Structure/function study of Lewis α3- and α3/4-fucosyltransferases: the α1,4 fucosylation requires an aromatic residue in the acceptor-binding domain

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All vertebrate α3- and α3/4-FUTs possess the characteristic acceptor-binding motif VxxHH(W/R)(D/E). FUT6 and FUTb enzymes, harboring R in the acceptor-binding motif, transfer fucose in α1,3 linkage, whereas FUT3 and FUT5 enzymes with W at the candidate position can also transfer fucose in α1,4 linkage—FUT3 being more efficient than FUT5. To determine the involvement of the W/R residue in acceptor recognition, we produced 34 variants of human FUT3, FUT5, FUT6, and ox FUTb Lewis enzymes. Among the FUT3 variants where W111 was replaced by the other amino acids, only enzymes with an aromatic residue at the candidate position kept about 50% of the α1,4 activity and showed no changes in Km values for GDP-Fuc donor and H-type 1 acceptor substrates. All other substitutions produced enzymes with less than 20% of the α1,4 activity. Thus the ability of α3/4-FUTs to recognize type 1 substrates involves the aromatic character of W in the acceptor-binding domain. The α1,3 activity of FUT6 and FUTb significantly decreased when their R residue was substituted by basic or charged residues. Moreover, FUT3 and FUT5 variants with W→R substitution had a better affinity for H-type 2 substrate and higher α1,3 activities. Therefore the optimal fucose addition in α1,3 linkage requires the R residue in the acceptor-binding motif of Lewis FUTs.

Key words: aromatic amino acid/acceptor substrate specificity/conserved peptide motifs/Lewis fucosyltransferase/site-directed mutagenesis

Introduction

All α3-fucosyltransferases (FUTs) transfer fucose from the GDP-fucose donor substrate to GlcNAc residues belonging to the core unit of N-glycans or peripheral lactosamine structures of glycoconjugates. Owing to its positions in glycans, fucose plays a significant role in many biological processes (for reviews, see Staudacher et al., 1999; Becker and Lowe, 2003). In Vertebrates, although several α3-fucosyltransferase genes exist in the same genome, all the corresponding enzymes transfer fucose in α1,3 linkage on peripheral GlcNAc of type 2 acceptor substrates (Galβ1,4GlcNAc). This is the case for Homo sapiens, where FUT3, 4, 5, 6, 7, and 9 correspond to α3-FUTs (Kaneko et al., 1999; Staudacher et al., 1999). However, these enzymes can be distinguished by their different tissue distributions and by their distinct affinities for type 2 substrates (Cameron et al., 1995; Kaneko et al., 1999; Mollicone et al., 1999). Among α3-FUTs, FUT3 and FUT5, two members of the Lewis FUT family, also transfer fucose in α1,4 linkage on type 1 substrates (Galβ1,3GlcNAc) and were consequently named α3/4-FUTs. They are involved in Lewis blood group antigen synthesis, such as Lea and Leb antigens (Costache et al., 1997a; Oriol, 1995). FUT5 enzymes have a predominant α1,4 activity, whereas FUT5 enzymes add fucose with the same efficiency on type 1 and type 2 acceptors (Costache et al., 1997b). In the animal kingdom, the ability to link fucose in α1,4 linkage has been proved for Primates (Dupuy et al., 2002), whereas this activity is widely spread in the plant kingdom (Costa et al., 2002; Fitchette et al., 1999). α1,4 fucosylation is even detected in the Helicobacter pylori strain UA948 (Rasko et al., 2000), but it would rather result from a lateral gene transfer.

The α3- and α3/4-FUTs have a putative common type II structure (Figure 1). Comparison of their peptide sequences localizes most of the amino acid differences in the amino-terminal part (variable segment), whereas numerous identities are present in the carboxy-terminal portion (constant segment). Four highly conserved sequences were described all along the peptide sequence (Figure 1). Motifs I and II are localized in the constant segment and are specific to α3- and α3/4-FUTs (Oriol et al., 1999). In the variable segment, motif III contains the first amino acids required for the correct protein folding and catalysis (Dupuy et al., 2002). Indeed, amino acid deletions occurring in motif III of human FUT3 and FUT5 gave inactive enzymes (Xu et al., 1996). Conversely, deletions upstream of motif III did not alter the activity (Xu et al., 1996), although mutations in this region disturbed the subcellular localization of the enzyme (Sousa et al., 2003). The fourth motif, called the acceptor-binding motif (abm), intercedes in acceptor substrate binding and specificity (Dupuy et al., 1999; Sherwood et al., 2002).

