Characterization of Phosphorylation-defective Mutants of Human P-glycoprotein Expressed in Mammalian Cells*

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To assess the role of phosphorylation of the human multidrug resistance MDR1 gene product P-glycoprotein for its drug transport activity, phosphorylation sites within its linker region were subjected to mutational analysis. We constructed a 5A mutant, in which serines at positions 661, 667, 671, 675, and 683 were replaced by nonphosphorylatable alanine residues, and a 5D mutant carrying aspartic acid residues at the respective positions to mimic permanently phosphorylated serine residues. Transfection studies revealed that both mutants were targeted properly to the cell surface and conferred multidrug resistance by diminishing drug accumulation. In contrast to wild-type P-glycoprotein, the overexpressed 5A and the 5D mutants exhibited no detectable levels of phosphorylation, either in vivo following metabolic labeling of cells with [32P]orthophosphate or in vitro in phosphorylation assays with protein kinase C, cAMP-dependent protein kinase, or a P-glycoprotein-specific protein kinase purified from multidrug-resistant KB-V1 cells. These results reconfirm that the major P-glycoprotein phosphorylation sites are located within the linker region. Furthermore, the first direct evidence is provided that phosphorylation/dephosphorylation mechanisms do not play an essential role in the establishment of the multidrug resistance phenotype mediated by human P-glycoprotein.

Multidrug resistance (MDR) is a major impediment to effective cancer chemotherapy. In many human cancer cells, cross-resistance to a variety of natural product cytotoxic drugs is associated with the overexpression of the multidrug resistance MDR1 gene that encodes P-glycoprotein (reviewed in Refs. 1–3). Highly homologous mdr or pgp genes have also been identified in rodents (reviewed in Refs. 1–3). Based on the amino acid sequence deduced from the MDR1 cDNA sequence, P-glycoprotein is predicted to consist of two similar halves, each of which contains a transmembrane domain and a nucleotide binding fold (4, 5). These structural elements identify the MDR1 gene product as a member of the superfamily of ATP-binding cassette transporters, which includes the cystic fibrosis transmembrane conductance regulator, and many other membrane-associated proteins from eukaryotic and prokaryotic origin (reviewed in Ref. 6). Gene transfer experiments involving MDR1 cDNA have corroborated that expression of P-glycoprotein is sufficient to endow drug-sensitive cells with multidrug resistance (reviewed in 2, 3). P-glycoprotein is an integral plasma membrane protein that functions as an energy-dependent drug efflux pump to reduce the intracellular accumulation of cytotoxic agents (reviewed in Refs. 1–3). P-glycoprotein interacts directly with a variety of anticancer drugs and transports them across the plasma membrane lipid bilayer. P-glycoprotein exhibits a substrate-stimulated ATPase activity (7, 8), suggesting that ATP hydrolysis may provide the energy required for the drug transport mechanism (reviewed in Refs. 1–3).

P-glycoprotein was described as a phosphoglycoprotein (9, 10), and several studies have corroborated that both native and recombinant P-glycoproteins are phosphorylated in vivo (11–20). Numerous studies have been conducted to address the importance of phosphorylation for the multidrug transporter activity of P-glycoprotein. Many multidrug-resistant cell lines were shown to express elevated levels of protein kinases, in particular protein kinase C (21–24) and changes in levels and/or activities of protein kinases, such as protein kinase C or cAMP-dependent protein kinase (protein kinase A) have been suggested to play a role in modulating levels of multidrug resistance mediated by P-glycoprotein. Attempts have been made to correlate the degree of phosphorylation of P-glycoprotein with its drug efflux activity (Refs. 11, 13, 16, 22, 25, and 26; reviewed in Ref. 27). Generally an increase in protein kinase activity and/or phosphorylation of P-glycoprotein has been associated with increased levels of multidrug resistance. Many of these studies involved the use of activators and/or inhibitors of protein kinases to modulate the state of phosphorylation of P-glycoprotein, but these regulatory molecules are not very specific and often cause multiple cellular effects. For example, several protein kinase inhibitors including staurosporine and derivatives thereof, calphostin C, or certain isoquinolinesulfonamide derivatives may directly interact with P-glycoprotein and affect its drug efflux activity independent of, or in addition to, their effects on P-glycoprotein phosphorylation (28–31). Various protein kinase agonists (e.g. 12-O-tetradecanoylphorbol-13-acetate or diacylglycerol) and antagonists (e.g. stauro-
tein has been demonstrated to be phosphorylated (37). Similarly, the linker region of the mouse P-glycoprotein mRNA (37). Three of these four serine residues appear to be phosphorylated (37). The two homologous halves of P-glycoprotein. This region, commonly referred to as the linker region, is characterized by a high content of charged amino acids (approximately 30–40%) and contains several consensus sequences for phosphorylation by protein kinases requiring basic amino acid residues near the phosphoacceptor group (e.g. protein kinase C, protein kinase A). A cluster of four serine residues was shown to be phosphorylated in vitro by protein kinase C (Ser-661, Ser-667, Ser-671) and/or protein kinase A (Ser-667, Ser-671, and Ser-683) (36, 37). Three of these four serine residues appear to be phosphorylated in vivo, namely Ser-661, Ser-667, and Ser-671 (26, 36, 37). Similarly, the linker region of the mouse mdrl P-glycoprotein has been demonstrated to be phosphorylated in vitro at analogous serine residues, namely Ser-669 by protein kinase C and Ser-681 by protein kinase A (38). Several protein kinase C and/or protein kinase A consensus phosphorylation sites are also present in the linker region of the mouse mdrl and the hamster pgl P-glycoproteins, but the actual sites of phosphorylation have not yet been described. In analogy with the R domain between the two halves of the cystic fibrosis transmembrane conductance regulator, a target for multisite phosphorylation by protein kinase A believed to regulate the CAMP-dependent cystic fibrosis transmembrane conductance regulator chloride channel activity, it has been hypothesized that the phosphorylatable linker region of P-glycoprotein might be a regulatory domain that controls its drug transport function (36, 38).

