loop (inset) at the asparagine residues 91, 94, and 99 contained in the consensus sequence Asn-X-Ser/Thr (X can be any amino acid but proline) (Kornfeld & Kornfeld, 1985). The introduced mutations (N→Q), which prevent glycosylation, are underlined (inset). Although not shown, 91, 94, and 99 N was also replaced with D or deleted altogether.

majority of WT P-gp protein migrates mainly at ~140–150 kDa, but a small portion also migrates at 170 kDa. This result, which is also supported by earlier observations in vaccinia virus infected human osteosarcoma cells, is attributed to incomplete glycosylation of WT P-gp due to a rapid rate of protein synthesis (Ramachandra et al., 1996).

Cyclosporin A treatment, which has been demonstrated to increase the localization of mutant P-gps at the cell surface that otherwise get trapped in the endoplasmic reticulum (Loo & Clarke, 1997), was not able to increase Gly<sup>-</sup> P-gp cell surface expression (*data not shown*). However, WT P-gp expression levels were elevated to some extent (10–15%) when grown in the presence of 10 μM cyclosporin A. These results suggest that reduced Gly<sup>-</sup> P-gp expression is not due to increased accumulation of the full-length molecule within the endoplasmic reticulum or intracellular vesicles. These findings are also supported by the immunoblot analysis of cellular levels of WT and Gly<sup>-</sup> P-gp (*see Fig. 3*).

#### ANALYSES OF DRUG TRANSPORT FUNCTION AND SUBSTRATE SPECIFICITY

The ability of the Gly<sup>-</sup> P-gp to transport drugs was assessed by fluorescence activated cell sorting (FACS). HeLa cells, expressing pTM1 (vector alone), WT P-gp, and Gly<sup>-</sup> P-gp were loaded with various fluorescent drugs or drug analogues for 40 min in the presence and absence of the P-gp specific inhibitor, cyclosporin A. As shown in Fig. 4, the glycosylation-deficient P-gp expressing HeLa cells accumulate more daunorubicin than the WT cells. The level of accumulation of fluorescent substrates parallels the level of the glycosylation-deficient protein at the cell surface (Fig. 2). The Gly<sup>-</sup> P-gp expressed at the cell surface is functional and capable of transporting drugs across the cell membrane. A wide range of drugs and drug analogues were then used to assess the effect of glycosylation on P-gp substrate specificity. As shown in the Table, a qualitative assessment of transport was made. The Gly<sup>-</sup> P-gp ex-

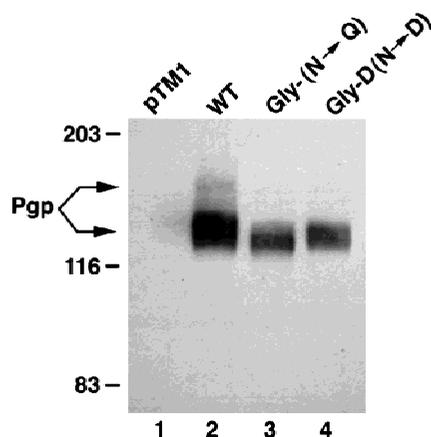


**Fig. 2.** Cell surface expression of wild-type and glycosylation-deficient P-gp. Infected-transfected HeLa cells were stained with the external epitope specific monoclonal antibodies, MRK16, UIC2 and 4E3, and subjected to FACS analysis, as described in Materials and Methods. HeLa cells were transfected with a vector containing pTM1 (----), pTM1-MDR1(H6) (—●—), or pTM1-MDR1-Gly<sup>-</sup>(H6) (—○—).

pressing HeLa cells compared to WT generally accumulate more of the substrate. This increased accumulation is consistent with the reduced level of expression. This result suggests that glycosylation is not required for the drug transport function of P-gp, and the lack of this post-translational modification does not cause any gross alterations in substrate specificity. These studies extend previous work demonstrating that the complete glycosylation of P-gp is not essential for its function (Beck & Cirtain, 1982; Ling et al., 1983; Germann et al., 1990; Kuchler & Thorer, 1992; Schinkel et al., 1993) (*see below*).

#### DRUG PHOTOAFFINITY LABELING OF P-gp

Drug photoaffinity labeling was performed to verify that the drug binding properties of the glycosylation-deficient

