Alteration of Substrate Specificity by Mutations at the His[61] Position in Predicted Transmembrane Domain 1 of Human MDR1/P-Glycoprotein[†]

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ABSTRACT: In CFTR, a member of the ABC superfamily and a chloride channel, amino acid substitutions in its transmembrane domains 1 and 6 (TM1, TM6) have been reported to modulate the anion selectivity or ion conductance of the ion channel. In P-glycoprotein, no amino acid substitution in TM1, but some in TM6, have been reported to modify the substrate specificity of this protein. In this work, we demonstrated the involvement of His[61], which is in the middle of the predicted TM1, in the function of P-glycoprotein. His[61] was replaced by all other amino acid residues, and each of the mutant cDNAs was introduced into drug-sensitive human carcinoma cells, KB3-1. The drug-resistance profile of cells stably expressing each mutated P-glycoprotein was investigated by comparing their relative resistance to vinblastine, colchicine, VP16, and adriamycin. The resistance to vinblastine was increased by replacing His[61] by amino acids with smaller side chains, while it was lowered by replacing by amino acids with bulkier side chains. The reverse effect was observed for resistance to colchicine and VP16. The resistance to adriamycin was increased by replacing by amino acids with bulkier side chains except Lys or Arg, which have a basic side chain. We also showed that the replacement of His[61] by Phe and Lys greatly impaired the efflux of calcein AM, while the replacement had no effect on the efflux of rhodamine 123. These results suggest that an amino acid residue at position 61 in TM1 is important in deciding the substrate specificity of P-glycoprotein.

P-glycoprotein functions as an ATP-dependent efflux pump that transports cytotoxic drugs out of the cells before the drugs reach their intracellular targets, so conferring multidrug resistance on the cells (Chen et al., 1986; Endicott & Ling, 1989; Gottesman & Pastan, 1993; Roninson, 1991). P-glycoprotein transports many structurally dissimilar cytotoxic drugs that act on different intracellular targets, such as the *Vinca* alkaloids, colchicine, actinomycin D, epipodophyllotoxins, paclitaxel, and anthracyclines. P-glycoprotein transports not only cytotoxic drugs but also calcium channel blockers (Saeki et al., 1993b), immunosuppresive agents (Saeki et al., 1993a), steroid hormones (Ueda et al., 1992), peptide antibiotics (Kino et al., 1996; Ueda et al., 1993, 1996) and others (Hirai et al., 1995; Okamura et al., 1993; Tanigawara et al., 1992).

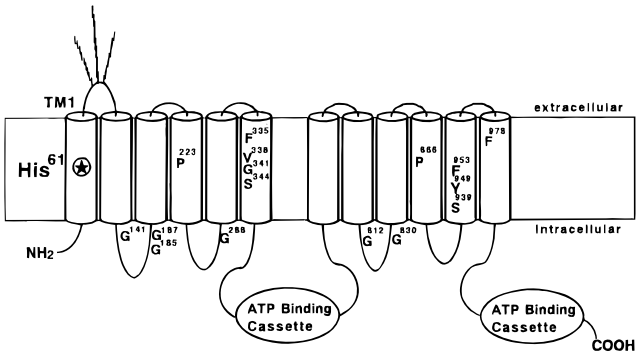

FIGURE 1: Location of His[61] and amino acid residues in P-glycoprotein that were reported to alter the substrate specificity by mutation (Choi et al., 1988; Hanna et al., 1996; Kajiji et al., 1993; Kioka et al., 1989; Loo & Clarke, 1993a,b, 1994a,b). The structural model is based on those proposed by Juranka *et al*. (Juranka et al., 1989) and Gottesman and Pastan (Gottesman & Pastan, 1988). Position numbers of amino acid residues are of human MDR1/P-glycoprotein except for Ser[939], Tyr[949], and Phe[953], which are of mouse mdr1a/P-glycoprotein. In hamster pgp1, a double mutation in TM6 (Gly[338] and Ala[339]) was also reported to alter the substrate specificity (Devine et al., 1992) (not shown in this figure).