The identification of the major sites of phosphorylation provides an opportunity to use site-directed mutagenesis to address the role of phosphorylation of P-glycoprotein for its drug efflux activity. Our approach was to substitute five consensus sites for phosphorylation by protein kinase C (Ser-661, Ser-667, Ser-671, Ser-675, Ser-683) within the linker region of P-glycoprotein by nonphosphorylatable alanine residues (5A mutant), or by aspartic acid residues to mimic permanently phosphorylated serine-like residues (5D mutant). The 5A and 5D mutants of P-glycoprotein were tested for their ability to confer multidrug resistance to drug-sensitive cells and were characterized for drug-binding capacity and state of phosphorylation.

### MATERIALS AND METHODS

#### Genetic Engineering of Expression Vectors for Mutants of P-glycoprotein

**To facilitate the construction of P-glycoprotein phosphorylation mutants, two unique restriction sites, Clal and Xbal, were introduced into the nucleotide sequence of the human MDR1 cDNA open reading frame at positions 1941 and 2060, flanking the sequences encoding the linker region.** The Clal and Xbal sites were created by a T→C transition at position 1943 and a G→A transition at position 2063. These changes in the nucleotide did not affect the encoded amino acid sequence. Three MDR1 cDNA fragments, an Apal-ClaI fragment encompassing nucleotides 1586–1946, a Clal-Xbal fragment encompassing nucleotides 1941–2065, and an Xbal-Apssl78 fragment encompassing nucleotides 2060–2780 were generated by the polymerase chain reaction using pMDR2000XS (39) as a template. Each of these three fragments was subcloned individually into the appropriate restriction sites of a pGem2-derived vector pEA CX 11, and the correctness of their placement was confirmed. Then the three subcloned PCR fragments were ligated together and inserted to replace the native Apal-Apssl78 MDR1 cDNA fragment in pSx-MDR1/A-wt, a pGem2-derived plasmid carrying the full-length MDR1 cDNA isolated from the multidrug-resistant KB-V1 cell line (40) as an Sstl-Xbal fragment. This control plasmid was named pSx-MDR1/A-wt-CX and contains novel Clal and Xbal restriction sites.

**For constructing P-glycoprotein variants in which five serine residues at positions Ser-661, Ser-667, Ser-671, Ser-675, and Ser-683 were replaced with either alanine or aspartic acid, modified Clal-Xbal fragments were generated by chemical synthesis of a series of eight oligodeoxynucleotide and overlapping oligodeoxynucleotides. Oligodeoxynucleotides UAG-114 (5'-CGATGCCCTGTACAA-3'), UAG-115 (5'-TCATTTGAGACGATTCTAAGG-3'), UAG-92 (5'-GTTCCTCAATATGTTCAAGTCCGCTCTA-3'), UAG-93 (5'-TCTTCTTTCTATATTAGGGCAGTCATGGAACAAAG-3'), UAG-94 (5'-ATAAGAAAAGAAGACCTCGTAGGGGTCTGCTCGAGCA-3'), UAG-95 (5'-GTCTTGGCCTGTGCTCCACGGACAGCCCTCAGGTTGC-3'), UAG-122 (5'-CAAGCCGCAAGAGAAGCTGTACAAAGAGGCT-3'), and UAG-123 (5'-CTAGAGCCCCCTTTGGTACAGGTTCTC-3') were designed for the 5A mutant, and UAG-114, UAG-115, UAG-116 (5'-GTCTTCAATAGTCAAGATCCGCTCTA-3'), UAG-117 (5'-TCTTTCTTTATATTAGCGGCTCTGGA-3'), UAG-94 (5'-ATAAGAAAAGAAGACCTCGTAGGGGTCTGCTCGAGCA-3'), UAG-95 (5'-GTCTTGGCCTGTGCTCCACGGACAGCCCTCAGGTTGC-3'), UAG-122 (5'-CAAGCCGCAAGAGAAGCTGTACAAAGAGGCT-3'), and UAG-123 (5'-CTAGAGCCCCCTTTGGTACAGGTTCTC-3') phosphorylated at the 5' end using T4 polynucleotide kinase according to standard procedures (41). Eight identical amounts of eight oligodeoxynucleotides were annealed in the presence of 10 mM MgCl₂ by heating to 80 °C and slow cooling to room temperature. Subsequently, the annealed oligodeoxynucleotides were introduced into Clal and Xbal double-digested pSx-MDR1/A-wt-CX, and their DNA sequences were confirmed. Finally, the wild type and two mutant MDR1 cDNAs were isolated as Sstl-Xhol fragments and placed under control of Harvey murine sarcoma virus long terminal repeat sequences in the pCO1 retroviral vector (42), to give the expression vectors pHA-MDR1/A-wt-CX (wild-type control), pHA-MDR1/A-wt-5A (encoding mutant carrying five alanine residues at positions 661, 667, 671, 675, and 683), and pHA-MDR1/A-wt-5D (encoding mutant carrying five aspartic acid residues at positions 661, 667, 671, 675, and 683).