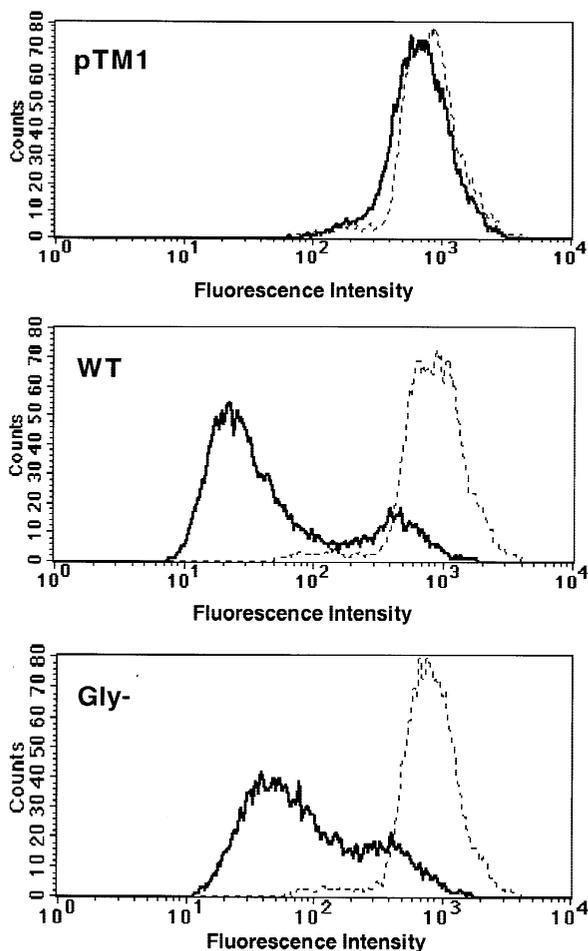


**Fig. 3.** Wild-type and glycosylation-deficient P-gp expression. HeLa cells were transfected with pTM1 (control, lane 1), pTM1-MDR1(H6) (WT P-gp, lane 2), pTM1-MDR1-Gly<sup>-</sup>(H6) (Gly<sup>-</sup> P-gp, lane 3) or pTM1-MDR1 Gly<sup>-</sup>D (H6) (Gly-DP-gp, lane 4) and infected with vaccinia virus. At 48 hr postinfection, the cells were harvested, and crude membranes were prepared. An immunoblot analysis of the crude membrane preparations (2  $\mu$ g protein/lane) was done using the monoclonal antibody, C219. The position of P-gp is indicated by an arrow.

P-gp were unaltered. The photoreactive prazosin analogue, IAAP, was used to label WT and Gly<sup>-</sup> P-gp from vaccinia virus infected HeLa cell crude membranes in the presence and absence of the inhibitor, cyclosporin A. Cyclosporin A is a P-gp reversing agent that is capable of inhibiting IAAP labeling (Dey et al., 1997). The labeled proteins were analyzed by SDS-PAGE and autoradiography. The vaccinia virus infected-transfected control cells, pTM1, show no IAAP labeling as the endogenous levels of P-gp are not high enough to be detected by photo-crosslinking at low concentration ( $\sim$ 5 nM) of IAAP (*data not shown*). Both the WT (Fig. 5, lanes 1) and Gly<sup>-</sup> P-gp were labeled by IAAP (lane 3). Furthermore, cyclosporin A completely inhibited IAAP labeling for both WT and Gly<sup>-</sup> P-gp (Fig. 5, lanes 2 and 4, respectively). The use of this P-gp inhibitor demonstrates that the labeling of both WT and Gly<sup>-</sup> P-gp with IAAP is specific. The lower level of IAAP labeling for Gly<sup>-</sup> P-gp results from its reduced level of expression. Thus, there seems to be no alterations in the drug binding properties of P-gp resulting from the absence of glycosylation.

#### ATPASE ACTIVITY OF WILD-TYPE AND GLYCOSYLATION-DEFICIENT P-gp

By measuring the release of inorganic phosphate from Mg-ATP, the ATPase activity of WT and Gly<sup>-</sup> P-gp was determined using the vaccinia virus expressed protein in crude membranes (Fig. 6A). The control membranes, prepared with pTM1, show a basal activity of approxi-



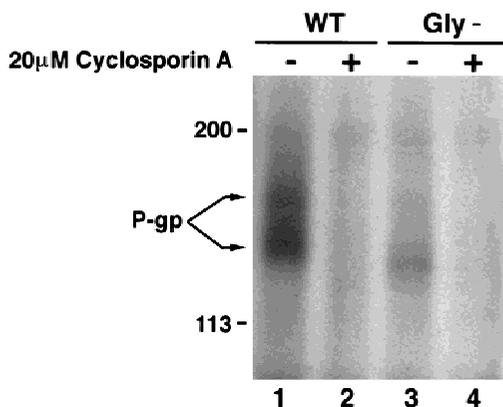
**Fig. 4.** Assessment of the drug transport function of wild-type and glycosylation deficient P-gp. The drug accumulation of vaccinia virus infected-transfected HeLa cells was determined by a FACS analysis. Cells were transfected with pTM1 (control), pTM1-MDR1(H6) (WT P-gp) or pTM1-MDR1-Gly<sup>-</sup>(H6) (glycosylation deficient P-gp). After 24 hr postinfection, cells were harvested, washed and loaded for 40 min with 3  $\mu$ M daunorubicin, in the absence (–) and presence of an inhibitor, 5  $\mu$ M cyclosporin A (---).

mately 6–9 nmoles/min/mg and exhibit no verapamil-stimulated ATPase activity (*data not shown*). The WT P-gp membranes have a basal activity of approximately 22.0 nmoles/min/mg and a verapamil stimulated activity of 39.3 nmoles/min/mg. Gly<sup>-</sup> P-gp membranes have a basal activity of 10.2 nmoles/min/mg and a verapamil-stimulated activity of 15.1 nmoles/min/mg protein. The lower levels of activity observed for the glycosylation deficient protein is most likely due to lower levels of expression of Gly<sup>-</sup> P-gp in the crude membrane preparation (Figs. 2 and 3). However, the fold increase in verapamil-stimulated ATP hydrolysis, which is reflective of P-gp activity, is not significantly altered in the glycosylation deficient P-gp (Fig. 6A). Moreover, Gly<sup>-</sup> D P-gp (N→D) mutant also exhibits verapamil-stimulated

**Table.** Assessment of the transport function of wild-type and glycosylation-deficient P-glycoprotein using fluorescent substrates