The mutations to amino acids that modulate substrate specificity have been located in TM4[1] (Loo & Clarke, 1993a), TM6 (Devine et al., 1992; Loo & Clarke, 1993b, 1994a), TM10 (Loo & Clarke, 1993a), TM11 (Gros et al., 1991; Hanna et al., 1996; Kajiji et al., 1993), TM12 (Loo & Clarke, 1993b), and the first (Choi et al., 1988; Kioka et al., 1989; Loo & Clarke, 1994b), second, and fourth cytoplasmic loops (Loo & Clarke, 1994b) (Figure 1). The residues located in these transmembrane domains have been considered to be involved in the binding and transporting mecha-

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[1] Abbreviations: TM, transmembrane domain; CFTR, cystic fibrosis transmembrane conductance regulator; His, histidine; Ala, alanine; Cys, cysteine; Asp, aspartic acid; Glu, glutamic acid; Phe, phenylalanine; Gly, glycine; Ile, isoleucine; Lys, lysine; Leu, leucine; Met, methionine; Asn, asparagine; Pro, proline; Gln, glutamine; Arg, arginine; Ser, serine; Thr, threonine; Val, valine; Trp, tryptophan; Tyr, tyrosine; Vbl, vinblastine; Col, colchicine; Adr, adriamycin; calcein AM, acetoxymethyl ester derivative of calcein.

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nisms of P-glycoprotein. However, the mechanism by which P-glycoprotein recognizes such structurally diverse compounds remains unclear.

CFTR (cystic fibrosis transmembrane conductance regulator) (Riordan et al., 1989), which functions as a chloride channel and malfunctions in cystic fibrosis (Riordan, 1993; Welsh et al., 1992), belongs to the ABC (ATP Binding Cassette) superfamily transporters, as P-glycoprotein (Hyde et al., 1990). The predicted topology of it, with the exception of the R domain, resembles that of P-glycoprotein. In this molecule, amino acid substitutions in TM1 and TM6 are found to modulate the anion selectivity or ion conductance of the ion channel (Akabas et al., 1994; Anderson et al., 1991; Sheppard et al., 1993; Tabcharani et al., 1993). Especially, three amino acids residues in TM1 of CFTR may be in the channel lining (Akabas et al., 1994). On the other hand, no amino acid substitution in TM1, but some in TM6, of P-glycoprotein has been reported to modify the function of this protein (Loo & Clarke, 1993b, 1994a).

We assumed that CFTR and P-glycoprotein might take similar tertiary configurations and expected that some amino acids in TM1 of P-glycoprotein would be directly involved in the substrate recognition. During our preliminary mutational analysis of TM1 of P-glycoprotein, we found that mutations of His^{61} alter the drug resistance pattern of P-glycoprotein (data not shown). In this work, we comprehensively examined the involvement of His^{61} in the functions of P-glycoprotein.

EXPERIMENTAL PROCEDURES

Materials. Vinblastine (Vbl), colchicine (Col), and adriamycin (Adr) were purchased from Wako Pure Chemical Industries, Ltd. VP16 (etoposide) and rhodamine 123 were from Sigma. Calcein AM was purchased from Molecular Probes. Monoclonal antibody C219 was from Centocor.

Construction of an Expression Vector. The human MDR1 cDNA ($Gly^{185} \rightarrow Val$) in pHaMAIRESneo (Metz et al., 1996) was replaced by the wild-type MDR1 cDNA isolated from a normal adrenal gland (Kioka et al., 1989). In this expression vector, the MDR1 cDNA was fused to the aminoglycosidase phosphotransferase (neomycin resistance: neo^R) gene with an encephalomyocarditis virus 5′ untranslated sequence, known as an internal ribosome entry site (IRES), which made it possible to coexpress the human MDR1 cDNA using cap-dependent translation initiation and the neo^R gene using cap-independent translation initiation from a bicistronic transcript (Aran et al., 1994).

Site-Directed Mutagenesis. Oligonucleotides were synthesized to generate the appropriate substitutions. The manufacturer's mutagenesis procedure (Sculptor in vitro mutagenesis system, Amersham) was used to replace histidine at position 61. Mutations were confirmed by DNA sequencing.

Transfection and Drug Resistance Assay. Human cultured cells KB3-1 were propagated in Dulbecco's modified Eagle's medium with 10% fetal bovine serum under 5% CO_2 at 37 °C. KB3-1 was transfected by MDR1 expression vectors and its mutated variants with LipofectAMINE (Gibco) according to the manufacturer's directions. Cells were first selected in the presence of 0.8 mg/mL geneticin (G418) for 10 days. the mass populations of geneticin-resistant colonies obtained were selected by Vbl (5 ng/mL) or Col (10 ng/mL). Cells transfected with an expression vector having the wild-type or every mutant cDNA yielded resistant colonies