**Cell Transfections, Drug Selection of Sublines, Colony Formation, and Drug Accumulation Assays—** Murine NIH 3T3 fibroblasts and human KB-3-1 carcinoma cells were maintained as monolayer cultures at 37 °C in 5% CO₂ using Dulbecco's modified Eagle's medium supplemented with 4.5 g/liter glucose, 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% (v/v) calf serum (NIH 3T3 cells) or 10% (v/v) fetal bovine serum (KB-3-1 cells). 100,000 NIH 3T3 or 50,000 KB-3-1 cells each were transfected with the pMDR1/A-wt-CX, pMDR1/A-wt-5A, pMDR1/A-wt-5D expression plasmids (10 μg each) as described previously (43). Stable transfectants were selected for drug resistance in the presence of vincristine (NIH 3T3 cells at 30 ng/ml, KB-3-1 cells at 3 ng/ml, or colchicine (NIH 3T3 cells at 60 ng/ml, KB-3-1 cells at 6 ng/ml). Mass populations of highly drug-resistant transfectants were selected in stepwise increasing concentrations of vincristine as follows. Approximately 200,000 cells were seeded per 10-cm dish, and increasing amounts of vincristine were added. Cells were grown for 7–14 days until colonies were visible to the eye. The highest vincristine concentration survived by all three different transfectants was chosen to adapt cells to these concentrations during two passages. Then the next step of selection was initiated as described above. Retrospectively, adaptation concentrations of vincristine for drug selection were 180 ng/ml, 600 ng/ml, and 2400 ng/ml for NIH 3T3 sublines, and 18 ng/ml, 48 ng/ml, and 300 ng/ml for KB-3-1 sublines.

**Drug resistance profiles of the NIH 3T3 and KB-3-1 parental cell lines and vincristine-selected transfectants were determined by measuring cell survival in colony formation assays as described (44). Average cloning efficiencies for these assays were 10–20% for NIH 3T3 sublines and approximately 50% for KB sublines. Drug accumulation assays were performed as described previously (26).**

Fluorescence-activated Cell Sorting (FACS) Analysis—Subconfluent cells from a 10-cm dish were harvested by trypsinization into phos-
Acid residues to mimic permanently phosphorylated serine-like residues. Serine residues are replaced by nonphosphorylatable alanine residues, whereas the 5D mutant encoded by pHaMDR1/A-wt-5D contains aspartic acid residues within the linker region (circled). In vivo phosphorilation of the linker region of human P-glycoprotein encoded by amino acids 633 and 692. Arrows point to serine residues targeted by site-directed mutagenesis.

Fig. 1. Design of mutants of P-glycoprotein with substitutions in linker region. A, the P-glycoprotein polypeptide chain consisting of 1280 amino acids is schematically represented as a line. Bars 1-12 refer to predicted transmembrane regions, and nucleotide binding folds (NB) are circled. The wild-type (WT) P-glycoprotein encoded by pHaMDR1/A-wt-CX harbors a cluster of five putative phosphorylation sites including Ser-661, Ser-667, Ser-671, Ser-675, and Ser-683 within the linker region (enlarged insert). In the 5A mutant encoded by pHaMDR1/A-wt-5A these serine residues are replaced by nonphosphorylatable alanine residues, whereas the 5D mutant encoded by pHaMDR1/A-wt-5D contains aspartic acid residues to mimic permanently phosphorylated serine-like residues. B, amino acid sequence of the linker region of human P-glycoprotein between amino acids 633 and 692. Enhanced chemiluminescence (ECL) as described by the manufacturer (Amersham).
with Koden XAR5 film at -70°C. The duplicate gel was transferred to nitrocellulose, and P-glycoprotein was detected by immuno-staining with C219 monoclonal antibody as described above.

RESULTS

Construction of 5A and 5D Mutant MDR1 cDNA Expression Vectors—Oligodeoxynucleotide-mediated mutagenesis was used to alter the major putative phosphorylation sites within the linker region of the human MDR1 gene product (Fig. 1). Five clustered serine residues at positions 661, 667, 671, 675, and 683, all representing protein kinase C and/or protein kinase A consensus sequences, were targeted for substitution with either nonphosphorylatable alanine residues or aspartic residues to mimic permanently phosphorylated serine-like residues. Four of these five targeted serine residues (Ser-661, Ser-671, Ser-683) have previously been demonstrated to be phosphorylated in vitro by protein kinase C and/or protein kinase A (36, 37).

