A sequence motif involved in the donor substrate binding by α1,6-fucosyltransferase: the role of the conserved arginine residues

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α1,6-Fucosyltransferase catalyzes the transfer of fucose to the innermost GlcNAc residue of an N-linked oligosaccharide. In order to identify the amino acid residue(s) which are associated with the enzyme activity and to investigate their function, we prepared a series of mutant human α1,6-fucosyltransferases in which the conserved residues in the region homologous to α1,2-fucosyltransferase had been replaced. These proteins were then characterized by kinetic analyses. The wild-type and mutant α1,6-fucosyltransferases were expressed using a baculovirus-insect cell system. The activity assay showed that replacement of Arg-365 by Ala or Lys led to a complete loss of activity while substitution of Ala or Lys for the neighboring Arg-366 decreased the activity to about 3% that of the wild type. Kinetic analyses revealed that the replacements of Arg-366 lead to an increase in the apparent $K_v$ value for both GDP-fucose and the acceptor oligosaccharide but did not markedly affect the apparent $V_{max}$. When these mutants were inhibited by GDP in a competitive manner with respect to the donor substrate, the $K_i$ values were found to be 50–100 times higher than the value in the wild type. On the other hand, in the inhibition by GMP, the $K_i$ values for the mutants were very similar to that of the wild type. These findings suggest that Arg-366 contributes to the binding of GDP-fucose via an interaction with the β-phosphoryl group of the GDP moiety of the donor, and that Arg-365 may also play an essential role in substrate binding. The results suggest that the motif common to α1,2- and α1,6-fucosyltransferases is critical for binding of the donor substrate, GDP-fucose.

Key words: fucosyltransferase/N-glycan

Introduction

α1,6-Fucosyltransferase is involved in the biosynthesis of Asn-linked oligosaccharides of glycoproteins and catalyzes the transfer of fucose from GDP-fucose to the innermost GlcNAc residue of the oligosaccharide of a glycoprotein, giving rise to an α1,6-fucosylated sugar chain (Wilson et al., 1976; Longmore and Schachter, 1982; Schachter, 1986). Fucosyltransferases are classified, according to the linkages catalyzed, into several families which include α1,2-, α1,3-, (α1,3/4-) and α1,6-fucosyltransferases. Each of these families, with the exception of α1,6, consists of several types of fucosyltransferases, which display a significant sequence homology (Costache et al., 1997; Breton et al., 1998). It is known that α1,6-fucosyltransferase is the only enzyme in this class which catalyzes the formation of α1,6-linked fucose in mammalians. However, homologous enzymes which transfer a fucose via an α1,6-linkage have been reported in other organisms (Stacey et al., 1994; Mergeret et al., 1996).

α1,6-Fucosyltransferase is widely distributed in mammalian tissues, as evidenced by activity assay and Northern hybridization (Miyoshi et al., 1997). While oligosaccharides that contain α1,6-fucose residues are frequently found in the N-glycans of a variety of glycoproteins, this structure is not found in the serum proteins biosynthesized by the liver, probably because of the very low activity of the enzyme in hepatocytes (Campion et al., 1989; Yamashita et al., 1989; Noda et al., 1998a). Nevertheless, α1,6-fucosyltransferase activity is increased in the liver for cases of certain diseased states such as hepatocellular carcinomas, and, as a result, the content of α1,6-fucose residues in the Asn-linked oligosaccharides of the serum proteins, produced in the liver, are significantly elevated in such cases (Aoyagi et al., 1985, 1993a,b; Hutchinson et al., 1991; Ohno et al., 1992; Noda et al., 1998b). It has been proposed that this structural alteration, which is associated with carcinogenesis, could be of value in the differential diagnosis of the malignant diseases.

