Amino Acid Residue at Codon 268 Determines Both Activity and Nucleotide-Sugar Donor Substrate Specificity of Human Histo-blood Group A and B Transferases

IN VITRO MUTAGENESIS STUDY*

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Histo-blood group A transferase produces A antigens and transfers GalNAC to the acceptor substrate, H structures of glycolipids and glycoproteins. B transferase transfers galactose in place of GalNAC to the same acceptor substrate to synthesize B antigens. We have previously identified four amino acid substitutions between human A and B transferases. Out of these four substitutions at the last two positions (codons 266 and 268) were found to be crucial for the different donor nucleotide-sugar specificities between A and B transferases as analyzed by gene transfer of chimeric A-B transferase genes.

In the present study, we have in vitro mutagenized codon 268 of these two transferase cDNA expression constructs (glycine and alanine in A and B transferases, respectively) and produced substitution constructs with every possible amino acid residue at this position. We examined the activity and specificity of each construct by gene transfer followed by immunodetection of A and B antigens and in vitro enzymatic assay. Amino acid substitution constructs on the A transferase backbone with alanine, serine, and cysteine expressed enzymes with A and B transferase activities. Weak A activity was detected with histidine and phenylalanine constructs while weak B activity was detected with asparagine and threonine constructs. All the other amino acid substitutions at codon 268 on the A transferase backbone showed neither A nor B activity. The glycine construct on the B transferase backbone expressed both A and B transferase activities. Some substitution constructs on the B transferase backbone maintained B activity while some other substitutions abolished the activity.

These results show that the side chain of the amino acid residue at 268 of the human A and B transferases is responsible for determining both activity and nucleotide-sugar donor substrate specificity and strongly suggest its direct involvement in the recognition of and binding to the sugar moiety of the nucleotide-sugars.

Glycoproteins and glycolipids are essential components of cellular membranes. Carbohydrate structures of these molecules are complex and exhibit great variety, and they are synthesized through a series of reactions catalyzed by the enzymes called glycosyltransferases (1, 2). The human histo-blood group ABO system offers one of the best systems to study the structural basis of specificity and activity of glycosyltransferases (3).

The functional alleles at the ABO locus encode glycosyltransferases to catalyze the final step of reactions to synthesize the carbohydrate antigens A and B (4). A1 alleles encode an enzyme (A1 transferase) to transfer N-acetyl-D-galactosamine (or GalNAc for short) from the donor substrate, UDP-GalNAc, to the acceptor substrate H structures of glycoproteins and glycolipids. B alleles encode another enzyme (B transferase) to transfer a different sugar, D-galactose from UDP-galactose, to the same acceptor substrate. Therefore, comparative structural analysis is needed to delineate the structural basis of the different specificities of A and B transferases. O alleles are incapable of producing functional enzymes, and the substrates remain unchanged. In addition to these three major alleles, there are many other minor subtypes. Although they are rare in the population, glycosyltransferases coded by these subtypic alleles offer clues about the structural basis of the enzymatic activity of glycosyltransferases.

We have cloned the cDNA for the human histo-blood group A transferase (5) and used this cDNA to screen cDNA libraries made with RNA from human cell lines showing different ABO phenotypes. We then co-related the differences in the nucleotide sequences with each of the three major (A1, B, and O) alleles (6). Four amino acid substitutions (RGLG and GSMA at codons 176, 235, 266, and 268 in A and B transferases, respectively) were identified, which discriminate between A and B transferases. O alleles were found to possess a single nucleotide deletion relatively close to the amino terminus of the coding sequence of the enzyme. We then analyzed A2 alleles, one of the A subtypes, and identified another single nucleotide deletion, which differs from the single nucleotide deletion of O alleles, is located at the carboxyl terminus of the A transferase coding sequence (7). This deletion changes the frame of codons and results in a transferase (A2 transferase) with an additional 21 amino acids at the carboxyl terminus. The presence of this domain was then proven to diminish the activity and confer restriction on the acceptor substrate usage of the enzyme. Sequence analysis of the alleles responsible for various minor subtypes (8, 9) and two interesting phenotypes, cis-AB (10) and B(A) (9), also revealed other mutations in the gene. We also determined the partial nucleotide sequences of ABO genes from some species of primates (11). Among the four amino acid substitutions that discriminate human A and B transferases,
amino acid residues at only the third and the fourth positions were to be conserved in the primeto ABO genes. All the information from these sequence analyses, in combination with functional assays of A and B transferases and their chimeras (12), indicate the importance of amino acid residues at 266 and 268 of the human A and B transferases for the activity and the different donor substrate specificities of these two enzymes.