To facilitate the construction of these mutations within the MDR1 cDNA, two unique Clal and XbaI restriction sites, flanking the linker region that encodes the phosphorylation site cluster, were introduced without affecting the encoded amino acid sequence. Wild-type and 5A and 5D mutant Clal-XbaI linker subfragments were generated by gene synthesis, reintroduced into full-length MDR1 cDNA, and cloned into a pCO1-derived retroviral expression vector under the control of long terminal repeats (LTRs) of the Harvey murine sarcoma virus. The three different retroviral expression constructs, termed pHaMDR1A-wt-CX, pHaMDR1A-wt-5A, and pHaMDR1A-wt-5D, encode the wild-type human MDR1 gene product with five serine residues at positions 661, 667, 671, 675, and 683 or two mutant forms of P-glycoprotein with five alanine (5A mutant) or aspartic acid residues (5D mutant) at the respective positions.

Biological Activity of 5A and 5D Mutants of P-glycoprotein—The pHaMDR1A-wt-CX, pHaMDR1A-wt-5A, and pHaMDR1A-wt-5D expression vectors were first tested for their ability to confer drug resistance to drug-sensitive murine NIH 3T3 or human KB-3-1 host cells upon transfection. Initial selection of transfected cell populations in the presence of cytotoxic concentrations of vincristine or colchicine indicated that all three constructs encode a functional multidrug transporter (Table 1). Similar numbers of vincristine-resistant colonies were obtained for all wild-type and mutant constructs, but the numbers of colchicine-resistant colonies were somewhat lower for pHaMDR1A-wt-5D and even more so for pHaMDR1A-wt-5A in NIH 3T3 host cells, when compared with the pHaMDR1A-wt-CX control.

Mass populations of stably transfected, drug-selected cells were grown in the presence of a stepwise increasing amounts of vincristine to achieve high levels of expression of the wild-type and mutant forms of P-glycoprotein, to compare relative resistance to different drugs of populations of transfectants, and for biochemical analysis. Generally, selection for cells exhibiting enhanced drug resistance was achieved easily for all three different types of transfectants. The pHaMDR1A-wt-5A transfectants were rate-limiting for all steps of selection of the NIH 3T3 sublines but not for all steps of selection of the KB-3-1 sublines. The concentration of vincristine in the growth medium was raised three times for adaptation of NIH 3T3 transfectants from 30 to 180 ng/ml, and then to 600 ng/ml, and finally to 2400 ng/ml. Similarly, highly drug-resistant KB-3-1 transfectants were selected in four steps by adaptation at 3 ng/ml, 18 ng/ml, 48 ng/ml, and 300 ng/ml vincristine.

Enhanced vincristine resistance of subpopulations is usually paralleled by an increase in the levels of expression of transfected MDR1 gene products. Western blot analysis of whole cell extracts was used to determine the overall P-glycoprotein content in various transfected sublines. As shown in Fig. 2, both the 5A and 5D mutant P-glycoproteins were found to exhibit electrophoretic mobility comparable with that of the wild type, and both 5A and 5D mutants were recognized by the C219 monoclonal antibody, which binds to an epitope near the nucleotide binding regions of P-glycoprotein (49). Fig. 2A reveals that the increased resistance of NIH 3T3 transfectants from 30 ng/ml to 2400 ng/ml vincristine was accompanied by a dramatic and comparable increase in levels of expression of wild-type or mutant P-glycoproteins. The increase was estimated to be

Table 1

Transfection efficiencies of MDR1 phosphorylation mutants

| Transfected DNA       | NIH 3T3 drug selection | KB-3-1 drug selection |
|-----------------------|------------------------|-----------------------|
|                       | Vincristine (30 ng/ml) | Colchicine (60 ng/ml) | Vincristine (3 ng/ml) | Colchicine (6 ng/ml) |
| None                  | 1                      | <1                    | 0                     | <1                    |
| Vector control        | <1                     | 0                     | 0                     | <1                    |
| pHaMDR1A-wt-CX        | 480                    | 156                   | 288                   | 138                   |
| pHaMDR1A-wt-5A        | 360                    | 7                     | 280                   | 58                    |
| pHaMDR1A-wt-5D        | 480                    | 86                    | 208                   | 94                    |

