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Amino acid substitutions in the first transmembrane domain (TM1) of P-glycoprotein that alter substrate specificity

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Abstract Recently, we showed that the amino acid at position 61 in TM1 of human P-glycoprotein is important in deciding the substrate specificity of this protein. In this work, we investigated whether the amino acids other than His^{61} in TM1 of P-glycoprotein are also essential in the function of this protein. Nine amino acids residues, from Ala^{57} to Leu^{65} in TM1, were independently substituted to Arg, and analyzed the drug resistance of cells stably expressing each of these mutant P-glycoproteins. The mutant P-glycoproteins $Ile^{60} \rightarrow Arg$, $His^{61} \rightarrow Arg$, $Ala^{63} \rightarrow Arg$, $Gly^{64} \rightarrow Arg$, and $Leu^{65} \rightarrow Arg$ were normally processed and expressed in the plasma membrane. Substrate specificities of mutant P-glycoproteins $Gly^{64} \rightarrow Arg$ and $Leu^{65} \rightarrow Arg$ were quite different from that of the wild type, and similar to that of the $His^{61} \rightarrow Arg$ mutant, while the $Ile^{60} \rightarrow Arg$ and $Ala^{63} \rightarrow Arg$ mutant P-glycoproteins showed similar substrate specificities to that of the wild-type P-glycoprotein, suggesting that not only the amino acid residue at position 61 but also those at position 64 and 65 are also important in deciding the substrate specificity of P-glycoprotein. These three amino acids His^{61}, Gly^{64}, and Leu^{65} would form a compact region on an α-helix arrangement of TM1. These results suggest that a region consisting of His^{61}, Gly^{64}, and Leu^{65} in TM1 would participate in the formation of the recognition site for substrates of P-glycoprotein.

Key words: P-Glycoprotein; Multidrug resistance; Substrate specificity; ABC transporter

1. Introduction

P-Glycoprotein, a member of ABC superfamily of transporters, is an active transporter which utilizes the energy of ATP hydrolysis to pump cytotoxic drugs out of cells, [1,2]. P-Glycoprotein can recognize and transport so broad range of structurally dissimilar compounds that its overexpression confers cells multidrug resistance. However, the mechanism by which P-glycoprotein can recognize such structurally diverse compounds remains to be elucidated. To improve our understanding of the molecular mechanism of P-glycoprotein, it is necessary to identify amino acid residues involved in the recognition and transport of its substrates. A number of amino acid that modulate substrate specificity of P-glycoprotein have been identified. They have been located in transmembrane domain (TM) 4 [3], TM6 [4–6], TM10 [3], TM11 [7–9], TM12 [5], and the first [10–12], second, and fourth cytoplasmic loops [12]. The residues located in these transmembrane domains have been considered to be involved in the binding and transporting mechanisms of P-glycoprotein. However, no amino acid has been identified to be directly involved in the substrate recognition.

We recently demonstrated that the replacements of His^{61}, which is in the middle of TM1 of P-glycoprotein, by other amino acids altered the drug resistance profile of P-glycoprotein [13]. Especially, the replacement of His^{61} by amino acids with bulkier side chain than His altered the drug resistance profiles drastically. 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 [14,15].

The cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel whose inherited malfunctions causes cystic fibrosis [16,17]. CFTR also belongs to the ABC superfamily, and the predicted topology of it, with the exception of the R domain, resembles that of P-glycoprotein. In CFTR, substitution and modification of amino acids in TM1 were reported to alter the relative anion permeability sequence of the channel [18], and it was suggested that three amino acid residues in TM1 are involved in forming part of the channel lining [19]. If the amino acid at position 61 of P-glycoprotein is involved in the formation of the recognition site for substrates, it would be expected that the amino acids located near His^{61} are also involved in the formation of the substrate recognition site and the mutations to those amino acids change the substrate specificity of P-glycoprotein. Then, nine amino acids residues, from Ala^{57} to Leu^{65} in TM1, were independently substituted to Arg, and analyzed the drug resistance of cells stably expressing each of these mutants of P-glycoprotein.