Without any α3- or α3/4-FUT structure, FUT3-FUT5-FUT6 Lewis enzymes were mainly studied to identify the key amino acids involved in enzyme specificity and activity (for review, see De Vries et al., 2001). Indeed, although these enzymes show more than 85% peptide sequence conservation, they possess different acceptor substrate affinities and specificities. Structure–function studies have demonstrated

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that some residues in the constant domain are involved in
GDP-fucose recognition (Holmes et al., 1995; Sherwood
et al., 2000), catalysis (Sherwood et al., 1998; Vo et al.,
1998), or in disulfide bonding (Holmes et al., 2000).
Alternatively, residues of the variable segment could determine
α3- and α3/4-FUT acceptor substrate specificity. Eleven
amino acids (Figure 1) were identified as potential can-
didates for the determination of the type 1 acceptor substrate
specificity of human FUT3 (Legault et al., 1995). Down-
stream of the motif III, two other residues, H73 and I74 of
human FUT3, were found to be necessary for fucose trans-
fer on type 1 acceptors (Nguyen et al., 1998). In a previous
work, we showed that among the residues belonging to the
acceptor-binding motif VxxHH(W/R)(D/E), where x corre-
sponds to a hydrophobic residue, the W residue conserved
for α3/4-FUTs and the R residue conserved for α3-FUTs
are involved in acceptor substrate specificity (Dupuy
et al., 1999). The single substitution of W111 by R in the
VxxHHWD motif of human FUT3 (primarily an α4-FUT)
changed the specificity of fucose transfer from H-type 1 to
H-type 2. This change is associated with affinity increase
and decrease for H-type 2 and H-type 1 acceptors, respec-
tively (Dupuy et al., 1999). Characterization of new primate
Lewis enzymes confirmed the relationship between the
amino acid composition of this motif and the Lewis FUT
acceptor specificity (Dupuy et al., 2002). Recently,
Sherwood et al. (2002) reported that the two conserved H
residues of this motif are necessary for optimal activity and
acceptor binding.

The present study focuses on the W/R residue that fol-
lowed the conserved H doublet in the highly conserved
acceptor-binding motif of vertebrate α3- and α3/4-FUTs.
To determine its involvement in α3- and α3/4-FUT activ-
ities, we produced, by site-directed mutagenesis at this posi-
tion, 34 mutated forms of human FUT3, FUT5, FUT6, and
ox FUTb. Characterization of these enzymes demonstrated
that significant α1,4 fucosylation requires an aromatic resi-
due at the position of W/R residue. Conversely, α1,3 fuco-
sylation is optimal only when an R residue is present at the
candidate position.

Results

Acceptor-binding motif of vertebrate α3- and α3/4-FUTs

Acceptor substrate specificities of α3- and α3/4-FUTs
partly depend on amino acids localized between motifs I
and III of these enzymes. The alignment of 15 vertebrate
α3- and α3/4-FUT sequences helped to define the VxHHH
(W/R)(D/E) consensus acceptor-binding motif (Dupuy
et al., 1999). Since then, several new α3- and α3/4-FUTs
from a broad panel of species have been identified on the
basis of their activities and their sequences (Table 1). For
this study, we cloned and characterized new simian Lewis-
like FUTs (underscored in Table 1). In all these new verte-
brate sequences, the characteristics of the acceptor-binding
motif were retrieved that is, a V residue, followed by
two variable hydrophobic amino acids, two conserved
H residues, a W or R residue, and an acidic residue. As
expected, enzymes transferring fucose only in α1,3 linkage
have R in their acceptor-binding motif, whereas enzymes
also able to transfer fucose in α1,4 linkage have W at the
same position.

Involvement of W residue of the acceptor-binding motif
in α1,3- and α1,4-FUT activities

FUT3 enzymes transfer fucose with α1,4 linkage and, less
efficiently, with α1,3 linkage. Their higher α1,4 activity
is explained by their better affinity for type 1 substrates.
To establish the role of W residue of the acceptor-binding
motif in acceptor substrate specificity, the W111 of human
FUT3 was alternately substituted by the 19 other amino
acids. Activities of wild-type and mutated enzymes were
determined using H-type 1 (Fuc(1→2)Galβ1,3GlcNAc)
and H-type 2 (Fuc(1→2)Galβ1,4GlcNAc) trisaccharides as
acceptor substrates, because they produce the best activity
(Costache et al., 1997b; Dupuy et al., 1999) and make it
possible to analyze only α1,4 and α1,3 fucose addition on
GlcNAc, respectively. Substitutions of W111 by aromatic
residues (Y or F) decreased the α1,4 activity of the enzyme
to 58% (W111→Y) and 42% (W111→F) of the wild-type
activity (Figure 2A). Four other mutants (W111→A, W111→M, W111→S, and W111→H) conserved 10–20% of the wild-type α1,4 activity, whereas replacements of W111 by E, D, or K produced enzymes with undetectable α1,4 activity. Alternatively, all changes generated enzymes with a weak α1,3 activity except the W111→R substitution, which changed type 1 acceptor specificity of human FUT3 toward type 2, giving an enzyme with preferential α1,3 activity (equivalent to 60% of wild-type α1,4 activity, Figure 2A). In all cases, the recombinant enzymes were efficiently expressed in COS-7 cells because wild-type and mutated forms of FUT3 were similarly detected by western blot analysis using anti-human FUT3 antibodies (Figure 2B).