Compound	WT P-gp	Gly <sup>-</sup> P-gp	Gly <sup>-</sup> D P-gp
		(N → Q)	(N → D)
Calcein-AM (0.5 $\mu$ M)	+++++	++++	++++
Bodipy-FL-Forskolin (0.5 $\mu$ M)	+++++	++++	++++
Bodipy-FL-Verapamil (0.5 $\mu$ M)	+++++	++++	++++
Bodipy-FL-Vinblastine (0.1 $\mu$ M)	+++++	++++	++++
Bodipy-558/568-Prazosin (0.5 $\mu$ M)	+++++	++++	ND
Bodipy-FL-Prazosin (0.5 $\mu$ M)	+++++	++++	ND
Bodipy-FL-Taxol (0.5 $\mu$ M)	+++++	+++++	ND
Daunorubicin (3 $\mu$ M)	+++++	++++	ND

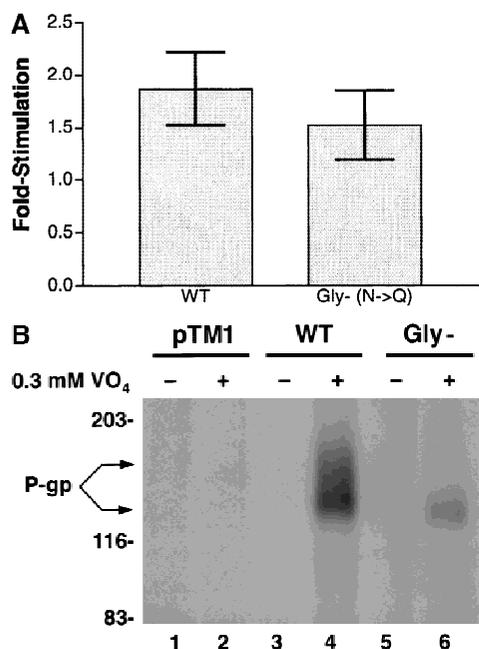
Transport of fluorescent substrates at concentrations as indicated in the parentheses, was assessed by FACS analysis as described in Materials and Methods (*see* Fig. 4). Unimpaired drug efflux (i.e., similar to WT) represented by +++++. Reduced drug efflux (60–90% of WT) represented by ++++ and ND, not determined.



**Fig. 5.** Photoaffinity labeling of wild-type and glycosylation-deficient P-gp with a prazosin analogue, [<sup>125</sup>I]-Iodoarylazidoprazosin (IAAP). Vaccinia virus infected-transfected HeLa cell crude membrane fractions were labeled with 2 nM IAAP in the absence (lanes 1 & 3) and in the presence of the P-gp inhibitor, 20  $\mu$ M cyclosporin A (lanes 2 & 4) as described in Materials and Methods. The position of P-gp is indicated by an arrow.

ATPase activity to a similar extent as Gly<sup>-</sup> P-gp (N→Q) (*data not shown*).

Due to the lower level of Gly<sup>-</sup> P-gp protein in crude membranes, 8-azido-[ $\alpha$ -<sup>32</sup>P]-ADP trapping in the presence of vanadate at 37°C was then performed to further quantitatively characterize the ATP hydrolysis of the mutant protein. Verapamil was added to the membranes to ensure that the ATP hydrolysis of P-gp was stimulated to the maximal level. Upon hydrolysis of [ $\alpha$ -<sup>32</sup>P]-8-



**Fig. 6.** Drug-stimulated ATP hydrolysis by wild-type and glycosylation-deficient P-gp. (A) Verapamil-stimulated ATP hydrolysis: Crude membranes of HeLa cells expressing WT or Gly<sup>-</sup> P-gp were incubated with 30  $\mu$ M verapamil, 10 mM MgCl<sub>2</sub>, 5 mM ATP in the presence and absence of 0.3 mM sodium orthovanadate at 37°C and the hydrolysis of ATP was measured as described in Materials and Methods. Vanadate-sensitive, verapamil-stimulated ATPase activities of WT and Gly<sup>-</sup> P-gp from three independent experiments are shown (mean  $\pm$  SD). (B) Vanadate-induced trapping of  $\alpha$ -<sup>32</sup>P-8-azido-ADP. Crude membranes were incubated with  $\alpha$ -<sup>32</sup>P-8-azido-ATP (25  $\mu$ M, 10  $\mu$ Ci), in the presence and absence of vanadate (0.3 mM). Verapamil (30  $\mu$ M) was added to the assay to ensure the stimulation of ATP hydrolysis. P-gp was immunoprecipitated using the polyclonal antibody, PEPG 13. SDS-PAGE and autoradiography were performed, as described in Materials and Methods and the autoradiogram is shown in the figure.

azido-ATP by P-gp, the phosphate analogue, vanadate, traps the labeled [ $\alpha$ -<sup>32</sup>P]-8-azido-ADP molecule in the P-gp active site where it becomes covalently cross-linked upon exposure to UV light (365 nm) (Urbatsch et al., 1995). As shown in Fig. 6B, the vanadate induced trapping of 8-azido-ADP (P-gp·MgADP·V<sub>i</sub>), revealed that the glycosylation-deficient mutant has a level of ATP hydrolysis that is ~30–40% of WT P-gp, consistent with the reduction in expression levels.

