Mutation of amino acids in the alpha 1,3-fucosyltransferase motif affects enzyme activity and $K_m$ for donor and acceptor substrates

Franziska Jost, Theodora de Vries, Ronald M.A. Knaettle, and Bruce A. Macher

Alpha 1,3-fucosyltransferases (FucT) share a conserved amino acid sequence designated the alpha 1,3 FucT motif that has been proposed to be important for nucleotide sugar binding. To evaluate the importance of the amino acids in this motif, each of the alpha 1,3 FucT motif amino acids was replaced with alanine (alanine scanning mutagenesis) in human FucT VI, and the resulting mutant proteins were analyzed for enzyme activity and kinetically characterized in those cases in which the mutant protein had sufficient activity. Two of the mutant proteins were inactive, six had less than 1% of wild-type activity, and four had ~10–50% of wild-type enzyme activity. Three of the mutant proteins with significant enzyme activity had substantially larger $K_m$ values for GDP-fucose than FucT VI wild-type enzyme. The fourth mutant protein with significant enzyme activity (S249A) had a $K_m$ at least 10 times larger than wild-type FucT VI for the acceptor substrate, with only a slightly larger (2–3 times) $K_m$ for GDP-fucose. Thus mutation of any of the amino acids within the alpha 1,3 FucT motif to Ala affects alpha 1,3-FucT activity, and substitution of Ala for some of the alpha 1,3 FucT motif amino acids results in proteins with altered kinetic constants for both the acceptor and donor substrates.

Secondary structure prediction suggests a helix–loop–helix fold for the alpha 1,3 FucT motif, which can be used to rationalize the effects of mutations in terms of 3D structure.

Key words: alanine scanning mutagenesis/alpha 1,3-fucosyltransferase motif/function/molecular modeling/structure

Introduction

$\alpha$1,3/4 Fucosyltransferases (FucTs) have been cloned from a variety of organisms, including human, mouse, and bacteria (De Vries et al., 2001, and references therein). Comparison of the amino acid sequences of the various FucTs from a single species reveals that there is a significant level of sequence conservation, especially in the catalytic domain (Oriol et al., 1999). For example, the six human FucTs share ~50% sequence identity within the catalytic domain and three of the human FucTs (III, V, and VI) have ~90% sequence identity. Comparison of FucT sequences among more divergent species demonstrates that there are fewer areas of sequence conservation and there is a single amino acid region in which 10 of 19 amino acids are conserved in all species (Martin et al., 1997). Martin and co-workers referred to this region as the alpha 1,3 FucT motif (Figure 1). Because all FucTs utilize a common donor substrate (GDP-fucose) but not the same acceptor substrate, it was hypothesized that the amino acids that make up the alpha 1,3 FucT motif may function in binding the donor substrate. However, there is little information regarding whether these residues are essential for enzyme activity or how they function. To investigate the importance of the amino acids of the alpha 1,3 FucT motif as described by Martin et al. (1997) we used the alanine scanning mutagenesis approach to determine if any of the alpha 1,3 FucT motif amino acids are required for enzyme activity and to obtain information on whether they are involved in substrate binding.

The alanine scanning mutagenesis approach was developed to elucidate the importance of the amino acid side chains that most strongly modulate the interaction between a protein and its ligand or substrates (Argos, 1988; Cunningham and Wells, 1989). Ala was chosen for scanning mutagenesis because it is the most abundant amino acid in proteins and is found both internally and at surface-exposed positions in proteins. In addition, Ala does not impose extreme electrostatic or steric effects. Ala eliminates the side chain beyond the $\beta$ carbon but does not change the main chain conformation. Cunningham and Wells (1989) have demonstrated that the substitution of a single Ala for an amino acid in a protein does not substantially alter the overall fold of the protein. Thus alanine scanning has been successfully used to evaluate the functional importance of single amino acid residues within a protein.

Applying the alanine scanning mutagenesis approach to an analysis of the alpha 1,3 FucT motif has shown that Ala substitution for residues within the motif with Ala results in a set of proteins that are either catalytically inactive proteins, have marginal fucosyltransferase activity, or have substantial enzymatic activity. Kinetic characterization of the mutant proteins with substantial enzymatic activity demonstrated that some of these proteins had altered $K_m$ values for the donor substrate, and one had an altered $K_m$ for the acceptor substrate. Thus, not all of the amino acids within...
the alpha 1,3 FucT motif affect the binding of the donor substrate.