The detection of two forms could correspond to two differently glycosylated FUT3 enzymes (Christensen et al., 2000).

Except for the rhesus macaque FUT5, which has R instead of W at the candidate position and only uses type 2 substrates (Dupuy et al., 2002), FUT5 enzymes use type 1 and type 2 acceptor substrates. Their substrate specificities differ from FUT3 ones in the sense that they more efficiently transfer fucose on H-type 2 acceptor substrates. Indeed, the α1,3 fucosylation of FUT5 enzymes is three times higher, compared to α1,4 fucosylation (Table II).

The peptide sequences of new vertebrate α3- and α3/4-FUTs are aligned by reference to human and ox Lewis enzymes, which are used in this study. The W/R residue, involved in the acceptor substrate specificity of vertebrate α3- and α3/4-FUTs (Dupuy et al., 1999), is in a white character on black background. The new simian Lewis-like sequences characterized for this study are underlined. nd, not determined.
The W124 in the acceptor-binding motif of human FUT5 was substituted by amino acids (which were brought out by the human FUT3 study) Y, F, and A to test modification in α1,4 activity, R to test modification in α1,3 activity, and V, which nearly cancels both activities. W124 substitutions by Y, A, or F residue produced, respectively, enzymes with 52%, 20%, and 17% of the wild-type α1,4 activity. Substitutions by R or V produced enzymes with undetectable α1,4 activity (Table II). The W124→Y/A/F replacements generated enzymes with similar (W→A) or weaker (W→Y/F) α1,3 activity compared to the wild-type one (Table II). Interestingly, the W124→V gave an enzyme with unchanged α1,3 activity, whereas the W124→R substitution produced an enzyme with a 3.8-fold increase of α1,3 activity (Table II). In these experiments, the expression levels of the modified proteins were similar to the wild-type one, as estimated by western blot (data not shown).

### Involvement of R residue of the acceptor-binding motif in α1,3-FUT activity

The involvement of R residue in α1,3 fucosylation was tested on the human α3-FUT FUT6. The R110 of human FUT6 was replaced by only two other basic residues (K and H), two charged amino acids (N and Q), and by W. Substitutions by H, N, K, and Q generated enzymes with less than 10% of the wild-type α1,3 activity and with undetectable α1,4 activity (Table III). These decreases in activity were not due to different expression levels of mutated enzymes in COS-7 cells (Figure 3). Replacement of R110

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>α1,4 Fucosylation (H-type 1 substrate)</th>
<th>α1,3 Fucosylation (H-type 2 substrate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FUT5</td>
<td>29</td>
<td>100</td>
</tr>
<tr>
<td>FUT5 W124→Y</td>
<td>15</td>
<td>59</td>
</tr>
<tr>
<td>FUT5 W124→A</td>
<td>6</td>
<td>92</td>
</tr>
<tr>
<td>FUT5 W124→F</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>FUT5 W124→V</td>
<td>0</td>
<td>102</td>
</tr>
<tr>
<td>FUT5 W124→R</td>
<td>0</td>
<td>380</td>
</tr>
</tbody>
</table>

Activities were measured after either 3 h or 1 h incubation with H-type 1 or H-type 2 acceptor substrates, respectively. 100% activity corresponds to 1382 pmol/h/mg protein and control to crude protein extract from COS-7 cells transfected by pcDNA1/Amp alone.
by W in human FUT6 led to an enzyme without α1,3 and α1,4 activities (Table III).

The same experiment was performed with FUTb, an α3-FUT encoded by the ox futb Lewis gene (Oulmouden et al., 1997). Indeed, futb evolved from an ancestor gene which duplicates in primate lineage to form the Lewis gene family (Dupuy et al., 2002; Oulmouden et al., 1997). Mutations of R115, in the VxxHHRE acceptor-binding motif, by H, N, K, or Q residue gave recombinant enzymes with less than 10% of wild-type α1,3 activity (data not shown), results quite similar to that obtained for mutated forms of human FUT6. The substitution by W led to poor α1,3 and α1,4 activities, as has already been described (Dupuy et al., 1999). Therefore, none of the amino acids tested at the position of the W/R in the acceptor-binding motif of α3-FUTs generated enzymes that efficiently transfer fucose in α1,3 and/or α1,4 linkages.