#### BIOSYNTHESIS AND STABILITY OF WILD-TYPE AND GLYCOSYLATION-DEFICIENT P-GLYCOPROTEIN

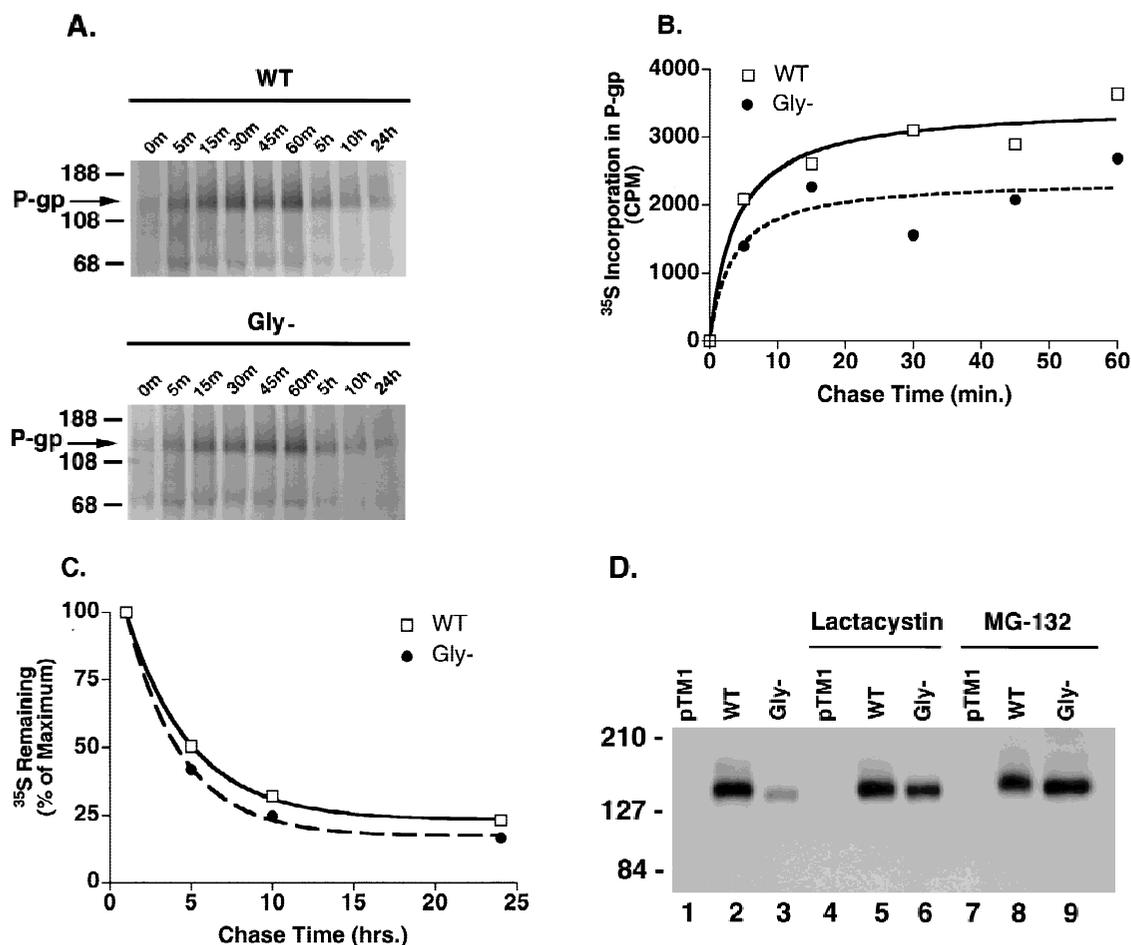
It was considered that the reduced levels of expression Gly<sup>-</sup> P-gp might be due to either reduced stability of the protein or alterations in P-gp biosynthesis. Therefore, <sup>35</sup>S pulse-chase labeling was performed using vaccinia

virus infected-transfected HeLa cells. HeLa cells were starved in DMEM without cysteine/methionine for 30 min, pulsed with <sup>35</sup>S-trans label (583–700  $\mu$ Ci/mL) for 5 min and chased to a maximum of 24 hr (48 hr postinfection). As shown in Fig. 7A, WT P-gp is predominantly expressed as the 140–150 kDa core glycosylated form, and very little of the fully glycosylated 170 kDa form is observed in vaccinia virus infected-transfected HeLa cells, as previously described (Ramachandra et al., 1996). As shown in Fig. 7B, the incorporation of <sup>35</sup>S in Gly<sup>-</sup> P-gp is 30–40% less than WT protein. Once the glycosylation-deficient P-gp is synthesized, its half-life of ~3 hr is approximately the same as the WT protein (see Fig. 7C). The 3 hr half-life of the vaccinia virus expressed WT P-gp was significantly shorter than that previously reported for drug resistant stable cell lines. For KB-V1 cells, the fully glycosylated P-gp was found to be synthesized in approximately 2–4 hr and has a half-life of 48–72 hr (Richert et al., 1988).

