Human α1,3/4-Fucosyltransferases

II. A SINGLE AMINO ACID AT THE COOH TERMINUS OF FucT III AND V ALTERS THEIR KINETIC PROPERTIES

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Loc Vo‡§, Stephanie Lee‡, Marie C. Marcinko‡, Eric H. Holmes¶, and Bruce A. Macher‡

From the ‡Department of Chemistry and Biochemistry, San Francisco State University, San Francisco, CA 94132 and ¶Northwest Hospital, Pacific Northwest Cancer Foundation, Seattle, Washington 98125

An analysis of the acceptor substrate specificity of domain swap mutants of human α1,3/4-fucosyltransferases (FucTs) III and V has been carried out. The results demonstrate that changing Aspαβγ of FucT III to Ala (as in FucT V) produced a protein (III/V1) with a reduced activity with a variety of acceptors. An analysis of the kinetic properties of FucT III and the III/V1 mutant demonstrated that III/V1 had a 40-fold reduction in its affinity for the H-type 1 acceptor substrate (Fucα1, 2Galβ1,3GlcNAc) and 4-fold reduction in its affinity for GDP-fucose when compared with FucT III. Further, the overall catalytic efficiency of III/V1 was ~100-fold lower than that of FucT III with an H-type 1 acceptor substrate. The complementary domain swap resulting from the change of Alaαβγ of FucT V to Asp (V/III1) produced a FucT that had higher enzyme activity with a range of acceptor substrates and had a higher affinity for an H-type 2 acceptor substrate (Fucα1,2Galβ1,4GlcNAc) with an 8-fold higher overall catalytic efficiency than that of FucT V. No significant change occurred in the Km for GDP-fucose for this protein when compared with FucT V. Kinetic parameters of two other FucT domain swaps (III/V and V/III), resulting in proteins that differed from FucT III and V at the NH2 terminus of their catalytic domain, were not significantly different from those of the parental enzymes when H-type 1 and H-type 2 acceptor substrates were utilized. Thus, substitution of an acidic amino acid for a nonpolar amino acid (i.e. Asp versus Ala) at the COOH terminus of FucTs produces an enzyme with enhanced enzyme activities. These results, together with the results presented in the accompanying papers (Nguyen, A. T., Holmes, E. H., Whitaker, J. M., Ho, S., Shetterly, S., and Macher, B. A. (1998) J. Biol. Chem. 273, 25244–25249; Sherwood, A. L., Nguyen, A. T., Whitaker, J. M., Macher, B. A., and Holmes, E. H. (1998) J. Biol. Chem. 273, 25256–25260), provide new insights into the structure/function relationships of human α1,3/4-FucT enzymes.

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† Present Address: Department of Molecular and Cell Biology, University of California, Davis, CA 95616.

§ To whom correspondence should be addressed: Northwest Hospital, Pacific Northwest Cancer Foundation, 120 Northgate Plaza, Suite 218, Seattle, WA 98125. Tel.: 206-368-3060; Fax: 206-368-3061; E-mail: eholmes@nwbio.org.

α1,3/4-Fucosyltransferases (FucTs)1 catalyze the synthesis of a variety of fucosylated glycoconjugates including Lea, Se, and Se (1). Five human α1,3/4-FucTs have been cloned and designated FucT III–VII (1–4, 6–8). Three of these enzymes (FucTs III, V, and VI) share a high level (>85%) of amino acid sequence homology, and the genes encoding each are located closely on human chromosome 19 (see Ref. 10 and references therein). It has been proposed that they were derived by gene duplication from a common precursor gene (1). We (11) and others (1, 6, 12, 16) have analyzed the acceptor substrate specificity of these highly homologous enzymes, and each has a unique acceptor substrate specificity. Thus, FucT III has predominantly α1,4-FucT activity with disaccharide acceptors (having some activity with selected type 2 acceptor substrates, e.g. H-type 2), whereas FucTs VI appears to be a true α1,3-FucT. The acceptor substrate specificity of FucT V is more complex and depends on the type of acceptor substrate utilized. For example, when simple disaccharides are used in a “so-called” Dowex assay as substrates, FucT V has very low α1,4-FucT activity (<5%) compared with α1,3-FucT activity. In contrast, FucT V catalyzes the transfer of fucose to a type 1 acceptor substrate with an aliphatic aglycone to a much greater extent than to the type 1 disaccharide (11, 12).