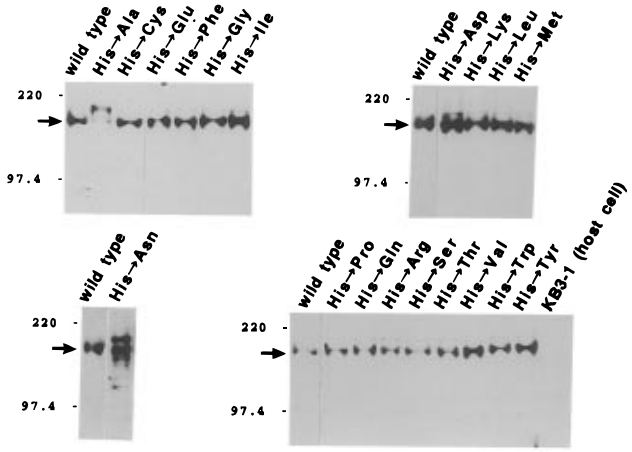

FIGURE 2: Immunoblots of membrane proteins from cells stably expressing wild-type and mutant P-glycoproteins. Membrane proteins were obtained from stable transformants maintained in 20 ng/mL vinblastine for mutants $His^{61} \rightarrow$ Phe, Met, Lys, Arg, Trp, or 30 ng/mL vinblastine for wild-type and the other mutants. Twenty micrograms of membrane proteins were resolved on a 7% SDS−PAGE gel and reacted with monoclonal antibody C219 as a probe for P-glycoprotein. The arrow indicates the position of the 170-kDa mature form of P-glycoprotein.

in the presence of Vbl and Col. However, the ratios of Vbl- and Col-resistant colonies emerging from cells transfected with some mutant P-glycoprotein cDNAs were different from that from cells transfected with the wild-type P-glycoprotein cDNA (data not shown). Then, we obtained cells expressing a readily detectable amount of mutant P-glycoprotein by further selection with stepwise increasing concentrations of Vbl. Cells transfected by $His^{61} \rightarrow$ Phe, Lys, Met, Arg, or Trp cDNA, which yielded more Col-resistant colonies than Vbl-resistant colonies, were selected with stepwise increasing concentrations (10, 20, 30 ng/mL) of Vbl and finally maintained in 20 ng/mL Vbl. Cells transfected with wild-type and the other mutant cDNAs, which yielded more Vbl-resistant colonies than Col-resistant colonies, were selected with stepwise increasing concentrations (10, 20, 40 ng/mL) of Vbl and finally maintained in 30 ng/mL Vbl. The IC_{50} (the drug concentration that inhibits cell growth by 50%) was measured by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from dose−response curves for increasing concentrations of Vbl, Col (0−300 ng/mL), VP16 (0−6 mg/mL), and adriamycin (0−900 ng/mL). Relative resistance to Vbl and Col of cells stably expressing mutant P-glycoproteins described below were as expected from the ratio of Vbl- and Col-resistant colonies emerging from geneticin-resistant cells, suggesting that the stepwise selection with Vbl did not alter the resistance profiles conferred by mutant P-glycoproteins.

Immunoblotting. Membrane proteins were prepared as described previously (Ueda et al., 1992) and resolved by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) on 7% gels. Immunoblotting was done as described previously (Ueda et al., 1992). Relative amounts of P-glycoprotein were measured by the density of the immunoreactive bands with a densitometer (Pharmacia LKB Ultro Scan XL) ($n = 2-4$).

Digital Fluorescence Microscopy. Each transformant and KB3-1 cells grown on glass coverslips in 3.5-cm dishes (Meridian P35GC) were placed on the stage (kept at 37 °C) of an inverted microscope (Zeiss Axiovert 100). Fluorescence images were collected by an intensified CCD videocamera (Attofluor). Images were digitized and stored every 1 and 5 min after the addition of calcein AM and rhodamine