**Kinetic parameters of mutated enzymes**

The kinetic parameters of human FUT3 and FUT5 wild-type enzymes, and some of their mutated forms that present strong activity, were determined for the nucleotide sugar donor substrate and for the best acceptor substrate (Table IV). The results showed that the apparent \( K_m \) values for GDP-fucose were similar for wild-type and mutated FUT3 and FUT5 enzymes. Consequently, aminoacid changes at the W position in the acceptor-binding motif did not modify the affinity of the mutated enzymes for the donor substrate. Significant changes in the \( K_m \) values for acceptor substrates were observed compared to the wild-type enzymes for some FUT3 and FUT5 variants. The FUT3 mutated enzymes with W111→Y or F substitutions had slightly higher \( K_m \) values for H-type 1 acceptor oligosaccharide, and the W111→A change generated a 10-fold increase of the \( K_m \) value for the same acceptor. In FUT5, the W124→Y mutation produced an enzyme with a \( K_m \) value for H-type 2 acceptor oligosaccharide close to that of the wild type. However, the mutated FUT5 enzyme with W124→R

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**Table III. α1,3- and α1,4-FUT activities of human FUT6 and its mutated forms**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>α1,4 Fucosylation (H-type 1 substrate)</th>
<th>α1,3 Fucosylation (H-type 2 substrate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FUT6</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>FUT6 R110→H</td>
<td>0</td>
<td>8.8</td>
</tr>
<tr>
<td>FUT6 R110→N</td>
<td>0</td>
<td>7.2</td>
</tr>
<tr>
<td>FUT6 R110→K</td>
<td>0</td>
<td>3.8</td>
</tr>
<tr>
<td>FUT6 R110→Q</td>
<td>0</td>
<td>3.8</td>
</tr>
<tr>
<td>FUT6 R110→W</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Activities were measured after either 3 h or 1 h incubation with H-type 1 or H-type 2 acceptor substrates, respectively. 100% activity corresponds to 1215 pmol/h/mg proteins and control to crude protein extract from COS-7 cells transfected by pcDNA1/Amp alone.

**Table IV. Kinetic parameters of human FUT3, FUT5, and some of their mutated variants**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Donor substrate (GDP-fucose)</th>
<th>Acceptor substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_m ) (µM)</td>
<td>( V_{max} ) (nmol/h/mg protein)</td>
</tr>
<tr>
<td>H-type 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FUT3</td>
<td>35</td>
<td>21</td>
</tr>
<tr>
<td>FUT3 W111→Y</td>
<td>34</td>
<td>10</td>
</tr>
<tr>
<td>FUT3 W111→F</td>
<td>37</td>
<td>10</td>
</tr>
<tr>
<td>FUT3 W111→A</td>
<td>46</td>
<td>1</td>
</tr>
<tr>
<td>H-type 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FUT5</td>
<td>38</td>
<td>1</td>
</tr>
<tr>
<td>FUT5 W124→Y</td>
<td>35</td>
<td>2</td>
</tr>
<tr>
<td>FUT5 W124→R</td>
<td>30</td>
<td>7</td>
</tr>
</tbody>
</table>

Kinetic constants were determined as described in Materials and methods. Incubation times were 1 h for wild-type and mutated FUT3 enzymes, 1.5 h for FUT5 and FUT5 W124→Y, and 30 min for FUT5 W124→R.
substitution had a 10-fold greater affinity for the H-type 2 substrate. Thus compared to wild-type enzymes, the affinities of FUT3 and FUT5 variants toward their best acceptor substrates are in close correlation with their levels of activity (Figure 2, Table II, and Table IV).

Discussion

Numerous species possess several distinct α3- and/or α3/4-FUTs, which transfer fucose with α1,3 and/or α1,4 linkage from the same nucleotide sugar donor, the GDP-fucose. However, along with other differences, they differ in their acceptor substrate specificities, which mainly depend on amino acids localized in their amino terminal segment (Legault et al., 1995; Nguyen et al., 1998; Sherwood et al., 2002; Xu et al., 1996). Peptide sequence alignment of these domains is a useful way to define conserved residues that are potentially involved in acceptor specificities. This strategy was successfully used to define the VxxHH(W/R)(D/E) conserved acceptor-binding motif (where x corresponds to a hydrophobic residue) in the NH2 domain of these enzymes (Dupuy et al., 1999). We demonstrated that W111 → R substitution conferred to human FUT3 the ability to use efficiently H-type 2 instead of H-type 1 acceptor substrate. This change in activity was related to affinity modifications toward acceptor substrates (Dupuy et al., 1999). Since then, new FUTs that add fucose in α1,3 and/or in α1,4 linkage on lacto- or neolacto-series structures have been isolated from Vertebrates.