Fig. 1. Expression of 5A and 5D mutants in stably transfected, vincristine-selected NIH 3T3 cells. Cellular extracts or crude membranes were prepared from parental NIH 3T3 cells (lane 1), pHaMDR1A-wt-CX-transfectants (lanes 2 and 5), pHaMDR1A-wt-5A-transfectants (lanes 3 and 6), and pHaMDR1A-wt-5D-transfectants (lanes 4 and 7) that were selected in the presence of 30 ng/ml (N3V30, N4V30, N5V30) or 2400 ng/ml (N3V2400, N4V2400, N5V2400) vincristine. A, total cellular proteins were size-fractionated by SDS-polyacrylamide gel electrophoresis and analyzed by Western blot using P-glycoprotein-specific C219 monoclonal antibody as described under “Materials and Methods.” B, crude membranes were prepared from the same cell lines and stained with Coomassie Blue after separation by SDS-polyacrylamide gel electrophoresis. Arrows indicate wild-type 5A and 5D mutant P-glycoproteins, and sizes of molecular mass markers are given in kDa.
100-fold based on densitometry scanning analysis. The NIH 3T3 populations maintained at 2400 ng/ml vincristine harboring pHaMDR1/A-wt-CX (N3V2400) cells, pHaMDR1/A-wt-5A (N4V2400) cells, and pHaMDR1/A-wt-5D (N5V2400) cells appeared to contain similar amounts of transfected MDR1 gene product based on the relative intensities of the immunoreactive bands. The levels of the transfected wild-type and mutant P-glycoproteins were high enough to allow detection by Coomassie Blue staining after SDS-polyacrylamide gel electrophoresis of crude membrane preparations of N3V2400, N4V2400, and N5V2400 cells (Fig. 2B). Cell populations grown at intermediate concentrations of vincristine contained intermediate levels of transfected MDR1 gene products according to their relative vincristine resistance (data not shown). Similar data were also obtained for the KB-3-1 transfectants, but overall levels of wild-type and mutant P-glycoprotein expression in vincristine-selected sublines were lower than in NIH 3T3 transfectants (data not shown). Generally, recombinant wild-type and mutant human P-glycoproteins expressed in murine NIH 3T3 transfectants had increased electrophoretic mobility.

![Graphs of cell surface expression of 5A and 5D mutants of P-glycoprotein](image)

**Fig. 3.** Cell surface expression of 5A and 5D mutants of P-glycoprotein. Parental NIH 3T3 cells, control KB-3-1 and KB-V1 cells, and NIH 3T3 transfectants expressing wild-type P-glycoprotein (N3V30, N3V2400), 5A mutant (N4V30, N4V2400), or 5D mutant (N5V30, N5V2400) were subjected to FACS analysis after staining with monoclonal human P-glycoprotein-specific antibody MRK-16 or IgG2a isotype control and FITC-labeled anti-mouse IgG2a antibody as described under "Materials and Methods." Transfectants were maintained at 30 ng/ml (N3V30, N4V30, N5V30) or 2400 ng/ml vincristine (N3V2400, N4V2400, N5V2400).
compared with P-glycoproteins expressed in human KB-3-1 transfectants, due to altered carbohydrate content.\textsuperscript{3}

A total of four different P-glycoprotein-specific detection reagents were used for Western blot analyses of N3V30, N4V30, N5V30, N3V2400, N4V2400, and N5V2400 cells, including C219 monoclonal antibody (49), 4007 antiserum (45), PEPG2 antiserum (46), and PEPG13 antiserum (46), and they all gave comparable results (Fig. 2, data not shown). The PEPG13 antiserum is specific for the human MDR\textsubscript{1} gene product and does not cross-react with human MDR\textsubscript{2} or mouse P-glycoproteins.\textsuperscript{4} Thus, these results indicate that the levels of the transfected wild-type or mutant human P-glycoproteins, but not the endogenous mouse P-glycoproteins, were increased by the vincristine selection of the various NIH 3T3 transfectants. Southern blot hybridizations of genomic DNAs isolated from NIH 3T3, N3V30, N4V30, N5V30, N3V2400, N4V2400, and N5V2400 cells with the pHDR.5 probe (50) indicate that drug selection of the transfectants was accompanied by amplification of the transfected human MDR\textsubscript{1} cDNAs (data not shown). Washes at low stringency did not indicate amplification of endogenous mouse mdr genes (data not shown).

FACS analysis of NIH 3T3 transfectants (Fig. 3, data not shown) and KB-3-1 transfectants (data not shown) using the human P-glycoprotein-specific monoclonal antibody MRK16 or an IgG2a isotype control, and an FITC-labeled anti-mouse IgG2a secondary antibody, confirmed the Western blot findings described above and indicated proper targeting of the 5A and 5D mutants to the cell surface. A comparable increase in cell surface fluorescence intensity of MRK16 signals was observed when increasingly resistant cell populations were investigated. Thus, similarly elevated levels of expression of the wild-type human P-glycoprotein and the 5A and 5D mutants at the cell surface of murine NIH 3T3 and human KB-3-1 transfectants correlated with their increased resistance to vincristine.

Colony formation assays in the presence of vinblastine, colchicine, and adriamycin were performed to demonstrate that the vincristine-selected pHaMDR1A-wt-CX-, pHaMDR1A-wt-5A and pHaMDR1A-wt-5D transfectants were cross-resistant to various cytotoxic drugs. As demonstrated by the killing curves in Fig. 4, both 5A and 5D mutants conferred multidrug resistance to the transfected cells, similar to wild-type P-glycoprotein. For all vincristine-selected transfectants, the rela-

\textsuperscript{4} U. A. Germann and S. V. Ambudkar, unpublished results.

\textbf{Fig. 4.} Drug survival characteristics of cell populations expressing 5A and 5D-mutants of P-glycoprotein. Colony formation assays were performed as described under “Materials and Methods” to determine the drug sensitivity of parental NIH 3T3 cells (dashed lines with diamonds) and of drug-selected NIH 3T3 transfectants maintained at a concentration of 30 ng/ml (open symbols) or 2400 ng/ml vincristine (filled symbols) that express wild-type P-glycoprotein (circles, N3V30 or N3V2400 cells), 5A mutant of P-glycoprotein (squares, N4V30 or N4V2400 cells), or 5D mutant of P-glycoprotein (triangles, N5V30 or N5V2400 cells). Drug survival was measured in increasing concentrations of vinblastine (A), colchicine (B), or adriamycin (C).