In order to investigate the molecular basis for oligosaccharide alteration associated with the hepatocarcinogenesis, we have purified α1,6-fucosyltransferase from pig brain and a human gastric cancer cell line, cloned their cDNAs, and analyzed the genomic structure of the human enzyme (Uozumi et al., 1996a; Yanagidani et al., 1997; Yamaguchi et al., 1999). Structural analyses of the cDNA clones indicates that the enzyme is a type II membrane protein, and that the domain structure of the enzyme is similar to those of other classes of fucosyltransferases, as well as other glycosyltransferases. However, when the complete amino acid sequence was compared with those of the other fucosyltransferases, no remarkable homology was found among them. Only a small region in the α1,6-fucosyltransferase was found to be significantly homologous to a portion of α1,2-fucosyltransferase, based on a comparison of their sequences (Breton et al., 1998). This suggests that the
motif would be involved in functional properties which are common to the α1,2- and α1,6-fucosyltransferases.

Of the numerous fucosyltransferases, the catalytic properties of the α1,3- or α1,3/4-fucosyltransferase involved in the biosynthesis of Lewis antigens have been intensively investigated in terms of kinetic properties and catalytic mechanism (Murray et al., 1996, 1997; Nguyen et al., 1998; Sherwood et al., 1998; Vo et al., 1998). On the other hand, although Glick et al. purified the α1,6-fucosyltransferase from cultured skin fibroblasts and characterized some of the kinetic properties of the enzyme (Voynow et al., 1991), the detailed catalytic mechanism remains unclear. Moreover, amino acid residues that are involved or essential for activity have not yet been identified. Information, as obtained by chemical modification studies or site-directed mutagenesis, would be highly desirable for the elucidation of the catalytic mechanism of the enzyme.

In this study, the residues conserved in the homologous motifs between α1,2- and α1,6-fucosyltransferases were selected as likely candidate residues which might play an essential or important role in the function of the enzyme, and then prepared mutant α1,6-fucosyltransferases in which these residues were replaced. These mutants, as well as the wild-type enzyme, were produced using a baculovirus–insect cell expression system, and were characterized by kinetic analyses to examine the role of the identified residues.

Results

Comparison of amino acid sequences of α1,2- and α1,6-fucosyltransferases

In order to identify the candidate amino acid residues which are essential for the activity of α1,6-fucosyltransferase, the entire amino acid sequences of human α1,2- and α1,6-fucosyltransferases were compared by dotplot analysis (Figure 1A). The analysis showed several small homologous regions, with the most significant being in residues 361–370 in the α1,6-fucosyltransferase. When this portion of the amino acid sequences from the α1,6-fucosyltransferases were aligned with the corresponding regions of other α1,6-fucosyltransferases from different species, as well as various α1,2-fucosyltransferases, as shown in Figure 1B, the alignment showed that the enzymes have perfectly conserved histidine and arginine residues, which correspond to His-363 and Arg-365 in the human α1,6-fucosyltransferase. Thus, mutant enzymes of human α1,6-fucosyltransferase in which these conserved residues had been replaced were prepared by the site-directed mutagenesis. The mutants with replacements at another arginine residue, Arg-366, were also prepared because this residue is conserved among the mammalian enzymes. The mutants examined in this study are summarized in Figure 1C.

Expression of the wild type and mutants of human α1,6-fucosyltransferase in insect cells

The mutant α1,6-fucosyltransferases were expressed using a baculovirus–insect cell system, since α1,6-fucosyltransferase activity was not detected in Sf21 cells, the insect cells used in this study. Expression of the wild-type and mutant enzymes were verified by immunoblot analysis of cell homogenates of the baculovirus-infected Sf21 cells using a specific antibody (Figure 2). The expression levels of mutants were very similar to that of the wild type, and, as a result, these comparable expression levels allowed us to compare the activities of the wild type and mutants using cell homogenates.

Fig. 1. Comparison of amino acid sequences from α1,2- and α1,6-fucosyltransferases. (A) Dotplot of the sequences from the α1,2- and α1,6-fucosyltransferases. The amino acid sequences of human α1,2- and α1,6-fucosyltransferases were compared by a window size of 15 residues and 40% of the identity using the computer software program Align. The numbers beside the matrix indicate the residue number of each enzyme. Diagonal plots show the homologous regions which satisfy the above conditions. The sequences of the most homologous region are given outside the matrix. (B) Amino acid sequence alignment of the homologous regions of human α1,2- and α1,6-fucosyltransferases. The amino acid sequences in the homologous region of α1,6-fucosyltransferases and α1,2-fucosyltransferases from various species were aligned by Clustal. The amino acid residues which are conserved in all mammalians are indicated by shaded boxes. The basic amino acids conserved and subjected to mutational analysis are indicated by the arrowheads. (C) The amino acid sequences of the α1,6-fucosyltransferase mutants. The mutated sequences of the region examined in this study are listed. Bold letters are substituted amino acids for the wild-type sequence.
Enzyme activities of the wild-type and mutant α1,6-fucosyltransferases