We have recently identified a second type of O allele that is devoid of the single nucleotide deletion found in all other O alleles previously analyzed and instead possesses two amino acid substitutions, one at codon 176 (arginine to glycine) and the other at codon 268 (glycine to arginine) of A transferase (13). The presence of this O allele in the Danish population has been reported (14). DNA transfection experiments have shown that the introduction of these two missense mutations to a functional A transferase expression construct completely abolished the enzymatic activity. Because the arginine to glycine substitution at codon 176 was incapable of changing activity or specificity of the enzyme (12), we speculated that the glycine to arginine substitution at codon 268 was responsible for the inactivation of the transferase. Sequence comparison of A and B transferases with bovine (15) and murine (16) α-1,3-galactosyltransferases confirmed the importance of codon 268 (rather than codon 266) of the human A and B transferases for different donor nucleotide-sugar specificities.

In order to test more directly this hypothesis and learn more about the structural specificities of these enzymes, we produced amino acid substitution constructs with every possible amino acid residue at position 268 in both A and B transferases. We then analyzed the effect of the amino acid residue at codon 268 on the nucleotide-sugar donor substrate specificity and enzymatic activity of A and B transferases.

EXPERIMENTAL PROCEDURES

Materials—The reagents for PCR1 were purchased from Perkin-Elmer, and reagents for DNA sequencing were from U.S. Biochemical Corp. (Cleveland, OH). Radioactive [3P]dATP was from DuPont NEN, and [3H]UDP-galactose and [14C]UDP-GalNAc were from Amersham Life Science (Arlington Heights, IL). Restriction enzymes and Lipopolysaccharide reagents were from Life Technologies, Inc. Frozen competent bacteria XL-1-Blue strain of Escherichia coli was from Stratagene (La Jolla, CA). Anti-A and anti-B mixtures of murine monoclonal antibodies were from Ortho Diagnostic Systems Inc. (Piscataway, NJ), and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin was from Sigma. β-Galactosidase assay kit was from Promega (Madison, WI). 2-Fucosyllactose, UDP-galactose, UDP-GalNAc, and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) were from Sigma, and Poly-Prep prefiltered AG1-X8 resin chromatography columns were from Bio-Rad. Degenerate oligodeoxynucleotides FY-78, FY-81, and PDM-1 to -6 were custom synthesized at BioSynthesis Inc. (Denton, TX). The nucleotide sequences of these oligodeoxynucleotide primers are as follows: FY-78, CCGATCCTGGTATTTGAAACGAC; FY-81, CGGAATCTGATGAGGTTTCTGGGGGAC; PDM-1, ATTTCTACATTGGGGNNNCTTCTGGGGGGCTG; PDM-2, ACCAGCC- CCCCCAAGAANNNCCCGGAGTGAAGAT; PDM-3, ATTTCTACATGGGGNNNCTTCTGGGGGGCTG; PDM-4, ACCAGCC- CCCCCAAGAANNNCCCGGAGTGAAGAT; PDM-5, ACCCCC- GGAAGACTTCCCCACCTGAAG; PDM-6, ACCCCCCGGAAAGGGTCAATGGCCTA-TCCCCCAGTATGA. The BamHI site of FY-78 and the EcoRI site of FY-81 are artificial. The capitalized underlined letter N denotes a mixture of four nucleotides (GATC) at the position.