Results

Martin et al. (1997) identified a 19-amino-acid region within alpha 1,3 FucTs that contains 10 residues that are conserved in all members of the family and defined this region as the alpha 1,3 FucT motif (Figure 1). In addition to these 10 amino acids, there are 2 amino acids in the alpha 1,3 FucT motif region as defined by Martin and co-workers that are conserved in most members of the family or have conservative amino acids substitutions at these sites in the sequence.

All studies were carried out with the catalytic domain of human FucT VI, which we refer to as wild type throughout this article. The motif’s amino acids as described by Martin et al. (1997) are spread over a segment 19 amino acids in length (residues 240–258) and a range of amino acids (including nonpolar, aromatic, basic, and acidic residues) are represented within the motif. Eight conserved residues occur at the N-terminus of the alpha 1,3 FucT motif, whereas four are located at the C-terminus, with four non-conserved amino acids (250–253) intervening. Four amino acids (E, F, K, and Y) occur twice in the motif, whereas others occur once.

Site-directed mutagenesis and expression of human FucT VI

To evaluate the functional importance of the amino acids of the alpha 1,3 FucT motif described by Martin et al. (1997), 12 mutant proteins were created by substituting Ala independently for each of the motif’s amino acids using site-directed mutagenesis. The proteins were purified and then characterized by western blot analysis as shown in Figure 2. Each mutant protein had ~ the same molecular weight as the wild-type enzyme (data not shown). Each mutant, except K241A, was produced at a level similar to that of FucT VI. The K241A mutant protein was produced at ~ one-tenth the level of wild-type enzyme (Figure 2).

Ala mutants of the alpha 1,3 FucT motif of human FucT VI have a range of enzymatic activities

The specific activity of each Ala mutant was quantified using LacNAc-C8 as an acceptor substrate under assay conditions that produce maximal activity for wild-type enzyme. As shown in Table I, the Ala mutant proteins could be subdivided into three categories based on their relative specific activity (i.e., specific activity of the mutant protein/specific activity of wild-type FucT VI multiplied by 100 and expressed as a percentage). No fucosyltransferase activity could be measured for two of the mutants; six had an enzyme activity that was less than 1% of wild-type enzyme; and four had activity ranging between ~ 10–50% of the wild-type enzyme.

Fig. 1. Alpha 1,3 FucT motif. The underscored amino acids represent the alpha 1,3 FucT motif as defined by Martin et al. (1997). Dashes represent amino acids in each sequence that are identical to those found in the human FucT III sequence, and dots indicate that there is no corresponding residue. In FucT VI the residues of the motif have the following residue numbers Y240 F242 L244 F246 E247 N248 S249 Y254 T256 E257 K258. Oriol et al. (1999) have designated the region from R239 to P270 as region II of the highly conserved sequences of alpha-3-fucosyltransferases (see Discussion). References for the various sequences are: Dupuy et al. (2002), Fabini et al. (2001), Kageyama et al. (1999), Lee et al. (1996), Letter et al. (1999), Martin et al. (1997), Patnaik et al. (2000), and Smith et al. (1996).

Fig. 2. Western blot analysis of Ala mutants of human α1,3 FucT VI.

Three separate western blots are shown, each contains a lane showing wild-type FucT VI for comparison. (A) Lane 1, wild-type FucT VI, lane 2, K241A, lane 3, F242A, and lane 4, Y254A. (B) Lane 1, wild-type FucT VI, lane 2, S249A, lane 3, T256A, lane 4, E257A. (C) Lane 1, wild-type FucT VI, lane 2, Y240A, lane 3, L244A, lane 4, F246A, lane 5, E247A, lane 6, N248A, lane 7, K258A.
Table I. Relative activity of FucT VI Alanine mutant enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>% Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>FucT VI Y240A</td>
<td>14</td>
</tr>
<tr>
<td>FucT VI K241A</td>
<td>&lt;1</td>
</tr>
<tr>
<td>FucT VI F242A</td>
<td>&lt;1</td>
</tr>
<tr>
<td>FucT VI L244A</td>
<td>7</td>
</tr>
<tr>
<td>FucT VI F246A</td>
<td>7</td>
</tr>
<tr>
<td>FucT VI E247A</td>
<td>&lt;1</td>
</tr>
<tr>
<td>FucT VI N248A</td>
<td>&lt;1</td>
</tr>
<tr>
<td>FucT VI S249A</td>
<td>57</td>
</tr>
<tr>
<td>FucT VI Y254A</td>
<td>0</td>
</tr>
<tr>
<td>FucT VI T256A</td>
<td>&lt;1</td>
</tr>
<tr>
<td>FucT VI E257A</td>
<td>&lt;1</td>
</tr>
<tr>
<td>FucT VI K258A</td>
<td>0</td>
</tr>
</tbody>
</table>

*Specific activity for each mutant was determined and compared to wild-type enzyme specific activity.

are a 3-h incubation) and using 10 times the standard protein concentration. In addition, these mutant proteins did not have detectable activity when assays were carried out with the H-type II acceptor, a substrate that is converted to product by wild-type FucT VI much more effectively (3 to 1) than LacNAc-C8.