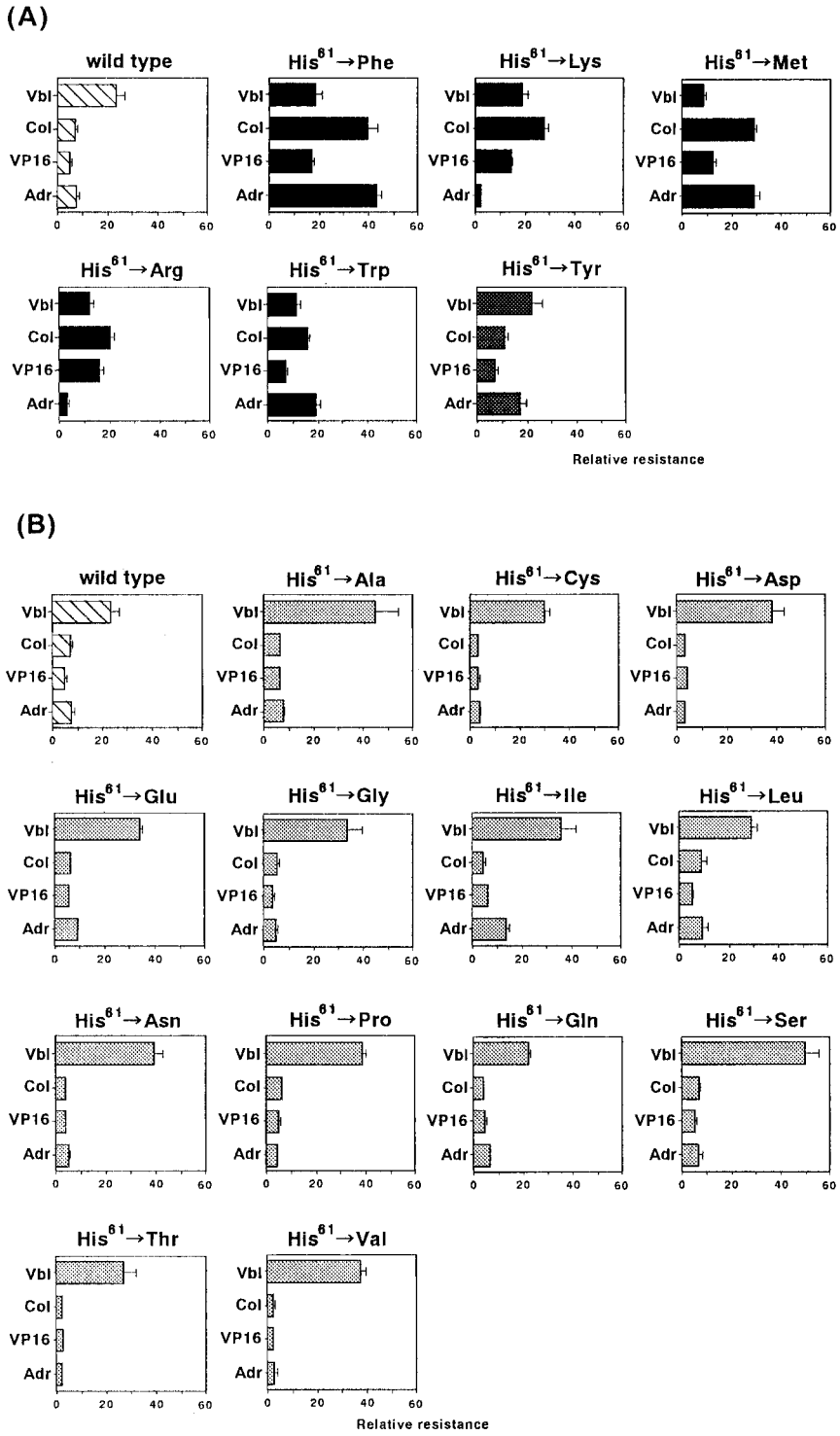

FIGURE 3: The drug resistance profiles of cells stably expressing wild-type or mutant P-glycoprotein in which His[61] was replaced by amino acids with bulkier side chains than His (A), and those of cells stably expressing wild-type or mutant P-glycoprotein in which His[61] was replaced by amino acids with smaller side chains than His (B). Relative resistance was calculated by comparing the IC_{50} (the drug concentration necessary to inhibit cell growth by 50%) for each stably transfected cells expressing the wild-type or mutant P-glycoprotein to the IC_{50} of the host cell KB3-1 and was then divided by the relative amount of P-glycoprotein in 20 μg of membrane protein. The relative amount of P-glycoprotein was measured as described in Experimental Procedures, and the amount of the wild-type P-glycoprotein was arbitrarily assigned a value of 1. Each value is the mean of three separate experiments. The IC_{50} for KB3-1 was 1.57 ng/mL for vinblastine (Vbl), 5.06 ng/mL for colchicine (Col), 173.6 ng/mL for VP16, and 9.35 ng/mL for adriamycin (Adr).

123, respectively. Fluorescence of individual cells were analyzed by an image-processing system (Ratiovision, Atto Instruments, Rockville, MD).