A high activity was found in the wild-type and H363A mutant enzymes, while significantly lower activities were detected in the Arg-366-substituted mutants (Table I). However, the mutants with substitutions at Arg-365 exhibited no activity, as well as control noninfected cells (Figure 3). Even when the homogenate which was added to the assay mixture was 100 times more concentrated, no activity was detected in the mutants. These results suggest that Arg-365 is required for enzyme activity, and that the neighboring arginine, Arg-365, also plays an important role in the activity even though it is not essential. Interestingly, the replacement of Arg-365 by Lys also resulted in a complete loss of activity even though a positive charge was retained after the substitution. This indicates the absolute requirement of the guanidino group of the arginine. It was also found that His-363 plays no significant role in catalysis in spite of the fact that it is perfectly conserved.

Kinetic analysis of the wild-type and mutant α1,6-fucosyltransferases

The R366A and R366K mutants, which retained activity, were subjected to kinetic analysis in order to explore the role of the arginine residue (Figure 4, Table II). The kinetic analysis showed that the replacement of Arg-366 led to the distinct kinetic properties from that of the wild type. Apparent $K_m$ values for both GDP-fucose as a donor and the oligosaccharide-Asn-PABA as an acceptor were increased by the replacements when the parameters were determined at the fixed concentration of either of the donor or acceptor. Since apparent $V_{max}$ determined by relatively high concentration of GDP-
fucose and various acceptor concentrations was not markedly changed by the replacements, it is suggested that the arginine residue would be involved in the binding of the substrates rather than the catalysis. In addition, since essentially no difference in the apparent parameters was observed between the R366A and R366K mutants, it seems unlikely that lysine can take the place of the arginine. When the kinetic analysis by varying the concentrations of both donor and acceptor substrates was carried out to assess the effects of the substitutions on the enzymatic properties by comparison of true $K_m$ values, it was found that the mutants follow the distinct reaction mechanism from that of the wild type; a ping-pong Bi-Bi mechanism for the wild type and a sequential mechanism for both mutants (data not shown). Since $K_m$ values based on the distinct reaction mechanisms have different meanings, the $K_m$ of the mutants could not be compared with that of the wild type in order to quantitatively argue the change in the binding affinity.

Inhibition studies by GMP and GDP

α1,2- and α1,6-Fucosyltransferases do not share a common oligosaccharide as their acceptor substrates, but utilize the common donor substrate, GDP-fucose. Therefore, it is more likely that the effects of the amino acid replacements at Arg-366 are primarily due to an impairment in the binding of the donor substrate but not of the acceptors. Hence, to further examine the roles of the arginine residue in interactions with the donor, the effects of its nucleotide moieties on the activity were investigated in terms of the competitive inhibition against the donor substrate. Both GDP and GMP inhibited the wild-type and the mutants with replaced Arg-366 in a competitive manner, as found in another class of fucosyltransferase (Murray et al., 1996) (Figures 5, 6). The $K_i$ values for these nucleotides were determined using variable concentrations of the donor and the inhibitors in the presence of a fixed concentration of acceptor (5 µM agalacto biantennary-Asn-PABA). The symbols used are solid circles for the wild type, open circles for R366A, and solid triangles for R366K. Velocities were normalized for a maximal velocity for each enzyme.