Mutant proteins with a relatively high level of enzyme activity were produced when Ala was substituted for alpha 1,3 FucT motif amino acids at the N-terminus (amino acid 240) and at the middle (amino acids 244, 246, and 249), but not at the C-terminus to the motif. The four C-terminal Ala mutants were either inactive or had 1% or less of wild-type enzyme activity, even though the substituted amino acids had a range of side chains (Y, T, E, and K). Three of the four mutants with relatively high activity were those in which Ala substituted for a nonpolar amino acid (Y, L, and F), the only exception being the substitution of Ala for Ser. In some cases, substitution of Ala for the same amino acid at two different locations within the alpha 1,3 FucT motif had substantially different effects on enzyme activity. For example, Y240A was the second most active mutant with 14% of wild-type activity, whereas Y254A was inactive. In addition, F242A had very low activity, whereas F246A had significant activity. In contrast, Ala substitution for E (247 and 257) or K (242 and 258) at the N- and C-termini of the motif resulted in proteins with very low activity. Finally, Ala substitution for two similar amino acids in the middle of the alpha 1,3 FucT motif, S249A and T256A, produced proteins with a dramatically different level (5% versus <1%, respectively) of fucosyltransferase activity.

To evaluate if Ala mutants of the alpha 1,3 FucT motif in another member of the fucosyltransferase family gave similar results to those for FucT VI, additional mutants were prepared by substituting Ala for two of the alpha 1,3 FucT motif amino acids (T257 and E258, homologous to T256 and E257) of human FucT III. The enzyme activities observed for these mutant enzymes were similar to those observed for FucT VI (i.e., <1% of wild-type activity).

Active Ala mutants of the alpha 1,3 FucT motif of human FucT VI have altered \( K_m \) values

Because all members of the alpha 1,3 FucT family use GDP-fucose as their donor substrate but use different acceptor substrates, it has been proposed that the amino acids of the alpha 1,3 FucT motif may participate in donor substrate binding. To evaluate this possibility, donor substrate saturation studies (Figure 3) were carried out with wild-type FucT VI and with each of the four Ala mutants (Y240A, L244A, F246A, and S249A) that had significant enzyme activity. To obtain an estimate of the \( K_m \) for the donor substrate of these two mutants it was necessary to use GDP-fucose concentrations of up to 0.5 mM, a concentration that is ~20 times the \( K_m \) for the wild-type FucT VI. The \( K_m \) for GDP-fucose for all of the four Ala mutants tested were higher than that for wild-type FucT VI, and three of the four had GDP-fucose \( K_m \) values that were ~5–15 times higher than that for wild-type enzyme (Table II). Only S249A had a GDP-fucose \( K_m \) value that was similar (two to three times) to that of the wild-type FucT VI.

In contrast, three of the Ala mutants with substantially higher GDP-fucose \( K_m \) had unaltered \( K_m \) values for the acceptor substrate (Table II; Figure 4). Among the four Ala mutants with significant enzyme activity, only S249A had an altered \( K_m \) for the acceptor substrate, LacNAc-C8. The \( K_m \) value is at least seven times higher than that for wild-type FucT VI, but the value reported in Table II is only an estimate because it was impossible to saturate the S249A with the acceptor substrate within its solubility range. A more accurate estimate of the \( K_m \) for H-type II acceptor was obtained for the S249A mutant (Figure 5). The \( K_m \) (102 ± 90 \( \mu \)M) for this acceptor was substantially different from that of the wild-type enzyme (80 ± 6 \( \mu \)M). Thus S249 is unique among the alpha 1,3 FucT motif amino acids evaluated in that it affects the \( K_m \) for the acceptor substrate but not that of the donor substrate. This enzyme had ~30% of wild-type activity when measured with an H-type II acceptor substrate concentration of 2.3 mM (i.e., the limit of solubility for this substrate, and two times \( K_m \) for the mutant).