RESULTS

To investigate the involvement of His[61] in the function of P-glycoprotein, His[61] was changed to 19 other amino acids. Each mutant or wild-type MDR1 cDNA was introduced into the drug-sensitive human carcinoma cells KB3-1 with an expression vector, in which the MDR1 cDNA was fused to the neo^R gene with a IRES. Cells were first selected in the presence of 0.8 mg/mL geneticin (G418) for 10 days, and then with stepwise increasing concentrations of vinblastine (Vbl) as described in Experimental Procedures, to obtain cells

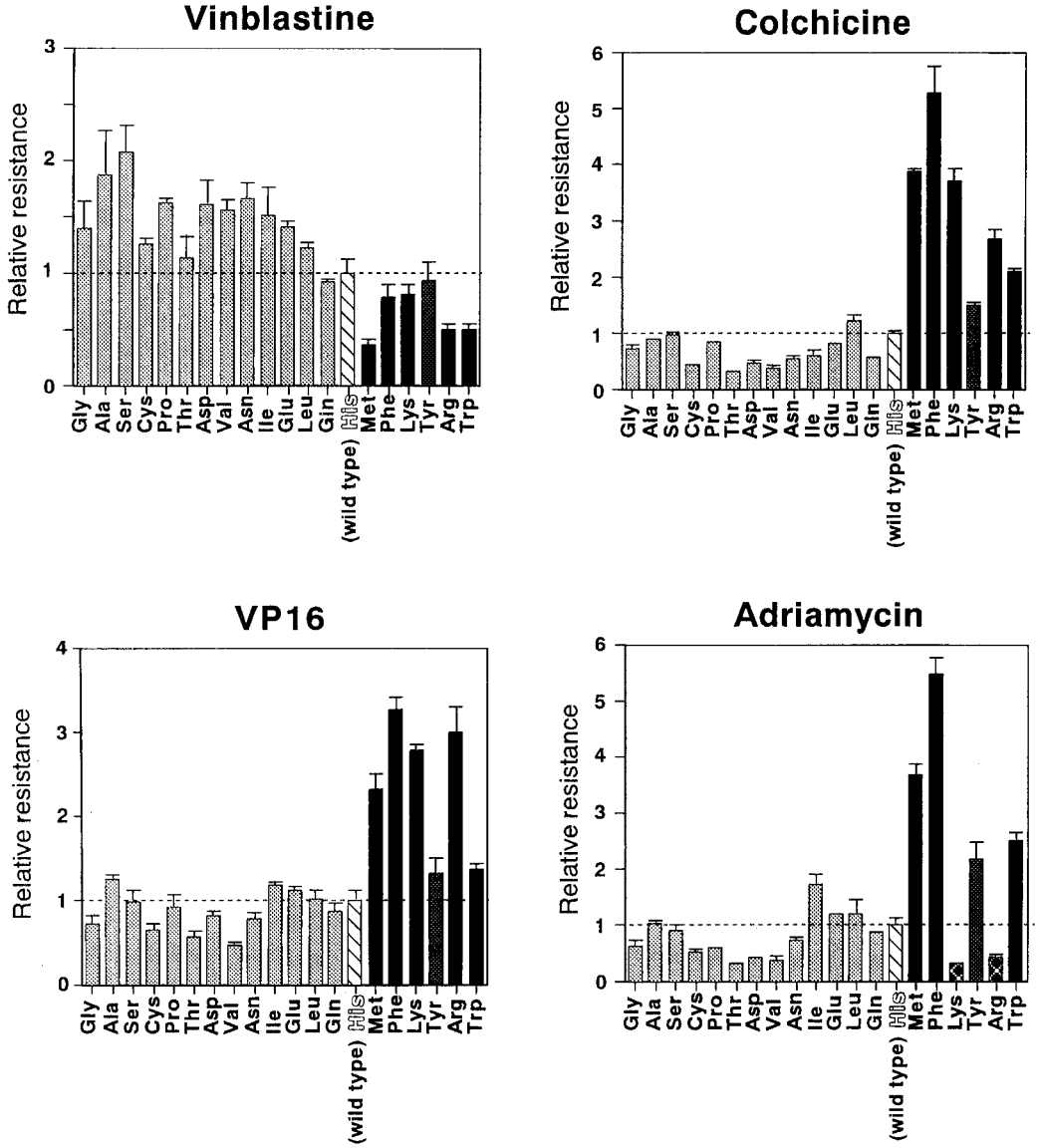

FIGURE 4: The relative ability of His^{61} mutants to confer resistance to each drug, Vbl, Col, VP16, and Adr. The results are presented relative to the ability to confer resistance to each drug of the wild-type P-glycoprotein, which is arbitrarily assigned a value of 1. Mutants are aligned in order of surface area of amino acids (Rose et al., 1985) at position 61.