\textbf{Fig. 5.} \([\text{H}]\text{Azidopine photoaffinity labeling of 5A and 5D mutants of P-glycoprotein.} \) Crude membranes (100 \(\mu\text{g of protein) from KB-V1 multidrug-resistant cells (V1, lanes 1 and 2), NIH 3T3 parental cells (3T3, lane 3), N3V2400 cells expressing wild-type P-glycoprotein (WT, lanes 4 and 5), N4V2400 cells expressing 5A mutant (5A, lanes 6 and 7), or N5V2400 cells expressing 5D mutant (5D, lanes 8 and 9) were labeled with 0.4 \(\mu\text{M[\text{H}]azidopine in the absence (lanes 1, 3, 4, 6, and 8) or presence (lanes 2, 5, 7, and 9) of 100 \mu\text{M vinblastine (VBL) as described by Bruggemann et al. (47). Arrows indicate wild-type and 5A and 5D mutant P-glycoproteins, and sizes of molecular mass markers are given in kDa.} \)
resistance to a variety of hydrophobic cytotoxic agents. P-glycoprotein, both 5A and 5D mutants conferred simultaneously 100–150-fold resistance. Similar data were obtained for cells maintained at the highest concentration of 2400 ng/ml when compared with the parental NIH 3T3 cell line, whereas effectants (N3V30, N4V30, N5V30 cells) was less than 10-fold relative resistance to vinblastine of the initially selected transfected NIH 3T3 cells (3T3, lane 1), N3V2400 cells expressing wild-type P-glycoprotein (WT, lane 2), N4V2400 cells expressing 5A mutant (5A, lane 3), N5V2400 cells expressing 5D mutant (5D, lane 4), drug-sensitive KB-3-1 cells (3-1, lane 5), and multidrug-resistant KB-V1 cells (V1, lane 6) were metabolically labeled with $^{32}$P orthophosphate (0.1 mCi/ml) for 4.5 h, and P-glycoprotein was immunoprecipitated with PEG13 antiserum as described under “Materials and Methods.” A $^{32}$P labeling of P-glycoprotein was visualized by autoradiography; exposure was for 24 h with an intensifying screen. A longer exposure of 5 days confirmed essentially no radioactive labeling of P-glycoprotein 5A and 5D mutants in lanes 3 and 4. B, P-glycoprotein was detected following SDS-polyacrylamide gel electrophoresis by immunoblotting with P-glycoprotein-specific C219 monoclonal antibody as described under “Materials and Methods.” Arrows indicate wild-type and 5A and 5D mutant P-glycoproteins, and sizes of molecular mass markers are given in kDa.

**FIG. 6.** Phosphorylation analysis of 5A and 5D mutants in intact cells in comparison with wild-type human P-glycoprotein. Parental NIH 3T3 cells (3T3, lane 1), N3V2400 cells expressing wild-type P-glycoprotein (WT, lane 2), N4V2400 cells expressing 5A mutant (5A, lane 3), N5V2400 cells expressing 5D mutant (5D, lane 4), drug-sensitive KB-3-1 cells (3-1, lane 5), and multidrug-resistant KB-V1 cells (V1, lane 6) were metabolically labeled with $^{32}$P orthophosphate (0.1 mCi/ml) for 4.5 h, and P-glycoprotein was immunoprecipitated with PEG13 antiserum as described under “Materials and Methods.” A $^{32}$P labeling of P-glycoprotein was visualized by autoradiography; exposure was for 24 h with an intensifying screen. A longer exposure of 5 days confirmed essentially no radioactive labeling of P-glycoprotein 5A and 5D mutants in lanes 3 and 4. B, P-glycoprotein was detected following SDS-polyacrylamide gel electrophoresis by immunoblotting with P-glycoprotein-specific C219 monoclonal antibody as described under “Materials and Methods.” Arrows indicate wild-type and 5A and 5D mutant P-glycoproteins, and sizes of molecular mass markers are given in kDa.

**FIG. 7.** In vitro phosphorylation analysis of 5A and 5D mutants and wild-type P-glycoprotein. Crude membrane preparations from N3V2400 cells expressing wild-type P-glycoprotein (WT, lanes 1–3), N4V2400 cells expressing 5A mutant (5A, lanes 4–6), or N5V2400 cells expressing 5D mutant (lanes 7–9) were phosphorylated in vitro by protein kinase C (lanes 2, 5, and 8) or V-1 kinase (lanes 3, 6, and 9) as described under “Materials and Methods.” P-glycoprotein was immunoprecipitated with PEG13 antiserum and analyzed by SDS-polyacrylamide gel electrophoresis. Transfer to nitrocellulose, and autoradiography (exposure 24 h). Lanes 1, 4, and 7 show membranes from N3V2400, N4V2400, and N5V2400 cells, respectively, incubated in reaction mixtures omitting protein kinases. Arrows indicate wild-type and 5A and 5D mutant P-glycoproteins, and sizes of molecular mass markers are given in kDa.