### Table I. Specific activities of the wild-type and mutant α1,6-fucosyltransferases expressed in the baculovirus-infected SF21 cells

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity $(\text{nmol/h/mg protein})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>ND$^a$</td>
</tr>
<tr>
<td>Wild type</td>
<td>280</td>
</tr>
<tr>
<td>H363A</td>
<td>190</td>
</tr>
<tr>
<td>R365A</td>
<td>ND$^a$</td>
</tr>
<tr>
<td>R366A</td>
<td>8.9</td>
</tr>
<tr>
<td>R365A/R366A</td>
<td>ND$^a$</td>
</tr>
<tr>
<td>R365K</td>
<td>ND$^a$</td>
</tr>
<tr>
<td>R366K</td>
<td>7.3</td>
</tr>
<tr>
<td>R365K/R366K</td>
<td>ND$^a$</td>
</tr>
</tbody>
</table>

$^a$Determined with 500 µM GDP-fucose and 5 µM agalacto biantennary-Asn-PABA.

$^b$Not detectable.

### Table II. Kinetic parameters for the wild-type and Arg-366-substituted mutants

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>GDP-fucose $^a$</th>
<th>Agalacto-biantennary-Asn-PABA $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$appV_{max}$ (nmol/h/mg)</td>
<td>$appK_m$ (µM)</td>
</tr>
<tr>
<td>Wild type</td>
<td>290</td>
<td>32</td>
</tr>
<tr>
<td>R366A</td>
<td>13</td>
<td>330</td>
</tr>
<tr>
<td>R366K</td>
<td>13</td>
<td>360</td>
</tr>
</tbody>
</table>

$^a$Parameters were determined in the presence of 5 µM of the acceptor substrate.

$^b$Parameters were assessed with 500 µM GDP-fucose.

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Fig. 4. Kinetic analysis of the wild-type and mutant α1,6-fucosyltransferases. (A) The activity was determined using variable concentrations of the donor substrate, GDP-fucose in the presence of a fixed concentration of acceptor (5 µM agalacto biantennary-Asn-PABA). (B) Assayed with variable concentrations of the acceptor and a fixed concentration of the donor, 500 µM GDP-fucose. The symbols used are solid circles for the wild type, open circles for R366A, and solid triangles for R366K. Velocities were normalized for a maximal velocity for each enzyme.
Substrate-binding residues in human α,1,6-fucosyltransferase

Inhibition of the wild-type and Arg-366-substituted mutant enzymes by GDP. Left panels, double reciprocal plots obtained with variable concentrations of GDP-fucose and 5 µM acceptor. The numbers in the panels indicate the inhibitor (GDP) concentration expressed in µM. Right panels, re-plots of $K_i/V_{max}$ for each concentration of GDP as the function of GDP. $K_i$ values for GDP were determined from the x-axis intercept.

**Table III.** $K_i$ values for GDP and GMP in the wild-type and Arg-366-substituted mutants

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>GDP (µM)</th>
<th>GMP (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1.2</td>
<td>3.8</td>
</tr>
<tr>
<td>R366A</td>
<td>140</td>
<td>2.1</td>
</tr>
<tr>
<td>R366K</td>
<td>52</td>
<td>0.92</td>
</tr>
</tbody>
</table>

Fig. 5. Inhibition of the wild-type and Arg-366-substituted mutant enzymes by GDP. Left panels, double reciprocal plots obtained with variable concentrations of GDP-fucose and 5 µM acceptor. The numbers in the panels indicate the inhibitor (GDP) concentration expressed in µM. Right panels, re-plots of $K_i/V_{max}$ for each concentration of GDP as the function of GDP. $K_i$ values for GDP were determined from the x-axis intercept.

Fig. 6. Inhibition of the wild-type and mutant enzymes by GMP. Left panels, double reciprocal plots obtained with variable concentrations of GDP-fucose and 5 µM acceptor. The numbers in the panels indicate the inhibitor (GDP) concentration expressed in mM. Right panels, re-plots of $K_i/V_{max}$ for each concentration of GMP as the function of GMP. $K_i$ values for GMP were determined from the x-axis intercept.

Discussion

In this study, Arg-365 has been identified as an essential residue for the activity of α,1,6-fucosyltransferase, and it is also shown that the neighboring arginine residue, Arg-366, would play an important role in the binding of the substrates. Furthermore, it is suggested that Arg-366 interacts with the β-phosphoryl group of GDP-fucose. It seems more likely that the essential residue, Arg-365, also functions at least as a substrate-binding residue in a similar manner to the neighboring arginine, even though the role(s) of the arginine could not be explored by the kinetic analyses because of the complete loss of activity in its mutants.