2. Materials and methods

2.1. Materials

Vinblastine (Vbl), colchicine (Col), and adriamycin (Adr) were purchased from Wako Pure Chemical Industries, Ltd. VP16 (etoposide) was from Sigma. Monoclonal antibody C219 was from Centocor.

2.2. Site-directed mutagenesis

Oligonucleotides were synthesized to generate the appropriate substitutions. The manufacturer's mutagenesis procedure (Sculptor in vi-

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Abbreviations: TM, transmembrane domain; CFTR, cystic fibrosis transmembrane conductance regulator; His, histidine; Ala, alanine; Gly, glycine; Ile, isoleucine; Arg, arginine; Vbl, vinblastine; Col, colchicine; Adr, adriamycin

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tro mutagenesis system, Amersham) was used to replace amino acids in TM1. Mutations were confirmed by DNA sequencing.

2.3. Transfection and drug resistance assay

The procedures of propagation of human cultured cells KB3-1 and of transfection by MDR1 expression vectors and its mutated variants were as described previously [13]. After cells were first selected in the presence of 0.8 mg/ml geneticin (G418) for 10 days, the obtained mass populations of geneticin-resistant colonies were selected by Vbl (5 ng/ml) or Col (10 ng/ml). From transformant cells that could be resistant to Vbl and/or Col, the author obtained cells expressing a readily detectable amount of mutant P-glycoprotein by further selection with stepwise increasing concentrations of Vbl. Cells transfected by $Ile^{60} \rightarrow Arg$, or $Ala^{63} \rightarrow Arg$ cDNA were selected with stepwise increasing concentrations (10, 20, 40 ng/ml) of vinblastine and finally maintained in 30 ng/ml Vbl. Cells transfected with $Gly^{64} \rightarrow Arg$, or $Leu^{65} \rightarrow Arg$ cDNAs were selected with stepwise increasing concentrations (5, 10, 20 ng/ml) of vinblastine and finally maintained in 20 ng/ml vinblastine. 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 Adr (0–900 ng/ml).

2.4. Immunoblotting

Membrane proteins were prepared as described previously [20] and resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 7% gels. Immunoblotting was done as described previously [20].

3. Results

To elucidate the function of TM1 of P-glycoprotein further, we replaced the amino acid residues from Ala^{57} to Leu^{65}, which are rather small and neutral amino acids, in TM1 independently by Arg, a bulky and basic amino acid, and investigated whether each of Arg substitutions altered the substrate specificity of P-glycoprotein as the substitution at His^{61}. In the previous study, we showed that the replacements of His^{61} by Arg changed the substrate specificity of P-glycoprotein drastically [13]. It reduced resistance to vinblastine (Vbl) and adriamycin (Adr), and increased resistance to colchicine (Col) and VP16, resulting in a resistance order of Col > VP16 ≈ Vbl > Adr, while that of the wild type was Vbl > Adr ≈ Col > VP16.

Each mutant or the 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, same as used previously [13]. Cells were first selected in the presence of 0.8 mg/ml geneticin (G418) for 10 days. To assess functionality of the mutants, the mass populations of geneticin-resistant colonies obtained were selected by Vbl (5 ng/ml) or Col (10 ng/ml). From cells transfected with the wild type and $Ile^{60} \rightarrow Arg$, $His^{61} \rightarrow Arg$, $Ala^{63} \rightarrow Arg$, $Gly^{64} \rightarrow Arg$, or $Leu^{65} \rightarrow Arg$ mutant cDNA, both Vbl- and Col-resistant colonies were obtained (Fig. 1). Cells transfected with the wild-type P-glycoprotein cDNA yielded slightly more resistant colonies in the presence of Vbl (5 ng/ml) than in the presence of Col (10 ng/ml). In contrast, cells transfected with mutants $His^{61} \rightarrow Arg$ slightly more resistant colonies in the presence of Col than in the presence of Vbl. Noticeably, cells transfected with mutant $Gly^{64} \rightarrow Arg$ or $Leu^{65} \rightarrow Arg$ yielded comparable number of Col-resistant colonies, but much less Vbl-resistant colonies, suggesting that the substrate specificities of these two mutants were altered from that of the wild type. To examine this possibility, we first obtained cells expressing a readily detectable amount of these five mutant P-glycoproteins by further selection with stepwise increasing concentrations of Vbl as described in Materials and Methods. Relative resistance to