Drug-binding Activity of 5A and 5D Mutants of P-glycoprotein—Photoaffinity labeling experiments were carried out to characterize the drug-binding capacity of the 5A and 5D mutant P-glycoproteins in more detail. These studies were performed with N3V2400, N4V2400, and N5V2400 sublines because of their high and similar expression of the transfected MDR1 gene products. As shown in Fig. 5, both 5A and 5D mutants were labeled specifically with $[^3]$H]azidopine, with efficiencies similar to wild-type P-glycoprotein. A 250-fold excess of nonradioactive vinblastine displaced the $[^3]$H]azidopine label completely from all three different forms of P-glycoprotein analyzed, indicating that both 5A and 5D mutants were capable of specific interactions with cytotoxic drug substrates.

State of Phosphorylation of 5A and 5D Mutants of P-glycoprotein—in the 5A and 5D mutants, five consensus sites of phosphorylation by protein kinase C and/or protein kinase A including the previously identified major phosphorylation sites of P-glycoprotein were substituted with the nonphosphorylatable amino acid residue alanine or aspartic acid. To exclude the presence of cytoxic phosphorylation sites within the mutant P-glycoprotein polypeptide chains, it was important to evaluate the state of phosphorylation of the 5A and 5D mutants by comparing it with that of wild-type P-glycoprotein. The N3V2400, N4V2400, and N5V2400 sublines were chosen for both in vivo and in vitro phosphorylation experiments, because they were the cell lines that expressed the highest and similar levels of transfected MDR1 gene products (Fig. 2). Phosphorylation analysis was studied in intact cells by measuring incorporation of $[^32]$P orthophosphate into the proteins. Immunoprecipitates were prepared from extracts of metabolically $[^32]$P-labeled cells using PEG13 antiserum. This polyclonal antiserum directed against amino acids 592–636 of the human P-glycoprotein was incubated in reaction mixtures omitting protein kinases. Arrows indicate wild-type and 5A and 5D mutant P-glycoproteins, and sizes of molecular mass markers are given in kDa.

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imately equal amounts of the various forms of human P-glycoprotein. These data suggest that the five serine residues that were substituted with alanine or aspartic acid in the 5A and 5D mutants include all major phosphorylation sites of human P-glycoprotein. No major cryptic phosphorylation site(s) was detectable in either the 5A or the 5D mutant of P-glycoprotein.

Additional in vitro phosphorylation studies were conducted using crude membrane preparations from N3V2400, N4V2400, and N5V2400 cells. As shown in Fig. 7, wild-type P-glycoprotein, but not the 5A or the 5D mutant, was phosphorylated by protein kinase C as well as by a novel, membrane-bound protein kinase (V-1 kinase) isolated from KB-V1 cells. Similar data were obtained using protein kinase A (data not shown). We also did not detect phosphorylation of the 5A and 5D mutant P-glycoproteins after immunoprecipitation with the monoclonal antibody C219 known to cross-react with all human and rodent P-glycoproteins (49). These results confirm earlier findings (see above and Fig. 3) indicating that only human P-glycoproteins (wild type or mutants) are overexpressed in the transfectants. No significant incorporation of radioactive phosphate was detected for the 5A and 5D mutants of P-glycoprotein, even in the presence of vanadate, a general inhibitor of phosphatases and ATPases (data not shown), confirming the results from the in vivo phosphorylation experiments described above.

Taken together, these data suggest that the major sites for in vitro and in vivo phosphorylation of P-glycoprotein are absent in the 5A and 5D mutants due to substitution by nonphosphorylable alanine or aspartic acid residues. Clearly, the analyzed phosphorylation- and dephosphorylation-defective mutants of P-glycoprotein confer multidrug resistance to drug-sensitive cells with efficiency comparable with the wild-type P-glycoprotein and have similar drug-binding capacity. Although we cannot exclude completely the possibility that the 5A and 5D mutants are phosphorylated at very low levels below detection limits, our data infer that phosphorylation of the major sites in the linker region is not essential for the drug efflux activity of human P-glycoprotein. Multidrug resistance can be mediated by P-glycoprotein in the absence of phosphorylation, as well as in a state mimicking stable phosphorylation, compensated by negatively charged residues.