The decreased incorporation of <sup>35</sup>S in Gly<sup>-</sup> P-gp (Fig. 7B) might be either due to decreased rate of synthesis or increased rate of degradation of improperly folded protein by the proteasome and/or endoplasmic reticulum-associated proteases (Jensen et al., 1995; Loo & Clarke, 1998a). To address this issue, HeLa cells were treated with proteasome inhibitors (Lee & Goldberg, 1998) such as lactacystin (5  $\mu$ M) and MG-132 (2 to 10  $\mu$ M) following transfection-infection for 16–20 hr. Although treatment with proteasome inhibitors resulted in an increase in cellular levels of Gly<sup>-</sup> P-gp (Fig. 7D), it did not enhance the level of mutant protein at the cell surface (*data not shown*). As described previously (Jensen et al., 1995), the WT P-gp level was not significantly affected by the treatment with lactacystin or MG-132. These data suggest that the decreased level of Gly<sup>-</sup> P-gp might be due to degradation of misfolded protein by the proteasome, as well as by endoplasmic reticulum-associated proteases (Loo & Clarke, 1998a).

#### SUSCEPTIBILITY TO TRYPSIN DIGESTION

Core glycosylated WT and mutant P-gps, expressed in HEK 293 cells, have been shown to have an increased susceptibility to trypsin digestion compared to the fully glycosylated molecules (Loo & Clarke, 1998b). To test whether unglycosylated P-gp is more susceptible to trypsin digestion, crude membranes of WT and Gly<sup>-</sup> P-gp were treated with trypsin at various concentrations. The extent of P-gp digestion was assessed by immunoblotting with the monoclonal antibody, C219. As shown in Fig. 8, Gly<sup>-</sup> P-gp was not more susceptible to trypsin degradation than the core-glycosylated WT P-gp. Both Gly<sup>-</sup> and WT P-gp, transiently expressed by the vaccinia virus in HeLa cells, are fully degraded at a protein:trypsin ratio of 50:1.



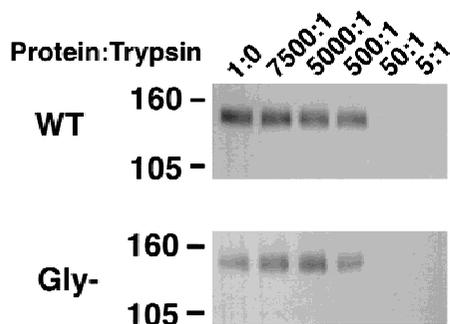
**Fig. 7.** Synthesis and degradation of wild-type and glycosylation deficient P-gp. Vaccinia virus expressed WT and Gly<sup>-</sup> P-gp expressing HeLa cells were pulsed for 5 min with <sup>35</sup>S methionine/cysteine (~700  $\mu$ Ci/mL) and chased for up to 24 hr. P-gp was immunoprecipitated with the polyclonal antibody PEPG-13, analyzed by SDS-PAGE, and autoradiography. Gly<sup>-</sup> P-gp (panel B) shows a reduced synthesis compared to WT P-gp (panel A). Panel B shows the quantitation of <sup>35</sup>S incorporation into P-gp. WT P-gp (—) is synthesized to a greater extent than Gly<sup>-</sup> P-gp (---), but the two proteins have similar half-lives of 3 hr (panel C). Vaccinia virus infected HeLa cells expressing WT and Gly<sup>-</sup> P-gp were treated for 20 hr with 5  $\mu$ M lactacystin or 10  $\mu$ M MG-132 (Panel D). Cell lysates were separated by SDS-PAGE, and P-gp was detected by immunoblot analysis with C219.

#### CONFORMATION-SENSITIVE UIC2 BINDING TO P-GLYCOPROTEIN

It has previously been shown that the reactivity of monoclonal antibody UIC2 with P-gp at cell surface is increased at 37°C in the presence of substrates or agents that induce ATP depletion (Mechetner et al., 1997). This enhanced antibody binding has been proposed to result from a conformational change induced by drug transport or by ATP depletion, resulting in greater UIC2 epitope accessibility. When HeLa cells expressing Gly<sup>-</sup> P-gp were pretreated with 5  $\mu$ M cyclosporin A prior to UIC2 staining at 37°C, a similar increase in UIC2 binding was observed (*data not shown*). This result suggests that during drug transport, Gly<sup>-</sup> P-gp, compared to WT P-gp, is able to undergo similar conformational changes.

#### Discussion

Although there are ten potential glycosylation sites in human P-gp, as determined by the presence of Asn-X-Ser/Thr residues in the primary amino acid sequence (Chen et al., 1986), only three sites in the first extracellular loop are glycosylated (Schinkel et al., 1993). There is a considerable body of evidence that argues against any major role for glycosylation in regulation of the transport function of P-gp: (i) Inhibition of N-linked glycosylation with tunicamycin has no effect on the MDR phenotype (Beck & Cirtain, 1982); (ii) MDR sublines can be selected from among lectin-resistant CHO mutants defective in N-linked glycosylation (Ling et al., 1983); (iii) mutants lacking all three N-linked glycosylation sites in the first extracellular loop of P-gp can confer



**Fig. 8.** Susceptibility of wild-type and glycosylation-deficient P-gp to trypsin treatment. WT and Gly<sup>-</sup> P-gp were subjected to trypsinization at 37°C using various protein to trypsin ratios, as given in the figure. After the termination of the reaction with trypsin inhibitor, the crude membrane preparations were subjected to immunoblotting with the monoclonal antibody, C219. The pattern of degradation for WT and Gly<sup>-</sup> P-gp is shown in the top and bottom panel (as indicated with WT and Gly<sup>-</sup> on the left), respectively.