Construction of Amino Acid Substitution Constructs—We employed polymerase chain reaction with degenerate oligodeoxynucleotides to introduce mutations at codon 268 of human A and B transferases (Fig. 1). Two DNA fragments were first amplified from the template plasmid pA3740 (or pB894) DNA (12), with two sets of oligodeoxynucleotide primers (FY-81 and PDM-2 (or -4) or FY-78 and PDM-1 (or -3), respectively). Oligo primers, PDM-1 and -2 (and PDM-3 and -4), possess degenerate nucleotide sequences (NNN) at codon 268 to represent all the possible 64 combinations. The two primers, PDM-1 and -2 (and PDM-3 and -4), are complementary to each other except at codon 268. Supplemented with the original plasmid DNA, the two amplified fragments overlapping another one at the primer sequence were used as templates to amplify the fragment bordered by the outer primers, FY-78 and -81. Oligo primer FY-81 contained a degenerate sequence, but the DNA portion containing this sequence was removed from the insert by SstI and BamHI double digestion before ligation with the remaining portion of A (or B) transferase expression construct. The ligated DNA was used to transform the XL-1-Blue strain of E. coli. Plasmid DNA was prepared from individual transformants and analyzed for the presence of inserts, and DNA containing the insert was denatured and used as a template for DNA sequencing reactions by Sanger’s dideoxy chain termination method using the Sequenase™ kit. Sequencing of the nucleotide sequence of codon 268 and its surrounding region (from nucleotide 695 to nucleotide 885) of 381 and 259 clones of A and B transferase amino acid substitution constructs, respectively, was determined for individual clones. Multiple clones with the correct sequence over the region were obtained for each amino acid substitution construct except for methionine chimeras. In order to obtain methionine substitution constructs at codon 268, we used primer PDM-5 (or -6) in place of PDM-2 (or -4). Primer PDM-5 has a methionine codon (ATG) at amino acid 268 in the A transferase sequence background whereas PDM-6 has a mixture of methionine and isoleucine codons (ATG/ATI) at this position in the B transferase sequence background. We used degenerate primer PDM-5 because the number of isoleucine constructs was small when this oligonucleotide primer was designed. Further DNA sequencing was performed over the entire coding region, which was amplified by PCR (from the SstI site to the termination codon between nucleotides 470 and 1065) in order to exclude the clones with any additional unexpected mutation(s). We have obtained at least one such clone for each amino acid substitution construct.

DNA Transfection, Immunostaining, FACS Analysis, and Enzymatic Assays—Two cell lines (HeLa and COS 1) were used as recipients of DNA transfection experiments. Lipopolysaccharide reagent (17) was used as per the manufacturer’s instructions. DNA from a single representative clone (whose coding sequence was confirmed to be correct over the PCR-amplified region) from each amino acid substitution construct was co-transfected with DNA from pSV-β-galactosidase control vector. Two days after transfection, HeLa cells were harvested and fixed. Portions of cells were immunostained with a mixture of murine anti-A or anti-B monoclonal antibodies and secondarily with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin antibody, and they were subjected to FACS analysis. The percentages of cells stained for β-galactosidase activity in situ (18) were used to normalize the different transfection efficiencies among constructs. DNA-transfected COS 1 cells were harvested and lysed, and the supernatant was used for A and B transferase assays in vitro. A and B transferase activities were analyzed by the transfer of the radioactivity from [3H]UDP-GalNAc and UDP-galactose to the acceptor substrate, 2-fucosylactose, respectively. The reaction was performed in a mixture of 100 mM sodium cacodylate (pH 6.5), 5 mM ATP (pH 6.5), 0.5 mM [32P]dATP, 0.1 mM UDP-GalNAc or UDP-galactose (containing 14C-labeled UDP-GalNAc or UDP-galactose, respectively), and 0.5 mM 2-fucosylactose. The reaction mixture containing the cell extract was incubated at 37 °C for 6 and 2.5 h for A and B transferase assays, respectively. Incorporation of the radioactivity was found nearly proportional to the incubation time for the original A and B transferase under these conditions. The reaction without the acceptor substrate was used as a negative control. Radioactivity was measured with a scintillation counter after separation of enzymatic reaction products from the donor nucleotide-sugar substrates through the AG1-X8 anion exchange column chromatography. The supernatant was also used for β-galactosidase enzymatic assay. Activity was determined for each sample by measuring hydrolysis of PDM-6 4-chloro-3-indolyl-β-D-galactoside, a substrate of β-galactosidase, and the resulting value was used to normalize DNA transfection efficiency.