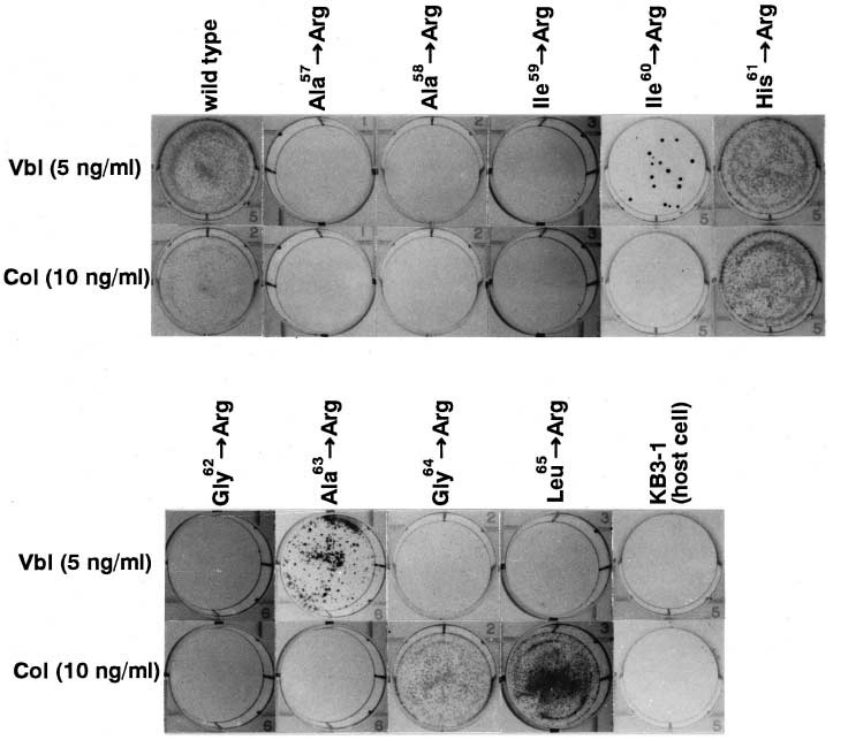

Fig. 1. Drug survival characteristics of geneticin-resistant mass populations of transfected cells. 1.0×10^5 cells from mass populations of geneticin-resistant cells stably transfected with either wild-type or mutant P-glycoprotein cDNAs, or untransfected KB3-1 control cells were plated in medium containing 5.0 ng/ml vinblastine or 10.0 ng/ml colchicine in 6-well plates. Plates were incubated for 7 days, stained with crystal violet (0.4% in methanol), and photographed.