MDR phenotype to the drug-sensitive cells (Schinkel et al., 1993); and (iv) recombinant P-gps produced in heterologous expression systems such as insect (Sf9, High Five) cells, and yeast, in which at most core-glycosylation can occur, exhibit either drug-stimulated ATPase activity or ATP-dependent drug transport (Sarkadi et al., 1992; Ruetz, Raymond & Gros, 1993; Ramachandra et al., 1998). However, mutant P-gps lacking all three glycosylation sites are less efficient in conferring drug resistance. Therefore, glycosylation may play a role in targeting P-gp to cell surface, in stabilizing the protein against proteolytic degradation at the cell surface (Schinkel et al., 1993), or in proper folding of the molecule within the endoplasmic reticulum during biosynthesis (*see below*). In this report, we have extended these studies by characterizing various properties such as drug transport, ATPase activity, drug binding, synthesis, degradation and gross conformation of glycosylation-deficient P-gp.

To determine the biochemical role of N-linked glycosylation in the generation of a MDR phenotype, a glycosylation deficient P-gp was expressed in HeLa cells using a vaccinia virus expression system. Transiently expressed WT P-gp in this system is predominantly core glycosylated, having a molecular weight of approximately 140–145 kDa. The core-glycosylation does not affect the cell surface expression or the transport function of P-gp (Hrycyna et al., 1998; Ramachandra et al., 1998). Upon removal of the glycosylation sites (Gly<sup>-</sup> P-gp, 91, 94, and 99 N→Q), P-gp does not get glycosylated and is expressed less compared to WT protein at the cell surface (Figs. 2 and 3). The replacement of Asn with negatively charged Asp or the deletion of all the three Asn residues did not result in an increase in the expression of the glycosylation-deficient P-gp. Therefore, these results demonstrate that the decrease in Gly<sup>-</sup> P-gp

expression did not result from the amino acid alterations but rather was due to the lack of glycosylation. Since mutants with N→Q, N→D or Δ N all gave a similar result, Gly<sup>-</sup> P-gp with N→Q mutations was used for further studies.

Although cell surface expression is reduced, the Gly<sup>-</sup> P-gp remains fully functional (Fig. 4). Furthermore, the glycosylation deficient protein retains its ability to transport a variety of anticancer drugs as well as other fluorescent substrates. Thus, our observations confirm the earlier findings (Schinkel et al., 1993) that glycosylation is not required for drug transport function and provide further evidence for the lack of its effect on the substrate specificity of P-gp (*see Table*). Similar observations have been reported for other membrane transport proteins as well. The unglycosylated GLYT1 (glycine) transporter exhibited decreased cell surface expression, but retained full activity when it was reconstituted (Olivares et al., 1995). In addition, the function and stability of the Na<sup>+</sup>/I<sup>-</sup> symporter, which is predicted to contain 13 transmembrane regions, is conserved in the absence of glycosylation (Levy et al., 1998).

Biochemical assays were performed to further elucidate the subtle alterations in P-gp-mediated activities caused by the lack of glycosylation. Biochemical characterization of the glycosylation mutant shows that there are no alterations in the drug binding properties (Fig. 5) and drug-stimulated ATPase activity of the protein (Fig. 6). The lower net verapamil-stimulated ATPase activity of Gly<sup>-</sup> P-gp can be attributed to its lower level of expression. However, the fold-stimulation of ATPase activity, an intrinsic property that reflects protein functioning, is essentially unaltered in the glycosylation-deficient mutant P-gp. Furthermore, the vaccinia virus expressed Gly<sup>-</sup> P-gp does not show an increased susceptibility to trypsin digestion compared to the WT (Fig. 8). Similarly, the conformation-sensitive monoclonal antibody, UIC2, exhibits substrate-induced increased binding with Gly<sup>-</sup> P-gp to the same extent as WT protein (Mechetner et al., 1997). The unaltered trypsin sensitivity and the reactivity with UIC2 of Gly<sup>-</sup> P-gp suggest that the gross conformation of the mutant protein is similar to that of the WT P-gp. These results also demonstrate that the drug binding properties, ATPase activity, and gross conformation of P-gp are not influenced by glycosylation.

To elucidate the role of glycosylation in the biosynthesis and stability of the protein, <sup>35</sup>S pulse-chase labeling was performed using the vaccinia virus expression system. Previously, it has been observed that glycosylation can significantly reduce the half-life of norepinephrine transporter (Melikian, 1996). Gly<sup>-</sup> P-gp appears to be synthesized at a similar rate as WT, but due to misfolding of some of the mutant protein, it is degraded by the proteasome resulting in lower levels of expression. The treatment with inhibitors of proteasome function,

such as MG-132 or lactacystin, results in increased intracellular levels of Gly<sup>-</sup> P-gp, without increasing its level at the cell surface (*data not shown*). These proteasome inhibitors do not have any significant effect on the level of WT protein (Jensen et al., 1995; Loo & Clarke, 1998a). These findings demonstrate that the proteasomal degradation of misfolded mutant protein may be the primary cause for the reduced level of Gly<sup>-</sup> P-gp. Thus, the glycosylation appears to affect proper folding of P-gp in the endoplasmic reticulum instead of facilitating its trafficking or increasing its stability at the cell surface, as proposed earlier (Schinkel et al., 1993).