RESULTS

Specificity and Activity of A Transferase Amino Acid Substitution Constructs—Two cell lines were used as recipients of DNA transfection. HeLa cells express type H antigens on the cell surface and have been used successfully in our previous studies as recipients of DNA transfection of A and B transferase expression constructs and their derivatives (7, 12, 13). The specificity and activity of each amino acid substitution con-
Fig. 1. Locations of primers. The locations of primers are schematically shown. Coding regions of the human A and B transferase cDNAs are shown by open bars. The location of the remaining intron in the construct is also indicated (not on the scale). The A residue and the methionine residue of the initiation codon of A and B transferases are numbered 1. There are four amino acid (aa) substitutions between A and B transferases, and their positions are indicated (AArg176-Gly235-Leu266-Gly268 or B Gly176-Ser235-Met266-Ala268) in B). Two overlapping fragments were PCR-amplified using two sets of oligodeoxynucleotide primers (FY-81 and PDM-2 (or -4) and FY-81 and PDM-1 (or -3)) and template plasmid (pAAAA or pBBBB). The entire long fragment was then amplified with the two outermost primers (FY-78 and FY-81), cleaved with SstII and BamHI, and ligated with the vector portion of similarly digested original plasmid. (There is a BamHI site in the vector pSG-5, which locates downstream of the A and B transferase cDNA inserts.) After DNA transformation, insert-containing clones were identified, and the partial nucleotide (nt) sequence around the codon 268 was determined. Those clones without additional mutations in the sequenced region were further sequenced to their entirety over the coding region amplified by PCR (from the SstII site to the termination codon) and used for DNA transfection experiments.

The original A transferase alanine construct expressed weak A transferase activity, and asparagine and threonine constructs showed very weak B activity. Several A transferase constructs with other amino acids at codon 268 showed no activity of A or B transferase.

The original B transferase alanine construct expressed only B activity in FACS analysis; however, very weak A transferase activity was measured by in vitro enzymatic assay. One B transferase glycine construct showed strong activities of both A and B transferases. Two B transferase substitution constructs (serine and cysteine) expressed strong B transferase activity comparable with that of the original alanine construct. Two constructs (asparagine and threonine) showed strong B activity and five (aspartic acid, histidine, leucine, proline, and valine) constructs expressed moderate B activity. Two (glutamine and phenylalanine) and one (methionine) constructs expressed weak and very weak B activity, respectively.

**DISCUSSION**

Comparison of Two Detection Methods (Immunological Assays of A and B Antigens on the Surface of HeLa Cells and Enzymatic Assays of A and B Transferases in COS 1 Cell Extracts)—It is logical to assume that the detection of A and B antigens (secondary enzymatic reaction products) is more sensitive than that of enzymes (primary gene product) if the enzymes are translocated to the appropriate locus (Golgi apparatus) and functional in vivo. As shown in Tables I and II, sensitivity was actually much higher with the detection of A and B antigens than with the in vitro enzymatic assay. The median intensities of fluorescence of positively stained cells are also indicated in parentheses. Based on all the experimental results including the data from the pilot experiments (data not shown), A and B transferase activities of each amino acid substitution construct were determined and shown in the two far right columns. The original A transferase alanine construct expressed very strong A transferase activity as expected. An A transferase alanine construct expressed strong A transferase activity, which was comparable with that of the glycine construct, but this alanine construct expressed weak B transferase activity as well. Two A transferase substitution constructs (serine and cysteine) expressed moderately strong A and B activities. Histidine and phenylalanine constructs showed very weak A activity, and asparagine and threonine constructs showed very weak B activity. Several A transferase constructs with other amino acids at codon 268 showed no activity of A or B transferase.