expressing a readily detectable amount of mutant P-glycoprotein. All of the mutant and wild-type P-glycoproteins expressed in transfected cells were detected as the 170-kDa mature form with the anti-P-glycoprotein monoclonal antibody C219 (Figure 2), suggesting normal processing of all the mutant P-glycoproteins into the plasma membrane. The expression levels of the mutant proteins in each of the transfectants were not identical, but similar. The difference in the level of expression of the wild-type and the mutant P-glycoproteins was within a 2-fold limit. KB3-1 host cells did not express the endogenous MDR1 gene, or the transcriptional activation of the endogenous MDR1 gene did not occur even when the cells were maintained in the medium containing Vbl or colchicine (Col) for several months, because the sequence of the wild-type P-glycoprotein was not found in the RT-PCR products from these cells (data not shown). Hence the drug-resistance profiles of the stable mutant cell lines are considered to represent substrate specificities of the transfected mutant P-glycoproteins.

The drug-resistance profiles of the cells expressing mutant P-glycoproteins were investigated by comparing their relative resistance to Vbl, Col, VP16, and adriamycin (Adr). Cells expressing the wild-type P-glycoprotein showed 23.8 ± 3.1-fold resistance to Vbl above that of host cells, KB3-1. These cells were also resistant to Col (7.6 ± 0.5-fold), VP16 (5.4 ± 0.5-fold), and Adr (7.9 ± 1.0-fold), resulting in a resistance order of Vbl > Adr ≈ Col > VP16. Because there was some variability in the level of expression of wild-type and mutant P-glycoproteins (Figure 2), the relative resistance of each transformant was compared after standardizing to the level of P-glycoprotein expression (Figure 3).

The most drastic alterations in the drug resistance profile were observed with His^{61} → Phe, Lys, Met, Arg, and Trp mutants, in which His^{61} is replaced by amino acids having bulkier side chains than His (Figure 3A). The replacement of His^{61} by Phe, Met, or Trp, which has a nonpolar and bulkier side chain than His, reduced resistance to Vbl and increased resistance to Col, Adr, and VP16, resulting in a resistance order of Adr ≈ Col > Vbl ≈ VP16. The replacement of His^{61} by Lys or Arg, which have a bulkier and basic side chain, reduced resistance to Vbl and Adr and increased resistance to Col and VP16, resulting in a resistance order of Col > VP16 ≈ Vbl > Adr. Cells expressing His^{61} → Tyr, which also has a bulkier side chain than His, had increased resistance to Adr and slightly increased resistance to Col and VP16 (Figure 3A). The replacement of His^{61} by