Discussion

The 3D structures for some glycosyltransferases have been elucidated by X-ray analysis of protein crystals, but none has been obtained for any alpha 1,3/4 FucT. Therefore, other approaches have been applied to identify amino acid residues that are important for the activity, substrate specificity, and substrate binding of these enzymes (see de Vries et al., 2001 for a recent review). Identification of functionally important amino acids in mammalian FucTs has primarily resulted from the opportunity to compare FucT amino acid sequences from enzymes within this family that have different properties (e.g., acceptor substrate specificity, sensitivity to chemical reagents), followed by correlating the particular enzyme property to an amino acid.
sequence difference(s). Domain swapping and site-directed mutagenesis approaches have been used to pinpoint amino acids that are responsible for the enzyme property differences. For example, we used these approaches to demonstrate that H87 and I89 of human FucT III are critical for determining type I acceptor substrate specificity in this protein (Nguyen, et al., 1998; Xu, et al., 1996), and Dupuy et al. (1999) demonstrated that R115 of bovine FucT is critical in determining type II acceptor substrate specificity in FucTs.

Fig. 3. $V_s$ versus [GDP-fucose] plots for wild-type FucT VI and alanine scanning mutant enzymes. (A) Wild-type FucT VI; (B) FucT VI Y240A; (C) FucT VI L244A; (D) FucT VI F246A; (E) FucT VI S249A.
Alanine scanning mutagenesis of the alpha 1,3 FucT motif

Table II. Kinetic parameters for FucT VI alanine mutant enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>(K_m) GDP-fucose ((\mu)M)</th>
<th>(K_m) LacNAc-C6 (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FucT VI (wild-type)</td>
<td>19 ± 5(^a)</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td>FucT VI Y240A</td>
<td>220 ± 45</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td>FucT VI L244A</td>
<td>336 ± 54</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td>FucT VI F246A</td>
<td>110 ± 23</td>
<td>0.15 ± 0.03</td>
</tr>
<tr>
<td>FucT VI S249A</td>
<td>69 ± 4.5</td>
<td>1.6 ± 0.40</td>
</tr>
</tbody>
</table>

The concentration of the substrate that was held constant in the experiment was added at a concentration known to be saturating for the wild type enzyme.  

\(^a\)Values listed are averages of duplicate experiments.

As additional FucTs sequences were elucidated, it also became possible to identify additional, functionally important amino acids within the FucT sequence. The cloning of bacterial FucTs provided an opportunity to narrow the search for functionally important sequences because the FucT sequences from these organisms share only limited sequence identity with those from other organisms. As a result, Martin et al. (1997) identified a series of amino acids within a short sequence of all FucTs that is identical and referred to this region as the alpha 1,3 FucT motif and proposed that amino acids in this motif may be involved in donor binding. However, this hypothesis has not been tested. Cunningham and Wells (1989) developed an alanine scanning mutagenesis approach to evaluate the functional importance of individual amino acids within a protein, and the approach has provided important information on a range of proteins. Therefore, we applied Ala scanning mutagenesis to the 12 amino acids within the alpha 1,3 FucT motif.

Ala mutants have a range of enzyme activity

The results shown in Table I and summarized in Figure 6 demonstrate that substitution of Ala for different amino acids within the alpha 1,3 FucT motif results in proteins with widely varying specific activities. Because the enzymatic activity levels of some of the mutants was more than 100-fold lower than the wild-type enzyme, it was necessary to alter the reaction conditions substantially to obtain an estimate of their activity. Thus, for those mutants with low activity it was necessary to carry out the incubation for up to five times as long, and with substantially more protein than for reactions carried out with wild-type enzyme. Therefore, the values listed in Table I are estimates at best of the true specific activities for the mutants.

These data demonstrate that many of the single amino acid substitutions cause an enormous change in enzymatic activity under the standard reaction conditions established for wild-type FucT VI. Results reported for other proteins that have been studied by Ala mutagenesis would suggest that this dramatic change in enzyme activity is not due to gross alterations in the protein fold or overall structure. However, additional studies are required to allow conclusions to be drawn regarding how Ala substitution for amino acids in the alpha 1,3 FucT motif cause the observed activity change. Nonetheless, an important observation from the activity measurements for these mutants is that substituting Ala for amino acids that lie close to one another in the linear sequence of the protein produced dramatically different outcomes in activity. For example, substitution of Ala for N248 versus S249 resulted in proteins that differ more than 50-fold in terms of enzyme activity. The mutant enzymes analyzed could be placed into three categories based on specific activity: no activity, low activity, and high activity (summarized in Figure 6).