Specificity and Activity of B Transferase Amino Acid Substitution Constructs—The results of DNA transfection experiments with B transferase amino acid substitution constructs are shown in Table II. The original B transferase alanine construct expressed only B activity in FACS analysis; however, very weak A transferase activity was measured by in vitro enzymatic assay. One B transferase glycine construct showed strong activities of both A and B transferases. Two B transferase substitution constructs (serine and cysteine) expressed strong B transferase activity comparable with that of the original alanine construct. Two constructs (asparagine and threonine) showed strong B activity and five (aspartic acid, histidine, leucine, proline, and valine) constructs expressed moderate B activity. Two (glutamine and phenylalanine) and one (methionine) constructs expressed weak and very weak B activity, respectively.
efficiently. We used only single in vitro enzymatic assay conditions. However, under these conditions, we were able to detect activity even less than 1% of the original A and B transferase constructs using extracts from COS 1 cells transfected with each amino acid substitution construct. For example, A transferase activity of A transferase serine substitution construct has 0.4% of that of the original A transferase glycine construct. COS 1 cells possessed low activities of A and B transferases, and small amounts of radioactivity incorporated into the reaction products (high background level) made it difficult to detect very weak activities of some substitution constructs. For example, A transferase activity of A transferase serine substitution construct has 0.4% of that of the original A transferase glycine construct.

### Table I

| Residue | HeLa cells FACS analysis | COS 1 cells enzymatic assay | Conclusion |
|---------|--------------------------|-----------------------------|------------|
|         | A+  | B+  | A+  | B+  | +++++ | ++ |
| Ala     | 78  | 18  | 16  | 1.6 | +++++ | ++ |
| Arg     | 0   | 0   | 0   | 0   | +    | -- |
| Asn     | 0   | 16  | 0   | 0   | +    | +  |
| Asp     | 0   | 0   | 0   | 0   | +    | -- |
| Cys     | 52  | 50  | 0   | 0   | ++   | ++ |
| Gln     | 0   | 0   | 0   | 0   | +    | -- |
| Glu     | 0   | 0   | 0   | 0   | +    | -- |
| Gly     | 100 | 0   | 100 | 0   | +++++| -- |
| His     | 0   | 33  | 0   | 0   | +    | -- |
| Ile     | 0   | 0   | 0   | 0   | +    | -- |
| Leu     | 0   | 0   | 0   | 0   | +    | -- |
| Lys     | 0   | 0   | 0   | 0   | +    | -- |
| Met     | 0   | 0   | 0   | 0   | +    | -- |
| Phe     | 9   | 0   | 0   | 0   | +    | -- |
| Pro     | 0   | 0   | 0   | 0   | +    | -- |
| Ser     | 78  | 86  | 0.4 | 0.7 | +++  | +  |
| Thr     | 0   | 10  | 0   | 0   | +    | +  |
| Trp     | 0   | 0   | 0   | 0   | +    | -- |
| Tyr     | 0   | 0   | 0   | 0   | +    | -- |
| Val     | 0   | 0   | 0   | 0   | +    | -- |
| Negative control | 0 | 0 | 0 | 0 | + | -- |

* Antigen plus cell relative percent (median intensity).
* Enzymatic activity relative percent.