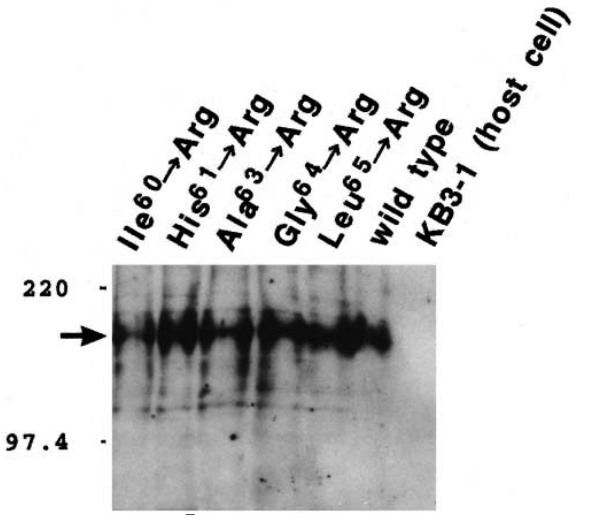

Fig. 2. Immunoblots of membrane proteins from cells stably expressing wild-type and mutant P-glycoproteins. Membrane proteins were obtained from stable transformants maintained in 20 ng/ml vinblastine for mutants $Gly^{64} \rightarrow Arg$ and $Leu^{65} \rightarrow Arg$, or 30 ng/ml vinblastine for wild type and the other mutants. Twenty μg of membrane proteins were resolved on a 7% SDS-PAGE gel and reacted with the monoclonal antibody C219 as a probe for P-glycoprotein. The arrow indicates the position of the 170-kDa mature form of P-glycoprotein.

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 (Fig. 1), suggesting that the stepwise selection with Vbl did not alter the resistance profiles conferred by mutant P-glycoproteins. The membrane fractions prepared from these transfectants were subjected to immunoblot with the anti-P-glycoprotein monoclonal antibody C219 (Fig. 2). From each of membrane fraction, a band migrating at about 170 kDa was detected, indicating that $Ile^{60} \rightarrow Arg$, $His^{61} \rightarrow Arg$, $Ala^{63} \rightarrow Arg$, $Gly^{64} \rightarrow Arg$, and $Leu^{65} \rightarrow Arg$ mutant P-glycoproteins were normally processed and expressed in the plasma membrane. 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 are maintained in the medium containing Vbl for several months, because the sequence of the wild-type P-glycoprotein was not found in the RT-PCR products from cells which were selected by Vbl after mutant MDR1 cDNAs were introduced (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.

However, from cells transfected with $Ala^{57} \rightarrow Arg$, $Ala^{58} \rightarrow Arg$, $Ile^{59} \rightarrow Arg$, or $Gly^{62} \rightarrow Arg$ mutant cDNA, no Vbl- or Col-resistant colonies were obtained (Fig. 1). It was reported [21] that no Vbl- or Col-resistant colonies were obtained from cells transfected with either $Gly^{54} \rightarrow Val$, $Ala^{58} \rightarrow Leu$, or $Gly^{62} \rightarrow Val$ mutant P-glycoprotein cDNA, and that the major products from these three mutant cDNAs were protein with an apparent mass of 150 kDa when their cDNAs were transiently expressed in HEK 293 cells, suggesting that these mutations in TM1 affected the proper folding and processing to the plasma membrane of P-glycoprotein. We also failed to detect the mature form of P-glycoprotein, when $Ala^{57} \rightarrow Arg$, $Ala^{58} \rightarrow Arg$, $Ile^{59} \rightarrow Arg$, and $Gly^{62} \rightarrow Arg$ mutant cDNAs were transiently expressed in HEK 293 cells (data not shown). These results suggested that these Arg mutations severely interfered with the proper processing of P-glycoprotein.

The drug-resistance profiles of the cells expressing $Ile^{60} \rightarrow Arg$, $His^{61} \rightarrow Arg$, $Ala^{63} \rightarrow Arg$, $Gly^{64} \rightarrow Arg$, and $Leu^{65} \rightarrow Arg$ mutant P-glycoproteins were investigated by comparing their relative resistance to Vbl, Col, VP16, and Adr (Fig. 3). The drastic alterations in the drug resistance profile were observed with $Gly^{64} \rightarrow Arg$ or $Leu^{65} \rightarrow Arg$ mutant as well as $His^{61} \rightarrow Arg$ mutant. The cells expressing these two mutant P-glycoproteins were more resistant to Col than Vbl, and showed the increased resistance to VP16, like cells expressing $His^{61} \rightarrow Arg$ mutant P-glycoprotein, while cells expressing the wild-type P-glycoprotein were more resistant to Vbl than Col, and showed low resistance to VP16. In contrast, the resistance profile of $Ile^{60} \rightarrow Arg$ or $Ala^{63} \rightarrow Arg$ mutant P-glycoprotein did not altered much from that of the wild type. These results suggest that the amino acid residues at positions 61, 64, and 65 have similar characteristic in deciding the substrate specificity.