Although it is entirely possible that the catalytic mechanism of α,1,6-fucosyltransferases is similar to that of α,1,2-fucosyltransferase and involves these conserved residues, it was found that Arg-366, a conserved residue, is not a significant participant in the catalysis of the enzyme. α,1,6-Fucosyltransferase shares only a common donor substrate, GDP-fucose, with α,1,2-fucosyltransferase, but not a common acceptor. Therefore, it can reasonably be argued that the residues play a significant role, primarily in interactions with the donor, rather than with acceptors.

The nature of the interaction of the arginine residues of α,1,6-fucosyltransferase with the nucleotide sugar is not clearly known. In general, enzymes which bind nucleotides, such as, for example, kinases, appear to frequently contain the positively charged amino acid residues, Lys and Arg in the nucleotide-binding site, and these residues interact directly with diphasphate or triphosphate groups of the substrates in an electrostatic manner. Furthermore in some enzymes or proteins, even a mutation which retains a positive charge, Arg-to-Lys or Lys-to-Arg, at such a residue, interaction with the nucleotide...
leads to the abolition of binding (Shen et al., 1991; Li et al., 1995; Chan and Gill, 1996; Tohgo et al., 1997; Kazuta et al., 1998). Thus, an electrostatic interaction would be the most likely interaction between the arginine residue identified in the enzyme and the nucleotide sugar. The character specific to arginine such as the capability of forming two hydrogen bonds, the bulkiness of the guanidino group, and a wider distribution of the positive charge may be of critical importance for interaction of the enzyme with the donor substrate.

In many glycosyltransferases requiring a metal ion such as Mn2+ for reaction, the metal ion is believed to allow the diphosphate moiety of the nucleotide sugar to coordinate and thus facilitate the binding to the enzymes (Powell and Brew, 1976a,b; Andree and Berliner, 1980; Boeggeeman et al., 1995). In addition, the divalent metal ion might possibly play a role, even as an electrostatic catalyst, stabilizing the negative charge which develops on the cleavage of the donor nucleotide. On the other hand, α1,6-fucosyltransferase, α1,2-fucosyltransferase, and certain other glycosyltransferases do not require a divalent cation for reaction, and thus it is conceivable that a positively charged residue, probably arginine in this case, substitutes for the divalent metal. The absolute requirement of Arg-365 could be explained by its involvement both in substrate binding and catalysis, and, in this case, the residue might possibly serve as a substitute for the divalent metal ion. A more detailed characterization of the Arg-365-substituted mutant of α1,6-fucosyltransferase may contribute to our understanding of the catalytic mechanism of divalent metal-independent glycosyltransferases.

Materials and methods

Materials

Restriction endonuclease and DNA modifying enzymes were purchased from Takara, TOYOBO and New England Biolabs. Oligonucleotide primers were synthesized by Greiner Japan. Other common chemicals were from Wako pure chemicals or Nacalai tesque.

Construction of transfer plasmids

Human α1,6-fucosyltransferase cDNA, which was previously cloned in our laboratory (Yanagidani et al., 1997), was excised from the pBluescript, and inserted into a transfer vector, pVL1393, by BamHI and EcoRI sites. The resultant plasmids were purified by a Qiagen plasmid purification kit, and then subjected to the transfection experiments.