Inactive mutants

K258 is the C-terminal residue of the motif, four amino acids downstream from Y254. Substitution of Ala for either of these residues generated proteins devoid of FucT activity. Consequently, it was impossible to determine if either of these residues altered the protein’s affinity for GDP-fucose. However, we previously evaluated the importance of the Lys that corresponds to K258 in FucT VI in two other FucTs (IV and VII) by mutagenesis (Sherwood et al., 1998, 2000). In these earlier studies, we prepared mutants of FucT IV and VII in which conservative (K300R in FucT IV and K240R in FucT VII) substitutions for the lysine residue were made. The FucT IV mutant was found to have low activity, whereas the FucT VII mutant was inactive. The FucT IV K300R mutant was shown to have a 10-fold higher \(K_m\) for GDP-fucose than the wild-type enzyme, demonstrating that GDP-fucose binding is altered by the Arg to Lys substitution in FucT IV. We anticipate that the homologous Lys residue in FucT VI is also involved in GDP-fucose binding and are currently testing this assumption by making the K258R mutant.

Low-activity mutants

Six of the alpha 1,3 FucT motif amino acids ([K241, F242] (E247, N248) (T256, E257]) occur in pairs and are distributed across the motif sequence, with two near the N-terminus, two near the center, and two near the C-terminus. Three of the six amino acids in this group are charged (one positively, two negatively), two are polar, whereas F242 is the only nonpolar amino acid in this group. All amino acids in this group have side chains of average size. Mutation of these amino acids to Ala produced enzymes with low activity levels (<1% compared to wild-type activity).

The first of these amino acids has been previously studied by Sherwood et al. (2000), who substituted Arg in FucT VII (homologous to K241 of FucT VI) with a Lys residue and found that the mutant enzyme had a twofold higher specific activity than wild-type enzyme and that the \(K_m\) for GDP-fucose was unchanged. This demonstrated that this amino acid was important for catalysis but not involved in donor binding. Thus the homologous amino acid in FucT VI would likely have a similar role.

A naturally occurring FucT VI mutant, E247K, which changes the functional group at this position from a
negatively charged to a positively charged one, has been shown to be inactive (Mollicone et al., 1994). We show that a mutation from glutamic acid to the nonpolar alanine produces a protein with minimal enzyme activity.

**High-activity mutants**

Four mutants, Y240A, L244A, F246A, and S249A, had activity levels of 14%, 7%, 7%, and 59%, respectively, of the wild-type specific activity. Except for S249, the amino acid...
acids in this group are nonpolar and are part of a hydrophobic cluster of six amino acids within the motif (Y240, F242, Y243, L244, F246, and L250). Mutating the three nonpolar amino acids, Y240, L244, and F246 to Ala maintains their hydrophobic character. It is possible that the main requirement for these amino acids is that their side chains are hydrophobic and the specific side chain is a result of evolutionary optimization. It is interesting to note that although our L244A mutant has less than one-tenth of wild-type activity, the naturally occurring mutant FucT VI L244V, described by Elmgren et al (2000), has a specific activity one-third that of the wild-type enzyme.

Substrate affinity

The four mutants (Y240A, L244A, F246A, and S249A) with the highest enzyme activity were kinetically analyzed. Our results demonstrate that three of these mutants, Y240A, L244A, and F246A, have acceptor substrate affinities that are similar to the wild-type enzyme. Thus, substituting Ala for any of these three nonpolar residues did not have a measurable effect on acceptor substrate binding. Elmgren et al. (2000) also found that the naturally occurring mutant L244V did not have an altered affinity for acceptor substrate.

In contrast, substituting Ala for the only polar residue among this group, S249, resulted in a protein with a much lower affinity for the two acceptor substrates, LacNAc-C₈ and H-type II-C₈. Although we were unable to obtain an accurate $K_m$ for LacNAc-C₈, we were able to determine that the mutant had a 10-fold lower affinity for H-type II-C₈. Some FucTs have a higher affinity for H-type II-C₈ than for LacNAc-C₈, and this is also the case for the S249A mutant. Thus, substituting Ala for S249 does not result in an altered acceptor substrate preference but results in an altered affinity for acceptor substrates in general. Therefore, other amino acids must account for the increased affinity of FucTs for H-type II-C₈ versus LacNAc-C₈.