4. Discussion

In this study, we examined whether the substitution of amino acids near His^{61} in TM1 affect substrate specificity of P-glycoprotein. Amino acid residues from Ala^{57} to Leu^{65} in TM1 were independently replaced by Arg, and stable transformants expressing each mutant P-glycoprotein were tried to be established. The mutant P-glycoproteins, in which either Ile^{60}, Ala^{63}, Gly^{64}, or Leu^{65} was replaced by Arg were successfully expressed in the plasma membrane of KB3-1 cells and were functional as drug transporters as the His^{61} mutants. The substrate specificities of $Gly^{64} \rightarrow Arg$ and $Leu^{65} \rightarrow Arg$ mutant P-glycoproteins were quite different from that of the wild type, and similar to that of $His^{61} \rightarrow Arg$ mutant, while Ile^{60} and $Ala^{63} \rightarrow Arg$ mutant P-glycoproteins showed similar substrate specificities to that of the wild-type P-glycoprotein. From the previous work [13], it was suggested that the size of the side chain of the residue at position 61 would play 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 [14,15]. The similarity among the substrate specificities of $Gly^{64} \rightarrow Arg$, $Leu^{65} \rightarrow Arg$, and $His^{61} \rightarrow Arg$ mutant P-glycoproteins suggest that the amino acid residues at position 64 and 65 are also important in deciding the substrate specificity.

Although all the mutant P-glycoproteins in which His^{61} was replaced by each of other 19 amino acids were properly sorted to the plasma membrane and functional as drug [13], the mutant P-glycoproteins, in which either Ala^{57}, Ala^{58}, Ile^{59}, or Gly^{62} was replaced by Arg, could not confer cells either Vbl- or Col-resistance. TM1 of P-glycoprotein has been suggested to be important for the formation of a functional configuration, because the mutations to Gly^{54}, Ala^{58}, and Gly^{62} in TM1 affected the proper folding and altered overall activity of P-glycoprotein [21]. It is most likely that replacement of Ala^{57}, Ala^{58}, Ile^{59}, or Gly^{62} by Arg also affects the proper folding