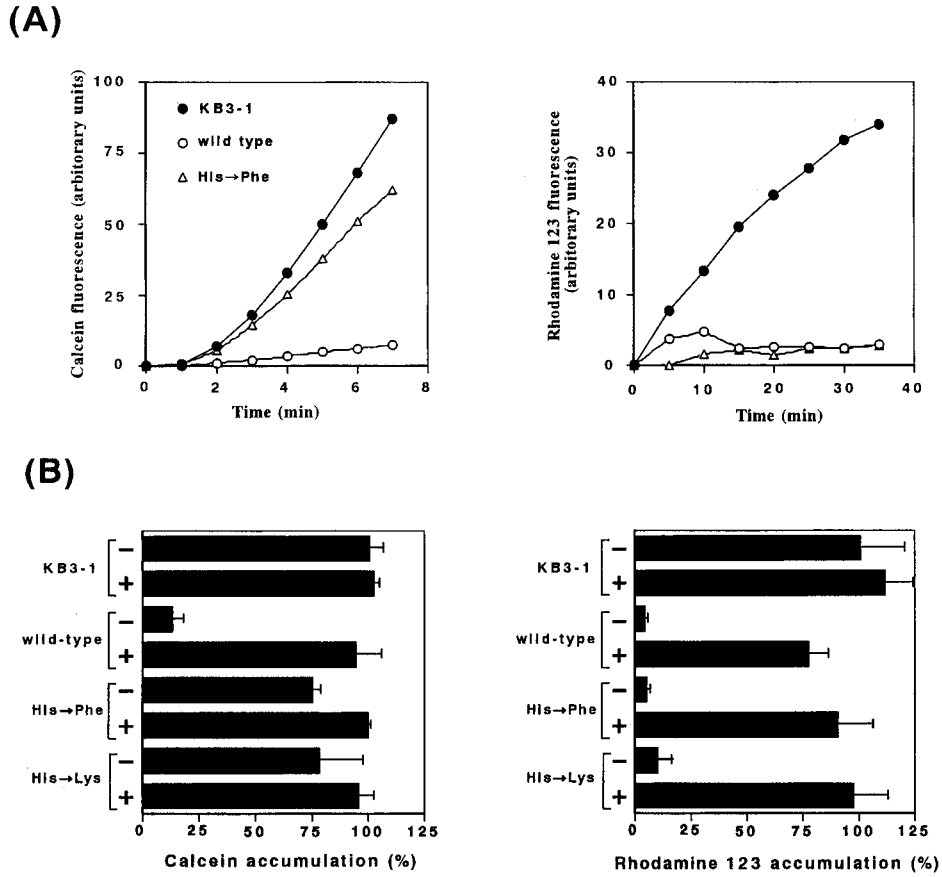

FIGURE 5: Calcein AM and rhodamine 123 uptake into KB3-1 and the transformant cells expressing wild-type or mutant (His[61] → Phe or Lys) P-glycoprotein. (A) Time dependence of accumulation of calcein, produced with cytoplasmic nonspecific esterases from calcein AM, and rhodamine 123 into KB3-1, transformants expressing the wild-type P-glycoprotein, or His[61] → Phe mutant P-glycoprotein. Cells were incubated in HBSS containing 0.1% glucose and 2.5 μM calcein AM (left panel) or 2.5 μM rhodamine 123 (right panel). The plots show calcein or rhodamine 123 fluorescence (in arbitary units) against time. Closed circle, KB3-1; open circle, cells expressing the wild-type P-glycoprotein; open triangle, cells expressing His[61] → Phe mutant P-glycoprotein. Each point is an average of 20 cells. Experiments were done at least three times. Data are from representative experiments. (B) The relative amount of accumulation of fluorescent indicators into KB3-1 or the transformants expressing wild-type or mutant P-glycoprotein. Cells were incubated in HBSS containing 0.1% glucose and 2.5 μM calcein AM for 15 min (left panel), or 2.5 μM rhodamine 123 for 120 min (right panel) in the absence (−) or presence (+) of 100 μM verapamil, and accumulations of fluorescence in 20 individual cells were measured. Each value is the mean and SD of three separate experiments. The results are presented relative to the accumulation of fluorescence into KB3-1 cells in absence of verapamil.

amino acids with smaller side chains than His increased resistance to Vbl and decreased resistance to Col, VP16, and Adr, in general (Figure 3B). Especially, cells expressing His[61] → Val and Thr mutant P-glycoproteins reduced the ability to confer resistance to Col, VP16, and Adr.

Figure 4 shows the standardized relative resistance of the transformants to each drug. Replacement of His[61] by amino acids with small side chains increased resistance to Vbl (MW: 811), which is one of the largest substrates for P-glycoprotein. Replacement by amino acids with large side chains generally increased resistance to the rather small substrates Col, VP16, and Adr, the molecular weights of which are 399, 588, and 580, respectively. These results suggest that the bulkiness of a side chain of the amino acid at position 61 is an important factor which decides the substrate specificity of this protein. However, the resistance to Adr of cells expressing mutants His[61] → Lys or Arg was specifically low, less than half of cells expressing the wild-type P-glycoprotein, among cells expressing mutants with side chains bulkier than His at position 61.

To determine whether the amino acid substitutions at position 61 have influence on the transport activity of P-glycoprotein, we compared the accumulation of two fluorescent indicators, calcein AM (MW: 995) and rhodamine 123 (MW: 381), in the cells expressing wild-type or mutant (His[61] → Phe or Lys) P-glycoprotein (Figure 5). Rhodamine 123 or fluorescent calcein, produced with cytoplasmic nonspecific esterases from calcein AM, accumulated in KB3-1 cells depending on time. However, these fluorescent indicators scarcely accumulated in cells expressing the wild-type P-glycoprotein. Cells expressing mutant His[61] → Phe or Lys accumulated about 70% of the calcein compared to KB3-1, while they scarcely accumulated rhodamine 123. In the presence of verapamil, a competitive inhibitor of P-glycoprotein, cells expressing the wild-type or mutant P-glycoprotein accumulated both fluorescent indicators as much as KB3-1 cells did. These results indicate that the reduced accumulation of these compounds were due to the efflux from cells by P-glycoprotein and that the rate of hydrolysis of calcein AM, which may affect the rate of calcein accumulation, is the same in the wild-type transfectants and in the mutants. These results, all together, suggest that amino acid substitutions at position 61 to Phe and Lys, which caused drastic changes in the multidrug resistance pattern, reduced the activity of transporting calcein AM, which is one of the largest substrates for P-glycoprotein, while these mutant P-glycoproteins transported rhodamine 123, which is one of the smallest substrates for P-glycoprotein, as efficiently as the wild-type P-glycoprotein.