P-gp has previously been shown to interact with the endoplasmic reticulum chaperon, calnexin. Mutants, which only become core glycosylated and have a half-life of 3 hr, associate with calnexin longer than WT P-gp (Loo & Clarke, 1994). This reduced half-life is similar to the half-life of the vaccinia virus expressed WT P-gp, which is predominately in the core glycosylated state (Fig. 7 A and B). Furthermore, the number and location of glycans has been shown to influence hemagglutinin interactions with calnexin and calreticulin by using microsomes after *in vitro* translation and expression in Chinese hamster ovary cells (Hebert et al., 1997). A complete lack of P-gp glycosylation may further prolong or inhibit its interaction with these chaperons and thus hinder its proper folding which is essential for its processing and routing to the plasma membrane. Cyclosporin A, which is able to promote folding of improperly folded P-gp molecules trapped in the endoplasmic reticulum or in intracellular vesicles (Loo & Clarke, 1997) was not able to increase the expression of Gly<sup>-</sup> P-gp. Thus, the Gly<sup>-</sup> P-gp does not appear to accumulate in intracellular vesicles and misfolded molecules are probably rapidly degraded by the proteasomal pathway. These results suggest that the glycosylation of P-gp may aid its interaction with chaperones, and therefore, assist proper maturation and routing to the plasma membrane. At present, we cannot rule out involvement of endoplasmic reticulum-associated proteases (Loo & Clarke, 1998a) in addition to proteasomal degradation of Gly<sup>-</sup> P-gp.

The N-linked glycosylation and the presence of complex sugars appear to interfere with the crystallization of membrane proteins and the sugars are typically removed from the purified protein by enzymatic digestion prior to the generation of crystals. In most cases, the prolonged enzymatic treatment results in inactivation of the protein. Thus, the availability of glycosylation-deficient functional P-gp is highly desirable for structural studies. Our studies demonstrate that the lack of glycosylation does not alter the function or the gross conformation of P-gp within the plasma membrane. Rather, the absence of glycosylation affects only the level of P-gp at the cell surface. To generate a large quantity of pure unglycosylated protein, we have expressed Gly<sup>-</sup> P-gp

(91, 94, 99N→Q) with (His)<sub>6</sub> tag at its carboxy terminus in a baculovirus expression system (J. Gribar, M. Ramachandra and S. Ambudkar, *unpublished data*) and this approach should facilitate the elucidation of the 2-D and 3-D structures of P-gp.

We thank Dr. Alfred Schinkel for providing the human *MDR1* cDNA, pRc/CMV-neo*MDR1* encoding the 91, 94, and 99 N→Q (Schinkel et al., 1993) mutant P-gp, Drs. Michael M. Gottesman and Ira Pastan for their support and advice and Drs. Kathleen Kerr and Zuben Sauna for useful comments on the manuscript.

## References

- Ambudkar, S.V. 1995. Purification and reconstitution of functional human P-glycoprotein. *J. Bioenerg. Biomembr.* **27**:23–29
- Ambudkar, S.V., Dey, S., Hrycyna, C.A., Ramachandra, M., Pastan, I., Gottesman, M.M. 1999. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. *Annu. Rev. Pharmacol. Toxicol.* **39**:361–398
- Bailey, J.L. 1967. Techniques in Protein Chemistry. pp. 340–341. Elsevier, New York
- Beck, W.T., Cirtain, M. 1982. Continued expression of *Vinca* alkaloid resistance by CCRF-CEM cells after treatment with tunicamycin or pronase. *Cancer Res.* **42**:184–189
- Chen, C.J., Chin, J.E., Ueda, K., Clark, D.P., Pastan, I., Gottesman, M.M., Roninson, I.B. 1986. Internal duplication and homology with bacterial transport proteins in the *mdr1* (P-glycoprotein) gene from multidrug-resistant human cells. *Cell* **47**:381–389
- Dey, S., Ramachandra, M., Pastan, I., Gottesman, M.M., Ambudkar, S.V. 1997. Evidence for two nonidentical drug-interaction sites in the human P-glycoprotein. *Proc. Natl. Acad. Sci. USA* **94**:10594–10599
- Earl, P.E., Cooper, N., Moss, B. 1991. Propagation of cell cultures and vaccinia virus stocks. *In: Current Protocols in Molecular Biology.* F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.C. Seidman and K. Struhl, editors. pp. 16.16.1–16.16.7. John Wiley and Sons, New York
- Germann, U.A., Chambers, T.C., Ambudkar, S.V., Licht, T., Cardarelli, C.O., Pastan, I., Gottesman, M.M. 1996. Characterization of phosphorylation-defective mutants of human P-glycoprotein expressed in mammalian cells. *J. Biol. Chem.* **271**:1708–1716
- Germann, U.A., Willingham, M.C., Pastan, I., Gottesman, M.M. 1990. Expression of the human multidrug transporter in insect cells by a recombinant baculovirus. *Biochemistry* **29**:2295–2303
- Gottesman, M.M., Pastan, I. 1988. The multidrug transporter, a double-edged sword. *J. Biol. Chem.* **263**:12163–12166
- Gottesman, M.M., Pastan, I. 1993. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.* **62**:385–427
- Gottesman, M.M., Pastan, I., Ambudkar, S.V. 1996. P-glycoprotein and multidrug resistance. *Curr. Opin. Genet. Dev.* **6**:610–617
- Hebert, D.N., Zhang, J.X., Chen, W., Foellmer, B., Helenius, A. 1997. The number and location of glycans on influenza hemagglutinin determine folding and association with calnexin and calreticulin. *J. Cell Biol.* **139**:613–623
- Higgins, C.F. 1992. ABC transporters: from microorganisms to man. *Annu. Rev. Cell Biol.* **8**:67–113
- Hrycyna, C.A., Ramachandra, M., Pastan, I., Gottesman, M.M. 1998. Functional expression of human P-glycoprotein from plasmids using vaccinia virus-bacteriophage T7 RNA polymerase system. *Methods Enzymol.* **292**:456–473