Each of the four highly active mutants had a lower affinity for GDP-fucose compared with wild-type enzyme. All three mutants (Y240A, L244A, F246A) in which Ala was substituted for a nonpolar amino acid had large (5–15 times wild type) changes in $K_m$ for GDP-fucose, with the L244A having the largest change relative to the wild-type enzyme. Interestingly, Elmgren et al. (2000) found that the naturally occurring L244V mutation in FucT VI did not change the affinity of the protein for GDP-fucose. Given the similarity of the amino acid substitutions (i.e., Ala or Val for Leu) in the two mutant forms of FucT VI, it is surprising that the mutants would vary substantially in their affinity for GDP-fucose.

In contrast to the three other highly active mutants, S249A had only a modest (~ three times wild-type) reduction in affinity for GDP-fucose. Because this mutant has a dramatically altered $K_m$ for acceptor and our donor saturation studies were carried out with LacNAc-C₈ as the acceptor substrate, the observed change in affinity for GDP-fucose must be considered only an estimated value.

Alpha 1,3 FucT motif predicted structure

To obtain a better insight in the 3D arrangement of mutated residues in the alpha 1,3 FucT motif, the secondary structures of FucT III and FucT VI were predicted using the Holley and Karplus (1989) method implemented in Quanta 2000. An $\alpha$-helical secondary structure is predicted for residues T236–N248 and I255–W267 of FucT VI, whereas the connecting residues 249–254 are predicted to have a random coil conformation. Similarly, $\alpha$-helices are predicted for residues T237–N249 and I256–W268 in FucT III with the surrounding residues predicted to be random coil.

A 3D model of the alpha 1,3 FucT motif was built using the Protein Design module in Quanta using standard $\alpha$-helical backbone geometry for the helical regions. The resulting model is depicted in Figure 7. The two predicted $\alpha$-helices place the hydrophobic residues whose mutation minimally affected the enzymatic activity of FucT VI (Y240, L244, and F246) on one side of the N-terminal $\alpha$-helix. S249 is located in the random coil region directly...
following the first helix. The concentration of hydrophobic residues on one side of this helix suggests that they may form a portion of the hydrophobic core of FucT VI and function to anchor the helix to the hydrophobic core of the enzyme. This is consistent with the observation that the mutation of these residues to a smaller but still hydrophobic Ala residue does not affect enzyme activity as substantially as other Ala substitutions within the motif. The generally more polar residues K241, F242, E247, and N248, whose mutation resulted in proteins with very low enzymatic activity, cluster on the opposite side of the helix. Their more polar nature and the substantial effect of their mutation on enzymatic activity suggest that these residues are solvent-exposed and possibly in direct contact with the substrates or products.

Similarly, the second helix projects Y254 and K258 toward one side of the helix and T256 and E257 toward the opposite side. The distinct loss of activity observed for the Y254A and K258A mutations suggest that amino acids on this side of the helix are crucial for catalysis. This agrees with the results reported by Sherwood et al. (1998) where conservative mutations of the residue homologous to K258 in FucT VI yielded inactive mutant enzymes. Aromatic residues like Y254 have been observed to participate in binding of the donor substrate in α1,3GalT (Boix et al., 2001; Gastinel et al., 1999) through aromatic stacking. The sensitivity of Y254 to mutation suggests this residue could be involved in direct stacking interactions with the donor substrate of FucT VI. The low activity observed for the proteins with the T256A and E257A mutations suggests that these residues may play a less direct role in the enzymatic function of FucT VI. Indeed, these residues are located on the more hydrophobic side of the second helix of our model, clustered near the hydrophobic residues L259, W260, L264, and W267. This clustering of hydrophobic residues on one side of the helix suggests again that T256A and E257A may face the hydrophobic core of the enzyme rather than being accessible to substrates. The negative charge of E257 could be masked by the formation of a salt bridge with the side chain of R261, which is adjacent to E257 on the same face of the α-helix (see Figure 7).

The mutational results do not provide any insight into the relative spatial orientation of the two α-helices with respect to one another. The presence of a Pro at position 252, however, suggests the presence of some form of turn between the two helices, bringing them in close proximity. A similar Pro residue is found in FucT III at position 253.

The 3D model also reveals that other amino acids (S238 and A245 in the first α-helix and I255, N262, and A266 in the second α-helix) are located on the same face as the amino acids that dramatically reduce FucT activity. Their placement spatially adjacent to residues now known to be important for enzyme activity makes them interesting candidates for future mutational studies.