Site-directed mutagenesis

Site-directed mutagenesis was carried out according to Kunkel (Kunkel, 1985), as described previously (Ikeda et al., 1995a). A 0.5 kb fragment obtained by digestion of human α1,6-fucosyltransferase cDNA with HindIII was subcloned into pBluescript KS+, and the resulting plasmid was used for transformation of CJ236 (ung, and the resulting plasmid was used for transformation of CJ236 with HindIII was subcloned into pBluescript GTCCATGTCGCGCGCACAGAC-3′ for the double replacement of Arg-365 by Ala and Arg-366 by Ala (R365A/R366A), 5′-GGAGTCCATGTCGCGCGCACAGACAAAGTG-3′ for the double replacement of Lys by Arg-365 and Lys by Arg-366 (R365L/R366L), and 5′-GGAGTCCATGTCGCGCGCACAGACAAAGTG-3′ for the double substitution of Lys for Arg-365 and Arg-366. The resulting mutations were verified by dyeoxy sequencing using a DNA sequencer (Applied Biosystems, model 373A), as were the entire sequences which had been subjected to mutagenesis. The corresponding region of the wild-type α1,6-fucosyltransferase cDNA was replaced by each mutant sequence. The transfer plasmids for these mutant enzymes were constructed in a manner similar to that of the wild-type enzyme, and used for transfection.

Cell culture and general manipulation of viruses

Spodoptera frugiperda (Sf) 21 cells were maintained at 27°C in Grace’s insect media (GIBCO-BRL) supplemented with 10% fetal bovine serum, 3.33 g/l yeastolate, 3.33 g/l lactalbumin hydrolysate, and 100 mg/l kanamycin. Recombinant viruses were manipulated as described (Piwnica-Worms, 1987).

Preparation of recombinant viruses

The purified transfer plasmids containing the wild-type or mutant α1,6-fucosyltransferase (1 μg) were cotransfected into 5 × 10^6 Sf21 cells with 10 ng of BaculoGold DNA (Pharmingen). Transfection experiments were carried out by the Lipofectin (GIBCO-BRL) method (Felgner et al., 1987), as described previously (Ikeda et al., 1995b,c). Media containing the recombinant viruses generated by homologous recombinations were collected 6 days after transfection. The recombinant viruses were further amplified to more than 5 × 10^7 plaque forming units/ml prior to use.

Electrophoresis and immunoblot analysis

SDS-PAGE analysis was carried out on 10% gels, according to Laemmli (Laemmli, 1970). The separated proteins were transferred onto PVDF membrane, and the resultant blot was blocked by 5% skim milk. The membrane was reacted with the anti-peptide antibody specific to porcine and human α1,6-fucosyltransferases, followed by reaction with a horseradish peroxidase–conjugated anti-rabbit IgG-antibody. The reactive bands were visualized by an ECL kit (Amersham).

Activity assay

α1,6-Fucosyltransferase activity was assayed using a fluorescein-labeled sugar chain substrate, according to the method of Uozumi (Uozumi et al., 1996b). An agalacto-biantennary sugar chain labeled with pyridylaminobutylamine was used as an acceptor substrate. Cell homogenates were incubated at 37°C with 5 μM of the acceptor substrate and 0.5 mM GDP-fucose as a donor in 0.1 M MES-NaOH, 1% Triton X-100 (pH 7.0). The reactions were terminated by boiling after an appropriate reaction time, and the mixtures were centrifuged at 10,000 × g in a microcentrifuge for 10 min. The resulting supernatants were injected to a reversed phase HPLC equipped with TSKgel, ODS 80TM (4.6 × 150 mm). The product and the
substrate were separated isocratically with 20 mM ammonium acetate buffer (pH 4.0) containing 0.15% n-butanol. Fluorescence of the column eluate was detected with fluorescence detector (Shimazu, model RF-10AXL) at excitation and emission wavelengths of 320 nm and 400 nm, respectively.

**Kinetic analysis and inhibition study with nucleotides**

Kinetic analysis was carried out under essentially the same conditions as used above with the exception of the concentration of the substrates. When the kinetic parameters for GDP-fucose were assessed, the concentration of the fluorescence-labeled acceptor, 5 μM, was used with variable concentrations of the donor. For the determination of parameters for the acceptor substrate, the concentration of GDP-fucose was fixed at 0.5 mM. Apparent kinetic parameters for these substrates and fixed concentration wavelengths of 320 nm and 400 nm, respectively.

**Protein determination**

Protein content was determined according to the method of Bradford (Bradford, 1976).

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**Abbreviations**

PABA, 4-(2-pyridylamino)butylamine; GDP-fucose, guanosinediphospho-fucopyranoside; GDP, guanosine diphosphate; GMP, guanosine monophosphate.

**References**