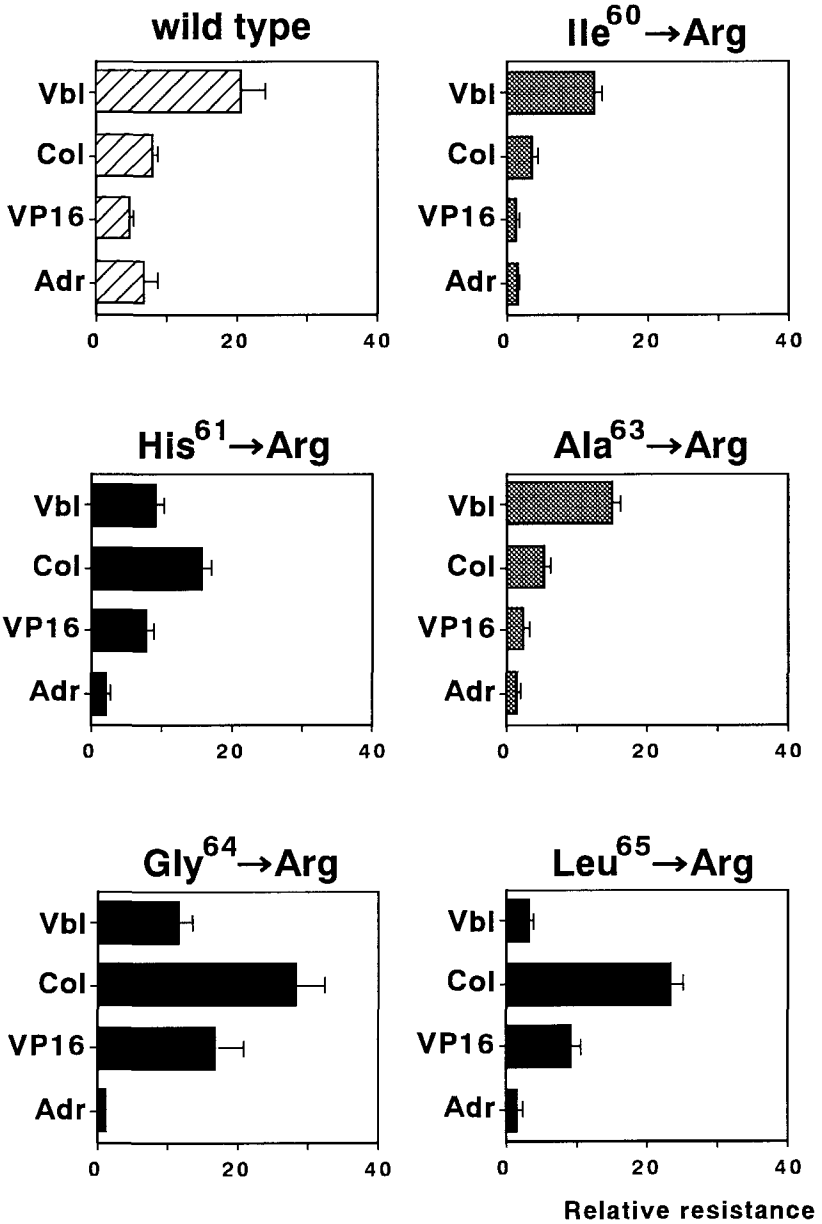

Fig. 3. The drug resistance profiles of cells stably expressing wild-type or mutant P-glycoprotein. Relative resistance was calculated by comparing the IC_{50} (the drug concentration necessary to inhibit cell growth by 50%) for each stably transfected type of cells expressing the wild-type or mutant P-glycoprotein to the IC_{50} of the host cell KB3-1. Each value is the mean of three separate experiments. The IC_{50} for KB3-1 was 1.20 ng/ml for vinblastine (Vbl), 4.60 ng/ml for colchicine (Col), 176.0 ng/ml for VP16, and 9.88 ng/ml for adriamycin (Adr).

and processing of P-glycoprotein. All together, these results suggest that some residues in TM1 would be important for the formation of a functional configuration and mutations to them would affect the proper folding and overall activity of P-glycoprotein.

According to the predicted model for secondary structure of human P-glycoprotein [22,23], supported by several experiments [21,24], it has been assumed that the secondary structure of the transmembrane domains of P-glycoprotein are α-helical. If it is a case, three amino acids His^{61}, Gly^{64}, and Leu^{65}, mutations of which had similar effects on the substrate specificity, would form an compact region on an α-helix arrangement of TM1 (Fig. 4). On the other hand, Ile^{60} and Ala^{63}, mutations of which did not greatly alter the drug resistance profiles of P-glycoprotein, would face away from this region on an α-helix arrangement. From these results, it is assumed that a region consisting of His^{61}, Gly^{64}, and Leu^{65} in TM1 would participate in the formation of recognition site for substrates of P-glycoprotein. The substrate specificities of the $Gly^{64} \rightarrow Arg$ and $Leu^{65} \rightarrow Arg$ mutants were similar to, but not exactly same with that of the $His^{61} \rightarrow Arg$ mutant. The cells expressing $Gly^{64} \rightarrow Arg$ or $Leu^{65} \rightarrow Arg$ mutant were more resistant to VP16 than to Vbl, while cells expressing the $His^{61} \rightarrow Arg$ mutant were more resistant to Vbl than to VP16 (Fig. 3). These results suggest that three amino acids, His^{61}, Gly^{64}, and Leu^{65} interact with substrates in different ways. Furthermore, Ala^{58}, Ile^{59}, and Gly^{62}, mutations of which abolished the function of P-glycoprotein, would face the other side on an α-helix arrangement of TM1. The region consisting of Ala^{58}, Ile^{59}, and Gly^{62} could be involved in the