**Other conserved sequences within alpha 1,3 FucT sequences**

Since Martin et al. (1997) proposed an alpha 1,3 FucT motif, FucT sequences from several other species have been identified. Based on an alignment of the FucT sequences, Oriol et al. (1999) have identified two highly conserved regions. One referred to as “highly conserved...”
sequence II of alpha-3-fucosyltransferases” is shown in Figure 1. This region includes the amino acids of the alpha 1,3 FucT motif, but extends further up- and downstream and includes one additional invariant residue (a proline residue near the C-terminus) and several other residues that are found in the FucT sequences of most species. These amino acids, as well as those found in the “highly conserved sequence I of alpha-3-fucosyltransferases” are also important candidates for analysis by mutagenesis (Oriol et al., 1999).

Finally, Dupuy et al. (2004) have recently used mutagenesis analysis to evaluate what they referred to as the acceptor-binding motif, VxxHH(W/R)(D/E), of FucTs. They have demonstrated that one of the key residues affecting acceptor substrate specificity (i.e., alpha 1.3 versus alpha 1,4) is the W/R residue of this motif. Holmes and co-workers (Sherwood et al., 2002) have shown that the His-His residues of this motif also alter acceptor substrate binding. Thus, amino acids within the alpha 1,3 FucT motif, the acceptor binding motif, and possibly region I described by Oriol et al. (1999) would appear to work in concert to regulate the acceptor and donor substrate binding in FucTs.

Materials and methods

Materials

The construct containing the full cDNA sequence encoding FucT VI was kindly provided by Dr. Eric Holmes (Division of Cell Surface Biochemistry, Northwest Hospital, Pacific Northwest Cancer Foundation, Seattle, WA). Lipofectamine 2000 was from Invitrogen (San Diego, CA). The pProta.AN vector was previously described (Nguyen et al., 2000) was from Invitrogen (San Diego, CA). The construct containing the full cDNA sequence encoding FucT VI was kindly provided by Dr. Eric Holmes (Division of Cell Surface Biochemistry, Northwest Hospital, Pacific Northwest Cancer Foundation, Seattle, WA). Lipofectamine 2000 was from Invitrogen (San Diego, CA). The pProta.AN vector was previously described (Nguyen et al., 2000). Oligonucleotide primers were synthesized by Operon Technologies (Alameda, CA). The QuickChange Site-Directed Mutagenesis Kit was purchased from Stratagene (La Jolla, CA). Restriction enzymes were acquired from either New England Biolabs (Beverly, MA) or Promega (Madison, WI). Dulbecco's modified Eagle's medium, penicillin, streptomycin, and fetal calf serum were purchased from either HyClone Laboratories (Logan, UT) or the University of California, San Francisco Cell Culture Facility (San Francisco, CA). 3-[N-morphinol propanesulfonic acid] (MOPS) was purchased from Gibco BRL Life Technologies (Grand Island, NY). Tris-HCl-polyacrylamide gels (4–20%), Kaleidoscope prestained protein standards, and trans-blotted nitrocellulose transfer membrane (0.45 μm) were purchased from Bio-Rad Laboratories (Richmond, CA). Nitro blue tetrazolium, 5-bromo-4-chloro-3-indoyl phosphate, and C18-Sep-Pak cartridges were purchased from Fisher Scientific (Fair Lawn, NJ). The methoxybenzylglycyl-dial glycoside acceptors, LacNAc-C8 and H-type II-C8, were generously provided by Dr. Ole Hindsaul (Department of Chemistry, University of Alberta). GDP-[1H]fucose was obtained from New England Nuclear Life Science Products (Boston, MA). All other chemicals were obtained from commercial sources and were of the highest purity available.

Truncated, wild-type FucT VI template

A truncated, wild-type FucT VI in the pPROTA vector was created and used as a template to generate the alanine mutants described in this study. The template encodes the wild-type catalytic domain (amino acid 40 to the C-terminus) of FucT VI with XmaI and XbaI restriction enzyme sites introducing silent mutations at nucleotides 611 and 789, respectively. This construct was used for cassette mutagenesis to produce the alanine mutants.