### Table II

| Residue | HeLa cells FACS analysis | COS 1 cells enzymatic assay | Conclusion |
|---------|--------------------------|-----------------------------|------------|
|         | A+  | B+  | A+  | B+  | A   | B   |
| Ala     | 0   | 100 | 0.3 | 100 | +   | ++++|
| Arg     | 0   | 0   | 0   | 0   | +   | ++  |
| Asn     | 0   | 91  | 0   | 0.7 | +   | ++  |
| Asp     | 0   | 64  | 0   | 0   | +   | ++  |
| Cys     | 0   | 86  | 0   | 1.8 | +   | ++++|
| Gln     | 0   | 45  | 0   | 0   | +   | ++  |
| Glu     | 0   | 0   | 0   | 0   | +   | ++  |
| Gly     | 85  | 95  | 12  | 25  | ++++| ++++|
| His     | 0   | 40  | 0   | 0   | +   | ++  |
| Ile     | 0   | 0   | 0   | 0   | +   | --  |
| Leu     | 0   | 55  | 0   | 0   | +   | ++  |
| Lys     | 0   | 0   | 0   | 0   | +   | --  |
| Met     | 0   | 8   | 0   | 0   | +   | --  |
| Phe     | 0   | 34  | 0   | 0   | +   | ++  |
| Pro     | 0   | 68  | 0   | 0   | +   | +++|
| Ser     | 0   | 91  | 0   | 15  | +   | ++++|
| Thr     | 0   | 55  | 0   | 0   | +   | ++++|
| Trp     | 0   | 0   | 0   | 0   | +   | --  |
| Tyr     | 0   | 0   | 0   | 0   | +   | --  |
| Val     | 0   | 55  | 0   | 0   | +   | +++|
| Negative control | 0 | 0 | 0 | 0 | + | -- |

* Antigen plus cell relative percent (median intensity).
* Enzymatic activity relative percent.

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saturation of A and B antigens on the cell surface when large numbers of enzymes are produced in vivo. We found one exception where no enzymatic reaction products were detected on HeLa cells, but A transferase enzymatic activity was measured in vitro in the COS 1 cell extract (B transferase original alanine construct). This result may not be real because the A transferase activity detected in vitro was so small (0.3% of that of original A transferase); however, a similar finding was previously reported using sera from blood group B individuals as an enzyme source (19).

An A Transferase Alanine Construct Expressed Strong A and Weak B Transferase Activities—We have previously identified two missense mutations in two cases of cis-AB alleles responsible for the cis inheritance of both A and B transferase activities by a single gene on a single chromosome (10). One of these mutations was found at the last position of the four amino acid substitutions that discriminate the human A and B transferases. cis-AB alleles contained the B-specific alanine at codon 268 in the A transferase backbone. Therefore, the A transferase alanine substitution construct corresponds exactly to previously published expression construct pAAAB. DNA transfection of this A transferase alanine construct has yielded a result showing weak B transferase activity, which is different from the previous result with pAAAB (12). The repeated analysis of the pAAAB indicates that discrepancy may be ascribed to antibody specificity issues. The same explanation can be applied to pBBB, another chimeric construct representing B(A) allele (9), which was previously found not to express A activity (12). Considering the fact that B(A) was initially identified with certain murine anti-A monoclonal antibody (20), different anti-A antibodies may detect A antigens on HeLa cells transfected with the pBBB construct.

Effects of Amino Acid Substitutions at Codon 268 on Specificity and Activity of A Transferases—Clearly positive A transferase activity was observed with six amino acid substitution constructs with the A transferase backbone. Those amino acid residues are glycine, alanine, serine, cysteine, histidine, and phenylalanine, and activity decreases in that order. Apparently, the strength of activity is reciprocally proportional to the size of side chain. The side groups of amino acid residues other than four (glycine, alanine, serine, and cysteine) may be too bulky to accommodate the GalNAc portion of the UDP-GalNAc nucleotide-sugar into the recognition and interacting site of a transferase. It is difficult to explain weak A activity of histidine and phenylalanine constructs only by the size of the side group; however, these amino acid residues may have special characteristics considering the fact that these amino acid substitution constructs on B transferase backbone also showed moderate B transferase activity, as will be discussed. B transferase activity was observed with serine, cysteine, alanine, asparagine, and threonine (all with small side chains) at codon 268 in A transferase constructs. The side chain of glycine (hydrogen) may be big enough to interact with GalNAc but too small to interact with galactose.