DISCUSSION

In this work, the involvement of His^{61} in the function of P-glycoprotein was examined. All of the examined mutant P-glycoproteins were expressed as a mature form (Figure 2) and were properly sorted to the plasma membrane, which was detected by immunostaining (data not shown). Furthermore all of them bound azidopine efficiently (data not shown) and were functional as drug transporters. These results were rather surprising, because it was suggested that mutations to Gly^{54}, Ala^{58}, and Gly^{62} in TM1 affected the proper folding and altered overall activity of P-glycoprotein (Loo & Clarke, 1996). These results, all together, suggest that although TM1 of P-glycoprotein is important for the formation of a functional configuration, amino acid substitutions at position 61 do not cause a major configurational alteration in P-glycoprotein; hence the amino acid residue at position 61 is considered not to be involved in the formation of the framework of P-glycoprotein, such as in the interaction between transmembrane α-helices. Instead, the replacements of His^{61} by other amino acids altered the drug resistance profile of P-glycoprotein. The mutations $His^{61} \rightarrow$ Phe and Lys impaired the efflux of calcein AM, while they did not affect the efflux of rhodamine 123. These results suggest that the amino acid at position 61 is important in the transport of substrates of P-glycoprotein and especially is involved in deciding the substrate specificity.

The size of the side chain of this residue is suggested to be an important factor that determined the substrate specificity. It appears that, as far as the drugs we examined, there is an inverse relationship between the size of the side chain of the amino acid at position 61 and the molecular weight of preferred substrates. These results suggest that the amino acid residue at position 61 or the region containing this residue plays a key role in deciding the preferential molecular size of the substrates, which have been suggested to be one of the important determinants for substrates to interact effectively with P-glycoprotein (Tang-Wai et al., 1993; Zamora et al., 1988). An attractive hypothesis is that this amino acid residue is involved in the formation of the substrate recognition site. If this is a case, the small side chain would make a large binding pocket fit for large substrates, while the large side chain would make a small binding pocket fit for small substrates. However, we cannot eliminate the possibility that substitutions of this residue cause allosteric effects and cause subtle structural changes in the protein at a distance.

The ability to confer resistance to Adr of mutant P-glycoproteins showed a characteristic dependence on the nature of the amino acids: although cells expressing mutants in which His^{61} was replaced by amino acids with side chains larger than His showed higher (more than 2-fold than that of the wild-type) resistance to Adr, cells expressing mutants in which His^{61} was replaced by Lys or Arg showed markedly lower resistance to Adr. This is most likely caused by the electrostatic force of repulsion between the positive charge on the bulky side chain of these amino acids and the positively charged aminosugar group of Adr. These results may support the idea that the amino acid residue at position 61 directly interacts with substrates.

The comprehensive nature of the experiments of this study showed rather directly that mutation of a single residue in P-glycoprotein can alter the pattern of drug resistance, increasing resistance to some drugs and decreasing resistance to others, whether or not P-glycoprotein expressing cells have been exposed to cytotoxic drugs. This provides a fairly direct refutation of the hypothesis that P-glycoprotein acts not as an ATP driven drug-efflux pump but rather by altering intracellular or intraorganellar pH and $\Delta\phi$, which indirectly alters the translocation and efflux of cationic drugs (Hoffman et al., 1996; Simon et al., 1994). Their hypothesis provides no explanation for the changing pattern of specificity to individual drugs observed in this study.

In CFTR, amino acid residues in TM1 may be involved in forming part of the channel lining (Akabas et al., 1994). We recently found that mutations at positions 64 and 65 have affects similar to mutation at position 61 (manuscript in preparation). If we assume that TM1 takes an α-helical conformation according to the predicted model for secondary structure of human P-glycoprotein (Gottesman & Pastan, 1988; Juranka et al., 1989), three amino acids His^{61}, Gly^{64}, and Leu^{65}, mutations of which have similar effect on the substrate specificity, would form an narrow region on an α-helix arrangement of TM1. It is intriguing that a region in TM1, not only in CFTR but also in P-glycoprotein, is important in substrate specificity.

In summary, the results of this study shed light on a role of TM1 in the substrate specificity of human P-glycoprotein. TM1 of CFTR is assumed to be involved in ion selectivity. This study suggests that P-glycoprotein and CFTR, both members of the ABC superfamily of transporters, might share a similar functional structure in spite of the difference in their functions as a transporter and an ion channel.

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