**DISCUSSION**

We have employed site-directed mutagenesis of predicted phosphorylation sites within the linker region of human P-glycoprotein to assess the importance of phosphorylation for its multidrug transport function and found that the activity of P-glycoprotein is not affected by its state of phosphorylation. Previously, analyzing P-glycoprotein in human KB-V1 cells, a total of three serine residues were found to be phosphorylated in vivo (26). Subsequent in vitro analyses identified these as Ser-661, Ser-667, and Ser-671 (36, 37). Our mutational analysis targeted these three predicted phosphorylation sites within the P-glycoprotein linker region as well as Ser-675 and Ser-683. The latter serine residue was included because it was found to be phosphorylated by protein kinase A in vitro (37). Ser-675 was mutagenized because it represents a nearby minimal consensus site for phosphorylation by protein kinase C that may become modified in the absence of the major phosphorylation sites. Two types of mutants were constructed: a 5A mutant, in which the five serine residues were replaced by nonphosphorylatable alanine residues, and a 5D mutant with five aspartic acid residues at the respective positions to mimic permanently phosphorylated serine-like residues. Stable transfection experiments with murine NIH 3T3 and human KB-3-1 cells demonstrated that both the 5A and 5D mutants of P-glycoprotein are expressed at the cell surface and endow the drug-sensitive host cells with resistance to a variety of cytotoxic drugs including Vinca alkaloids, anthracyclines, and colchicine. Subpopulations overexpressing high levels of transfected wild-type or mutant P-glycoproteins were selected to facilitate biochemical analyses. Highly drug-resistant murine transfecants were found to specifically overexpress the transfected MDR1 gene products, and there was no evidence for increased expression of endogenous mouse P-glycoproteins that could account for increased levels of multidrug resistance. In contrast to wild-type P-glycoprotein, no significant levels of phosphorylation were detected for the 5A and 5D mutants by phosphorylation studies performed in vitro and in vivo, despite their high levels of expression. Thus, in agreement with the biochemical data reported by Chambers et al. (36, 37) our mutational analysis confirmed that all the detectable phosphorylation sites are located in the linker region of human P-glycoprotein.

Interestingly, our phosphorylation studies performed with the 5A and 5D mutants did not indicate any other major cryptic phosphorylation site(s) present within or outside of the P-glycoprotein linker region, although the possibility of nondeletable minor phosphorylation sites cannot be completely excluded. Studies with additional P-glycoprotein mutants containing four or fewer alanine or aspartic acid substitutions at positions 661, 667, 671, 675, and 683 are ongoing to determine the exact in vivo phosphorylation sites by mutational analysis. As there is no evidence for any phosphorylatable residue(s) within the 5A and 5D mutants, they should serve as useful tools to reevaluate the effects of protein kinase activators and inhibitors on levels of multidrug resistance. They may also help to clarify the mechanisms of action of protein kinase modulators.

The 5A and 5D mutants mimic a phosphorylation-deficient and permanently phosphorylated P-glycoprotein, respectively. We found that both the 5A and 5D mutants of P-glycoprotein confer multidrug resistance to drug-sensitive cells. Our data imply that phosphorylation and dephosphorylation of P-glycoprotein is not essential for its drug efflux activity. A similar conclusion was implied in earlier work by Buschman and Gros (51), who showed that a chimeric mouse mdr1-P-glycoprotein containing the mouse mdr2 linker region was functional as a drug transporter. Since the mouse mdr2 linker region lacks the classic RXRS recognition sequence that is present in the mdr1 gene product, the argument was made that phosphorylation of this site was dispensable, at least for the multidrug transport activity of mouse P-glycoprotein (51).

Recently, a study involving site-directed mutagenesis of eight serine/threonine consensus sites for protein kinase C in the linker region of human P-glycoprotein has suggested that protein kinase C-mediated phosphorylation of P-glycoprotein may function to regulate the activity of an endogenous chloride channel (52). Despite the fact that confirmatory phosphorylation studies of the P-glycoprotein mutants were not performed, the eight sites mutated included the five sites targeted by us. Therefore, the P-glycoprotein mutants studied by Hardy et al. (52) were most likely phosphorylation-defective. Thus, one possible role for P-glycoprotein phosphorylation may be to affect indirectly the activity of another protein or proteins.

Our data suggest that phosphorylation and dephosphorylation of P-glycoprotein may not be essential for its overall ability to interact with different cytotoxic agents. It is possible, however, that phosphorylation of P-glycoprotein may contribute to regulation of its drug substrate specificity, as has been suggested by Bates et al. (11). Although there is no obvious pattern that correlates the state of phosphorylation mimicked by the 5A and 5D mutants with levels of resistance to a certain drug, it is interesting to note that both mutants in comparison
with wild-type P-glycoprotein appear to have a somewhat re-
duced capacity to confer resistance to colchicine, and adriamy-
cin, but not Vinca alkaloids. Our results from both the initial
drug selection of transfected cell populations and the drug
resistance profiles of the highly drug-resistant transfectants
indicated this tendency. Additional studies with donated sub-
lines and P-glycoprotein mutants in which not all phosphoryl-
sites are mutagenized should address a possible role of
phosphorylation in regulating substrate specificity.

Phosphorylation of P-glycoprotein has also been suggested to
influence the kinetics (velocity) of drug transport (25) or to
modulate multidrug resistance by affecting the half-life of P-
glycoprotein (34). The 5A and 5D mutants of P-glycoprotein
represent good models to address these issues. Ultimately,
experiments involving functional reconstitution of purified
wild-type and phosphorylation- or dephosphorylation-defective
mutants of P-glycoprotein in phospholipid vesicles may es-

Phosphorylation mechanism. One intriguing idea, however, is
that phosphorylation of P-glycoprotein may be less important
influencing the kinetics of drug transport (25) or to
influence the kinetics (velocity) of drug transport (25) or to
regulate the transport function of the multi-
drug transporter may be regulated by a phosphorylation/de-
phosphorylation mechanism. One intriguing idea, however, is
that phosphorylation of P-glycoprotein may be less important
for drug efflux than for the regulation of the transport of yet
to be determined physiologic substrate(s).

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