In the present work, we showed that all these vertebrate enzymes are characterized by the consensus VxxHH(W/R)(D/E) acceptor-binding motif and their α1,3 or α1,3/4 activities are correlated with the presence of W or R, respectively. Although required for optimal activity, amino acids in this motif are not equally involved in the acceptor substrate binding. For example, the V and the first H residues of human FUT4 α3-FUT are not directly involved in type 2 substrate binding (Sherwood et al., 2002). This suggests flexibility in the amino acid composition of the motif. Such flexibility is observed for Caenorhabditis elegans lactosamine α3-FUT (DeBose-Boyd et al., 1998) where the potential acceptor-binding motif is VLIAMD (Table V). Conservation of hydrophobic amino acids underscores their crucial role, potentially in protein folding. Also, the presence in C. elegans enzyme of M at the strictly conserved position of W/R residue could reflect the ability of this enzyme to transfer fucose to a wider variety of lacto- or neolacto-series structures (DeBose-Boyd et al., 1998), compared to vertebrate ones. For bacterial enzymes using type 1 and type 2 acceptor substrates (Ge et al., 1997; Rasko et al., 2000), no corresponding acceptor-binding motif is found. Ma et al. (2003) showed that amino acids involved in acceptor substrate specificity of Helicobacter pylori enzymes are located in their carboxy-terminal domain. Moreover, the bacterial α3/4-FUTs seem unrelated in evolution to the vertebrate ones.

In plants, α1,4 fucosylation and Leα structures are present. Several plant α4-FUTs have been cloned and characterized (Bakker et al., 2001; Léonard et al., 2002; Wilson, 2001), and their potential acceptor-binding motif identified (VAYKWD for A. thaliana, Table V). As in vertebrate α3/4-FUTs, the presence of W and D residues in the plant motif correlates with an α1,4-FUT activity of these enzymes. Conservative changes are observed for the hydrophobic residues—and the conserved H residues are substituted by the other basic amino acids, R or K. However, this motif is located closer to the amino-terminal end compared to animal enzymes. The hypothesis of a potential soluble enzyme devoid of transmembrane domain has been proposed and could account for this peculiar position in the sequence (Léonard et al., 2002). Although the exact role of

<table>
<thead>
<tr>
<th>Enzyme name species</th>
<th>GenBank Accession number</th>
<th>Acceptor-binding motif composition</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vertebrates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FUT3 human</td>
<td>X55578</td>
<td>101 VIVHHWD</td>
<td>α1,3/4</td>
</tr>
<tr>
<td>FUT5 human</td>
<td>M81485</td>
<td>102 VIVHHWD</td>
<td>α1,3/4</td>
</tr>
<tr>
<td>FUT6 human</td>
<td>L01698</td>
<td>103 VIVHHRE</td>
<td>α1,3</td>
</tr>
<tr>
<td>FUT1b ox</td>
<td>X87810</td>
<td>104 VIVHHRE</td>
<td>α1,3</td>
</tr>
<tr>
<td>Invertebrate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CEFT-I C. elegans</td>
<td>CAA91285</td>
<td>105 VLIAMD</td>
<td>α1,3</td>
</tr>
<tr>
<td>Plants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FUT4 A. thaliana</td>
<td>Q9C8W3</td>
<td>62 VAYKWD</td>
<td>α1,4</td>
</tr>
<tr>
<td>FUT4 L. esculentum</td>
<td>CAC85740</td>
<td>63 STFKWD</td>
<td>α1,4</td>
</tr>
<tr>
<td>FUT4 B. vulgaris</td>
<td>CAC44377</td>
<td>58 GAFFWD</td>
<td>α1,4</td>
</tr>
</tbody>
</table>

The invertebrate and plant sequences are aligned by reference to human Lewis FUT3 enzyme. The putative acceptor-binding motif of C. elegans enzyme was revealed by DIALIGN software, whereas the corresponding motif of plant enzyme was adjusted manually.

Table V. Peptide alignment of the putative acceptor-binding domain of Caenorhabditis elegans and plant α3- and α4-FUTs
the acceptor-binding motif in activity and substrate-binding in invertebrate and plant enzymes remains to be established, its involvement in acceptor substrate recognition by α3-, α3/4- and α4-FUTs is supported by its lack in core α3-FUTs, which use different acceptor substrates (Bakker et al., 2001; Fabini et al., 2001; Leiter et al., 1999).