Alanine scanning mutagenesis

The QuickChange Site-Directed Mutagenesis Kit was used to generate the alanine point mutations. Twelve alanine mutations were created using the following upper primers: Y240A (5'-GATGAGACCTCTGAAGATCCGCTATCG-3'); K241A (5'-GATGAGACCTCTCGAGGATCGTATCGT-G-3'); F242A (5'-CTCTCGACACTAAGAGAGTATCCGCTGAGTTCCAAACCCGAC-3'); E247A (5'-CTCTCGAATTCCGCTGAGTCTGCACTACAC-3'); N248A (5'-CTCTCGAATTCCGCTGAGTCTGCACTACAC-3'); S249A (5'-CTCTCGAATTCCGCTGAGTCTGCACTACAC-3'); L244A (5'-ACGCGTAATCCGCTGAGTCTGCACTACAC-3'); F246A (5'-GAGTTCCGCTGAGTCTGCACTACAC-3'); P247A (5'-GAGTTCCGCTGAGTCTGCACTACAC-3'); T256A (5'-GAGTTCCGCTGAGTCTGCACTACAC-3'). Lower primers were the same length as and complementary to the upper primers. Following polymerase chain reaction, the mixture was treated with DpnI to digest the template DNA. To excise the cassette, the mutant constructs were double-digested with XmaI and XbaI and the 178-bp DNA products were gel purified and subeloned into the truncated, wild-type FucT VI-pPROTA construct from which the XmaI and XbaI cassette had been removed. Each resulting alanine mutation and the truncated wild-type were propagated in the JM109 strain of Escherichia coli. The nucleotide sequence of each mutant construct was confirmed by completely sequencing both strands of the coding region (Microchemical Core Facility at San Diego State University).

Enzyme expression

The truncated, wild-type construct and the alanine mutant constructs were transfected into 90% confluent COS-7 cells using the lipofectamine method and grown in Dulbecco's modified Eagle medium containing 10% fetal calf serum. The enzymes were expressed as soluble proteins that have an N-terminal protein A, IgG binding domain (De Vries et al., 1995). The protein A–FucT VI chimeric, soluble proteins were harvested on day 2 and 3 posttransfection and purified from the cell culture media using IgG-agarose affinity beads.

Quantitative western blot analysis

Expressed chimeric enzymes were detected and quantified by western blot analysis (Xu et al., 1996). Briefly, the proteins were separated on 4–20% Tris-HCl polyacrylamide gels by western blot analysis (Xu et al., 1996).
gels and transferred to nitrocellulose membranes. The protein A-IgG binding domain of the chimeric proteins was detected by incubating the blot with anti-goat IgG–alkaline phosphatase conjugate and developing the blot with 5-bromo-4-chloro-3-indoyl phosphate and nitro blue tetrazolium. Quantification of the amount of FucT was accomplished by comparing the band intensities of samples to those obtained for known quantities of IgG.

**FucT assays**

The enzyme activities of wild-type and mutant FucT enzymes were determined as previously described (Nguyen et al., 1998) using soluble enzyme in media or IgG-agarose bound enzyme. The standard reaction mixture contained 50 mM MOPS-NaOH, pH 6.5; 40 mM MnCl₂, 0.05% bound enzyme. The standard reaction mixture contained 20 volumes of enzyme (total volume of 20 μl). For kinetic studies, the donor or acceptor concentrations were varied. The reactions were incubated for time periods ranging from 15 min to 20 h and stopped by dilution with 380 μl water. The reaction products were separated from substrate by reverse-phase chromatography (Sep-Pak C₁₈) and quantified as described previously (Xu et al., 1996). The results obtained were analyzed by fitting the initial rate data to the Michaelis-Menten equation using nonlinear regression analysis (KaleidaGraph 3.51, Synergy Software, Reading, PA). When Km values were determined for Ala mutants, the concentration of the second substrate was added at a level that is known to be saturating for wild-type FucT VI.

**Secondary structure prediction and molecular modeling**

The secondary structures of FucT III and FucT VI were predicted using the Holley-Karplus method (Holley and Karplus, 1989) implemented in Quanta 2000 (Accelrys, San Diego, CA). On the basis of the predicted secondary structure, 3D models of the FucT motifs were built using the Protein Design module in Quanta 2000 using standard α-helical backbone geometry for the helical regions and an extended conformation for loop regions.

**Acknowledgments**

We thank Melissa Comstock for helpful technical assistance and the preparation of FucT III mutants. This work was supported by a National Science Foundation Grant, MCB-9816780, and Grant P20 MD000262 from the National Center on Minority Health and Health Disparities to B.A.M.

**Abbreviations**

The abbreviations used are: FucT, α1,3-fucosyltransferases; LacNAc-C₈, Galβ1,4GlcNAc-O-(CH₂)₈COOCH₃; H-type-II, Fucα1,2Galβ1,4GlcNAc-O-(CH₂)₈COOCH₃; type-I, Galβ1,3GlcNAc; type-II, Galβ1,4GlcNAc.

**References**