Effects of Amino Acid Substitutions at Codon 268 on Specificity and Activity of B Transferases—Strong A transferase activity was detected only in the glycine construct with the B transferase backbone. The methionine at codon 266 of B transferase constructs is bigger than the leucine residue of A transferase constructs, and this bigger size may compensate the small hydrogen side chain of glycine at codon 268 and enable the B transferase glycine mutant to interact with and transfer both GalNAc and galactose. Strong B activity was observed with six B transferase constructs (alanine, glycine, serine, cysteine, asparagine, and threonine). In addition, five (aspartic acid, histidine, leucine, proline, and valine), two (glutamine and phenylalanine), and one (methionine) constructs showed moderate, weak, and very weak B activity, respectively. Comparison of B antigen-positive cell percentages among constructs with a neutral polar amino acid at codon 268 (glycine > serine > threonine > tyrosine = 0) indicates the importance of the size of side chain as shown above with A transferase substitution mutants. Comparison among four amino acid substitutions (asparagine > aspartic acid > glutamine > glutamic acid = 0) suggests that the presence of an acidic side chain decreased enzymatic activity. Not only the size but also the basic charge of the side groups may be responsible for the loss of activity in arginine and lysine constructs. However, the construct with histidine, a basic amino acid with an imidazole group, showed moderate B activity. Comparison among constructs with neutral hydrophobic side chains (alanine > valine > leucine > isoleucine = tryptophan = 0) shows again the importance of the size of the side chain for the strength of activity. The phenylalanine construct, however, showed weak B transferase activity. The construct with a proline residue, in which the nitrogen atom of the amino group is incorporated into a ring, introducing a bend in a peptide, still possessed B activity. In addition, more amino acid substitutions were functional in B transferase backbone than those in A transferase backbone. This implies that the amino acid residues at 266 and possibly 267 as well are also important in both the activity and the specificity of the transferases and that the size of the side chain at codon 268 is not the only issue. We hope that three-dimensional structural analysis by x-ray crystallography and molecular modeling of these transferases will clarify these ambiguities in the future.

O Alleles with Amino Acid Substitution Other Than Arginine at Codon 268—We have previously identified a second type of O allele, which contains two amino acid substitutions (arginine-to-glycine at 176 and glycine-to-arginine at 268) (13). The A transferase arginine construct showed no activity of A or B transferase suggesting that the glycine-arginine substitution at codon 268 is sufficient alone to abolish A transferase activity in the protein coded by this rare type of O allele. Single nucleotide substitutions at the first and the second positions of codon 268 (nucleotides 802 and 803) of the A transferase coding sequence result in five amino acid substitutions at codon 268 (from GCC: glycine to GAG: glutamic acid, GTG: valine, GCG: alanine, AGG and CCG: arginine, or TGG: tryptophan). Out of these five, alanine was the only substitution that did not spoil the enzymatic activity as shown in Table I. Similarly, single nucleotide substitutions at those positions of the B transferase coding sequence result in six amino acid substitutions (from GCC: alanine to ACG: threonine, TCG: serine, CCG: proline, GGG: glycine, GAG: glutamic acid, or GTG: valine). As shown in Table II, B transferase alanine to glycine substitution resulted in an enzyme with both A and B transferase activities while the glutamic acid substitution abolished the B transferase activity. All the other amino acid substitutions (threonine, serine, proline, and valine) did not abolish but weakened B transferase activity.

In all, five single base substitutions at codon 268 in the A transferase backbone and one in the B transferase backbone abolish the activity and result in phenotypically O alleles. Of these six possible mutations only an arginine substitution at codon 268 with the A transferase backbone has been discovered in O alleles in the human population so far (13, 14), in addition to the single nucleotide deletion that distinguishes most O alleles (6). Our present results, however, suggest the possible existence of O alleles with other amino acid residues at codon 268 in the A or B transferase backbone. ABO genotyping has become popular (6, 14, 21–24); however, the presence of O
alleles without the single nucleotide deletion (frameshift mu-

tation) and possibly of A and B alleles with an amino acid

residue at codon 268 other than glycine and alanine, respect-

ively, may lead to unexpected conflicting results between ABO

phenotypes and genotypes. Therefore, we must bear in mind

that genotyping based on partial sequence information may not

be 100% accurate.

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