In this study, we demonstrate that the ability of FUT3 enzymes to use type 1 substrates, and consequently their capacity to transfer fucose with α1,4 linkage, involves the aromatic character of the W residue in their acceptor-binding domain. Indeed, only enzymes with an aromatic residue (W, Y, or F) at the candidate position had significant α1,4 activity and high affinities for GDP-Fuc donor and H-type 1 acceptor substrates. Alternatively, we showed that the optimal α1,3 activity of Lewis FUTs could depend on the presence of R residue in the acceptor-binding motif. FUT3 improved its α1,3 activity only when W111 was substituted by R (Figure 2). In the same way, the only increase in α1,3 activity and affinity for H-type 2 acceptor of FUT5 was observed when W124 was replaced by R (Tables II and IV). Moreover, α1,3 activities of FUT6 and FUTb decreased when their R residue was substituted by basic or charged ones.

The correlation between acceptor substrate specificity and amino acid composition of the acceptor-binding motif is less obvious for FUT5 enzymes. They show W in their acceptor-binding motif but preferentially use H-type 2 trisaccharide substrates (Costache et al., 1997b). The mutated FUT5 with another aromatic residue (F or Y) at the candidate position had a lower α1,3 activity compared with the wild-type enzyme, whereas other substitutions (W124→A/V) allow the conservation of the original α1,3 activity. It is worth noticing that, as for W111→Y/F substitutions in FUT3, the W124→Y substitution in FUT5 gave an enzyme with around 50% of its wild-type α1,4 activity. We concluded that the type 1/type 2 substrate recognition by FUT5 enzymes involves amino acids of the acceptor-binding motif and one or several other residues of the catalytic domain. This hypothesis could also explain the lack of FUT6 α1,3/4 activities, when R110 was substituted by W.

The type 1 and 2 disaccharides have different molecular topographies. The minimum energy conformations of type 1 and 2 structures invert the relative orientation of Gal and GlcNAc residues of disaccharides by approximately 180°. The key polar groups of lacto- and neolacto-series acceptor substrates are hydroxyl groups at C-3 or C-4 of GlcNAc and C-6 of Gal (De Vries et al., 1997; Du and Hindsagul, 1996; Gosselin and Palcic, 1996). Because C-6 of Gal is necessary for activity whatever the acceptor substrate is, a correctly positioned Gal residue appears to be essential for enzyme activity. The active site of Lewis FUTs could consecutively form a pocket, which preferentially binds the GlcNAc residue of type 1 or type 2 acceptor substrates. It has been proposed that recognition of carbohydrate acceptors by glycosyltransferases occurred through hydrogen bonds and stacking of the sugar ring with aromatic amino acids (Hindsagul et al., 1991; Matsui et al., 1994; Vyas et al., 1991). Although W/R residue could also be involved in the protein folding, we hypothesize that the binding of type 2 acceptor substrates by α3-FUTs would partially rely on a hydrogen bond between the R residue of their acceptor-binding domain and a key polar group of the GlcNAc residue. Conversely, the presence of W residue in the acceptor-binding domain of α3/4-FUTs could allow the recognition of type 1 and type 2 substrates by the stacking of the acceptor GlcNAc ring with the cycle of W residue. The particular affinities of FUT5 enzymes toward type 1/type 2 substrates could be explained by a crucial interaction between a residue in FUT5 enzymes, which remains to be identified, and perhaps a reactive group of the acceptor substrate.

Genes encoding FUTs with α1,4 activity arose by duplications of an α3-FUT ancestor gene (for review, see Javaud et al., 2003). Among the different events that conduced to the appearance of α3/4-FUTs, the switch of the basic R to the aromatic W residue in the acceptor-binding motif was certainly a crucial point. The presence of W residue in α3/4-FUTs could be explained by a single point mutation changing R codon in W codon (CGG→TGG). The new data presented in this study gave support to our model of Lewis gene evolution in Primates (Dupuy et al., 2002), which proposed that α1,4 fucosylation appeared first in FUT3 and latterly in FUT5 independently. The differences between wild-type and mutated FUT3 and FUT5 enzymes concerning their proper α1,3/4 activities could then be explained by an independent process conferring the α1,4 function. Sequence comparison of a broad panel of Lewis enzymes is now needed to point out the amino acids, other than the W, in the acceptor-binding motif, conferring the original α1,4 fucosylation in the primate lineage.