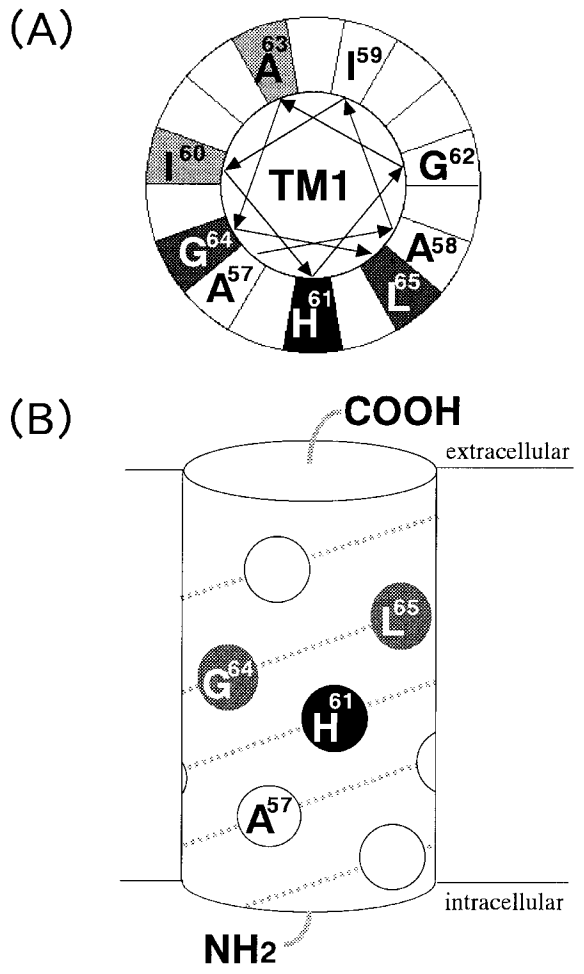

Fig. 4. Helical wheel representation of the residues from Ala[57] to Leu[65] in TM1. The helical wheel is viewed from its C-terminus (the extracellular side) (A) and is viewed from a side, to which His[61], Gly[64], and Leu[65], mutations of which drastically altered the substrate specificity of P-glycoprotein, would face. Amino acid residues are indicated in single-letter code followed by position number. Residues, mutations of which to Arg drastically altered the substrate specificity, are indicated with white letters in black (His[61]) or dark gray (Gly[64], and Leu[65]) backgrounds. Residues, mutations of which to Arg did not much alter the substrate specificity (Ile[60] and Ala[63]), are indicated with black letters in gray backgrounds. Residues, mutations of which to Arg abolished the function of P-glycoprotein, are indicated with black letters in white backgrounds.

formation of framework of P-glycoprotein, such as in the interaction between transmembrane α-helices.

In CFTR, it was suggested that three residues in TM1 participate in the formation of channel, through which ions would move across the membrane [19]. It may be presumed that three amino acids, His[61], Gly[64], and Leu[65], in P-glycoprotein also face to a pore or a cavity like CFTR, where these amino acids form a part of the substrate recognition site. It is interesting that a region in TM1, not only in CFTR but also in P-glycoprotein, could be involved in the interaction with substrate. P-Glycoprotein and CFTR, both members of the ABC superfamily, 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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