- Jensen, T.J., Loo, M.A., Pind, S., Williams, D.B., Goldberg, A.L., Riordan, J.R. 1995. Multiple proteolytic systems, including the proteasome, contribute to CFTR processing. *Cell* **83**:129–135
- Kornfeld, R., Kornfeld, S. 1985. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **54**:631–664
- Kuchler, K., Thorner, J. 1992. Functional expression of human *mdr1* in the yeast *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **89**:2302–2306
- Lee, D.H., Goldberg, A.L. 1998. Proteasome inhibitors: valuable new tools for cell biologists. *Trends Cell Biol.* **8**:397–403
- Levy, O., De la Vieja, A., Ginter, C.S., Riedel, C., Dai, G., Carrasco, N. 1998. N-linked glycosylation of the thyroid  $\text{Na}^+/\text{I}^-$  symporter (NIS). Implications for its secondary structure model. *J. Biol. Chem.* **273**:22657–22663
- Ling, V., Kartner, N., Sudo, T., Siminovitch, L., Riordan, J.R. 1983. Multidrug-resistance phenotype in Chinese hamster ovary cells. *Cancer Treat. Rep.* **67**:869–874
- Loo, T.W., Clarke, D.M. 1994. Prolonged association of temperature-sensitive mutants of human P-glycoprotein with calnexin during biogenesis. *J. Biol. Chem.* **269**:28683–28689
- Loo, T.W., Clarke, D.M. 1997. Correction of defective protein kinesis of human P-glycoprotein mutants by substrates and modulators. *J. Biol. Chem.* **272**:709–712
- Loo, T.W., Clarke, D.M. 1998a. Quality control by proteases in the endoplasmic reticulum. Removal of a protease-sensitive site enhances expression of human P-glycoprotein. *J. Biol. Chem.* **273**:32373–32376
- Loo, T.W., Clarke, D.M. 1998b. Superfolding of the partially unfolded core-glycosylated intermediate of human P-glycoprotein into the mature enzyme is promoted by substrate-induced transmembrane domain interactions. *J. Biol. Chem.* **273**:14671–14674
- Mechetner, E.B., Schott, B., Morse, B.S., Stein, W.D., Druley, T., Davis, K.A., Tsuruo, T., Roninson, I.B. 1997. P-glycoprotein function involves conformational transitions detectable by differential immunoreactivity. *Proc. Natl. Acad. Sci. USA* **94**:12908–12913
- Melikian, H.E., Ramamoorthy, S., Tate, C., Blakely, R. 1996. Inability to N-glycosylate the human norepinephrine transporter reduces protein stability, surface trafficking, and transport activity but not ligand recognition. *Mol. Pharmacol.* **50**:266–276
- Olivares, L., Aragon, C., Gimenez, C., Zafra, F. 1995. The role of N-glycosylation in the targeting and activity of the GLYT1 glycine transporter. *J. Biol. Chem.* **270**:9437–9442
- Ramachandra, M., Ambudkar, S.V., Chen, D., Hrycyna, C.A., Dey, S., Gottesman, M.M., Pastan, I. 1998. Human P-glycoprotein exhibits reduced affinity for substrates during a catalytic transition state. *Biochemistry* **37**:5010–5019
- Ramachandra, M., Ambudkar, S.V., Gottesman, M.M., Pastan, I., Hrycyna, C.A. 1996. Functional characterization of a glycine 185-to-valine substitution in human P-glycoprotein by using a vaccinia-based transient expression system. *Mol. Biol. Cell* **7**:1485–1498
- Richert, N.D., Aldwin, L., Nitecki, D., Gottesman, M.M., Pastan, I. 1988. Stability and covalent modification of P-glycoprotein in multidrug-resistant KB cells. *Biochemistry* **27**:7607–7613
- Ruetz, S., Raymond, M., Gros, P. 1993. Functional expression of P-glycoprotein encoded by the mouse *mdr3* gene in yeast cells. *Proc. Natl. Acad. Sci. USA* **90**:11588–11592
- Sarkadi, B., Price, E.M., Boucher, R.C., Germann, U.A., Scarborough, G.A. 1992. Expression of the human multidrug resistance cDNA in insect cells generates a high activity drug-stimulated membrane ATPase. *J. Biol. Chem.* **267**:4854–4858
- Schinkel, A.H., Kemp, S., Dolle, M., Rudenko, G., Wagenaar, E. 1993. N-glycosylation and deletion mutants of the human P-glycoprotein. *J. Biol. Chem.* **268**:7474–7481
- Senior, A.E., Al-Shawi, M.K., Urbatsch, I.L. 1995. The catalytic cycle of P-glycoprotein. *FEBS Lett.* **377**:285–289
- Urbatsch, I.L., Sankaran, B., Weber, J., Senior, A.E. 1995. P-glycoprotein is stably inhibited by vanadate-induced trapping of nucleotide at a single catalytic site. *J. Biol. Chem.* **270**:19383–19390