Materials and methods

Cloning of simian Lewis-like coding sequences

The gorilla (Gorilla gorilla), orangutan (Pongo pygmaeus), and gibbon (Hylobates lar) Lewis-like coding sequences were obtained by a one-step polymerase chain reaction strategy (Dupuy et al., 2002) on genomic DNA. This DNA was isolated from blood samples obtained at the Centre de Primatologie (Université Louis Pasteur, Strasbourg, France). The monoexonic open reading frames were amplified using sense 5'-CTCTTCTCCCAGCTACTCTGACCCATG-3' and antisense 5'-GCCCAAGCCCCATGCCGGCCTCTC-3' primers. They were inserted into the pcDNA3.1/Amp mammalian expression vector (Invitrogen, Carlsbad, CA), associated with the ABI Prism 310 Genetic Analyser (Perkin Elmer, Norwalk, CT). Nucleotide sequence data are available in GenBank under the accession numbers AF515436 and AF515437 for G. gorilla FUT5 and FUT6 genes, AF515438 and AF515439 for P. pygmaeus FUT3 and FUT5 genes, and AF515440 for H. lar FUT5 gene.

Peptide alignment of α3-, α3/4-, and α4-FUTs

Alignments were performed with DIALIGN (http://bioweb.pasteur.fr/seqanal/interfaces/dialign2.html) and refined with further manual adjustments using the ED program (Philippe, 1993) of the MUST 2000 package (http://sorex.snv.jussieu.fr/must2000.html).
Site-directed mutagenesis

The different FUT coding sequences were inserted into the mammalian expression vector pcDNA1/Amp (Invitrogen) and directly used for polymerase chain reaction–based mutagenesis. All mutations were performed with the Quick-Change Kit (Stratagene, La Jolla, CA). The primers used for mutagenesis are summarized in the Table VI. The open reading frames containing mutations were completely checked as described.

Expression of wild-type and mutated enzymes

High pure recombinant plasmids (plasmid midi Kit, Qiagen, Hilden, Germany) were used to transiently transfect COS-7 cells during 48 h (SuperFect Transfection Reagent, Qiagen). These cells lack endogenous lactosamine α1,3- and α1,4-FUT activities (Clarke and Watkins, 1999). COS-7 proteins were extracted in 1% (v/v) Triton-X 100, 10 mM sodium cacodylate (pH 6), 20% (v/v) glycerol, 1 mM dithiothreitol under shaking for 2 h at 4°C. Cell debris was eliminated by centrifugation, and the protein supernatant content was determined (Bradford, BioRad, Hercules, CA).

FUT assays

Blood group H-type 1 (Fucα1,2Galβ1,3GlcNAc-sp-biotin) and H-type 2 (Fucα1,2Galβ1,4GlcNAc-sp-biotin) trisaccharide acceptor substrates were purchased from Synthesome (Munich, Germany). FUT assays were performed in 60 μl volume containing 0.1 mM acceptor substrate, 25 mM sodium cacodylate (pH 6.5), 5 mM ATP, 20 mM MnCl2, 10 mM α-L-fucose, 3 μM GDP-[14C]–fucose (310 mCi/mmol; Amersham Pharmacia Biotech, Little Chalfont, U.K.) and 20 μg protein extracts from transfected COS-7 cells. Activities were measured as previously described (Dupuy et al., 1999): the reaction was stopped by addition of 3 ml cold water, and the reaction mixture was then applied to a conditioned Sep-Pack C18 reverse chromatography cartridge (Waters Millipore, Bedford MA). Unreacted GDP-[14C]–fucose was washed off with water. The radiolabeled reaction product was eluted with ethanol and counted with Biodegradable Counting Scintillant (Amersham Pharmacia Biotech) in a liquid scintillation beta counter (Liquid scintillation analyzer, Tri-Carb-2100TR, Packard, USA).

Western blot analysis

Thirty micrograms of soluble proteins from COS-7 cells were boiled for 3 min after the addition of β-mercaptoethanol (5% v/v) and bromophenol blue (0.02% w/v). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out on Tris/Tricine-10.5% sodium dodecyl sulfate–polyacrylamide gel. Separated proteins were electro-transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The immunoblots were

Table VI. Primers used in polymerase chain reaction for site-directed mutagenesis

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Mutations</th>
<th>Primers (5’→3’)</th>
<th>Positions</th>
</tr>
</thead>
<tbody>
<tr>
<td>FUT3</td>
<td>W11→F/Y/C</td>
<td>s CGTCTACGCTGCCACACCATDCGATATACGTCCAGCC 314-349</td>
<td>314-349</td>
</tr>
<tr>
<td></td>
<td></td>
<td>as GTGATGGACATGATCTGGAAGTGCTGACGGAGCC 349-314</td>
<td></td>
</tr>
<tr>
<td></td>
<td>W11→L/P/H/Q</td>
<td>s CGTCTACGCTGCCACACCATGGAAGTGCTGACGGAGCC 314-349</td>
<td>314-349</td>
</tr>
<tr>
<td></td>
<td></td>
<td>as GTGATGGACATGATCTGGAAGTGCTGACGGAGCC 349-314</td>
<td></td>
</tr>
<tr>
<td></td>
<td>W11→M/S/T/N/R/I/K</td>
<td>s GTGATGGACATGATCTGGAAGTGCTGACGGAGCC 314-349</td>
<td>314-349</td>
</tr>
<tr>
<td></td>
<td></td>
<td>as GTGATGGACATGATCTGGAAGTGCTGACGGAGCC 349-314</td>
<td></td>
</tr>
<tr>
<td></td>
<td>W11→V/A/D/E/G</td>
<td>s GTGATGGACATGATCTGGAAGTGCTGACGGAGCC 314-349</td>
<td>314-349</td>
</tr>
<tr>
<td></td>
<td></td>
<td>as GTGATGGACATGATCTGGAAGTGCTGACGGAGCC 349-314</td>
<td></td>
</tr>
<tr>
<td>FUT5</td>
<td>W2M→F/Y</td>
<td>s CGTCTACGCTGCCACACCATDCGATATACGTCCAGCC 353-388</td>
<td>353-388</td>
</tr>
<tr>
<td></td>
<td></td>
<td>as GTGATGGACATGATCTGGAAGTGCTGACGGAGCC 388-353</td>
<td></td>
</tr>
<tr>
<td></td>
<td>W2M→R</td>
<td>s CGTCTACGCTGCCACACCATGGAAGTGCTGACGGAGCC 353-388</td>
<td>353-388</td>
</tr>
<tr>
<td></td>
<td></td>
<td>as GTGATGGACATGATCTGGAAGTGCTGACGGAGCC 388-353</td>
<td></td>
</tr>
<tr>
<td></td>
<td>W2M→A/V</td>
<td>s CGTCTACGCTGCCACACCATGGAAGTGCTGACGGAGCC 353-388</td>
<td>353-388</td>
</tr>
<tr>
<td></td>
<td></td>
<td>as GTGATGGACATGATCTGGAAGTGCTGACGGAGCC 388-353</td>
<td></td>
</tr>
<tr>
<td>FUT6</td>
<td>R10→W</td>
<td>s CGTCTACGCTGCCACACCATGGAAGTGCTGACGGAGCC 311-326</td>
<td>311-326</td>
</tr>
<tr>
<td></td>
<td></td>
<td>as CGGATGCTGACATGATCTGGAAGTGCTGACGGAGCC 326-311</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R10→K/H/Q/N</td>
<td>s GTGATGGACATGATCTGGAAGTGCTGACGGAGCC 311-346</td>
<td>311-346</td>
</tr>
<tr>
<td></td>
<td></td>
<td>as GTGATGGACATGATCTGGAAGTGCTGACGGAGCC 346-311</td>
<td></td>
</tr>
</tbody>
</table>

Primer bases in bold designate substituted nucleotides. Degenerated bases are annotated according to IUB abbreviations. Positions of primers are given with the adenine residue of the initiation codon of each coding sequence assigned as base 1. s: sense primer; as: antisense primer.
processed by chemiluminescence detection (Chemiluminescence Blotting Substrate [POD], Roche Molecular Biochemicals, Mannheim, Germany). Anti-human FUT3 and anti-ox FUTb antibodies were produced in rabbit using truncated proteins of FUT3 (P45–T361) and FUTb (R35–Q36), which were obtained in Escherichia coli BL21 (DE3). The blot was first incubated with rabbit anti-human FUT3 antibodies (4 µg/ml) for recombinant FUT3, FUT5, and FUT6 enzymes or rabbit anti-ox FUTb antibodies (2 µg/ml) for recombinant FUTb, then with the secondary antibody, a pig anti-rabbit IgG conjugated to horseradish peroxidase (dilution 1:1000) (Dako, Denmark).

Kinetic constant determination

The apparent $K_m$ values for GDP-fucose were determined using 10–200 µM GDP-fucose including 8 µM GDP-[14C]fucose in each reaction, 20–40 µg proteins, and 2 mM H-type 1 or H-type 2 acceptors. The incubation time was between 30 and 90 min. The apparent $K_m$ values for H-type 1 and H-type 2 acceptors were determined with 0.05–3 mM acceptor and 200 µM GDP-fucose, including 8 µM GDP-[14C]fucose and incubation times of 30–90 min.

Acknowledgments

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References

Bakker, H., Schijlen, E., de Vries, T., Schiphorst, W.E., Jordi, W., Lommen, A., Bosch, D., and van Die, I. (2001) Plant members of the α1,3/4-fucosyltransferase gene family encode an α1,4-fucosyltransferase, potentially involved in Lewis(a/b) biosynthesis, and two core α1,3-fucosyltransferases. FEBS Lett. 507, 307–312.