In a previous study (13), we demonstrated that the catalytic domain of FucT III and V contained approximately 300 amino acids. These experiments utilized recombinant chimeric forms of the enzymes missing portions of the cytoplasmic, transmembrane, and stem regions expressed as fusion proteins with the protein A Ig-binding domain. Analysis of the enzymatic and substrate specificity properties of these chimeric enzymes indicated they behaved very similarly to both full-length enzymes and the truncated form without the protein A domain (11). This information, coupled with the ease of purifying the expressed chimeric enzyme via IgG-agarose beads, makes this system very convenient for studying functional aspects of amino acid alterations in the catalytic domain on enzyme activity. In this earlier study (13), we also demonstrated that swapping a segment of FucT III, containing eight of the amino acids unique to FucT III, into the analogous location in the FucT V catalytic domain produced a chimeric protein with substantial α1,4- as well as α1,3-FucT activities. Lowe and co-workers (14) have also identified similar segments of the FucT III amino acid sequence that produces α1,4-FucT activity. Thus, the acceptor substrate specificity of these FucTs can be altered by mutating a few amino acid residues (13–15). Thus far, the amino acids that have been shown to affect acceptor substrate specificity are located near the NH2 terminus of the catalytic domain of the FucTs. In the current study, we demonstrate that a single
amino acid at the COOH terminus of FucTs III and V is important for the binding of H-type 1 and 2 acceptor substrate and their conversion to product.

EXPERIMENTAL PROCEDURES

**Materials**—Rabbit IgG-agarose, anti-goat IgG-alkaline phosphatase conjugate, goat IgG, 5-bromo-4-chloro-3-indolyl phosphate, and nitro blue tetrazolium were obtained from Sigma. Trypsine, yeast extract, and agar were purchased from Difco. Plasmids utilized in the domain swaps were previously described (13). Wizard Mini and Maxi prep Purification Systems were purchased from Promega (Madison, WI). Fetal calf serum was obtained from Hyclone Laboratories Inc. (Logan, Utah). C18-SepPak cartridges were purchased from Fisher. Trans-blot transfer medium (0.45 μm), kleidoscope prestained standards, 12% Trisglycine gels, and Dowex AG 1-X2 resin, 200–400 mesh CI− form were obtained from Bio-Rad. The 8-methoxyxarboxyloctyl glycoside acceptors and GDP-fucose were kindly provided by Dr. Ole Hindsgaul (Department of Chemistry, University of Alberta). GDP-[3H]fucose (7.57 Ci/mmol) was purchased from NEN Life Science Products. All other reagents were of the highest purity commercially available.

**Recombinant Enzymes**—Domain swaps between FucT III and V were prepared as described previously (13) or as described here. Parental recombinant FucT III and V coding sequences were truncated catalytic domain forms cloned into the pPROTA vector (11). Domain swaps were designed based on the presence of conserved restriction enzyme sites in FucTs III and V. The coding sequences for FucT III and V were each treated with the enzyme HaeII. This produced fragments encoding amino acids 62–131 and 132–361 of FucT III and amino acids 76–146 and 147–374 of FucT V. Digest fragments were separated by agarose gel electrophoresis and purified with a QIAEX gel extraction kit. The fragments were ligated to give the chimeric FucTs (III/V1 and V/III1) that contain fucosyltransferase activity to investigate the question of which of the amino acid differences that occur between FucT III and V account for their unique substrate specificities. Each was characterized with respect to pH optimum, metal ion requirements, and other reaction condition variables and found to resemble FucTs III and V in their optimal reaction conditions (results not shown). One set (IIIIV and VIII/V) has proven useful for probing amino acids that account for the observed difference in type 1 and type 2 acceptor substrate specificities (see accompanying article (15)). Another set (III14/V and V14/III) has been partially characterized and shown2 to catalyze the transfer of fucose to H-type 1 and 2 acceptor substrates but not to other neutral or sialylated acceptor substrates. The third set of chimeric FucTs (III/V1 and V/III1) contain a single amino acid change near the COOH terminus (Fig. 1); III/V1 contains an Asp to Ala substitution at amino acid 336 of the FucT III sequence, and V/III1 has the complementary substitution (i.e., Ala to Asp at amino acid 349 of the FucT V sequence). Preliminary analyses of these proteins, at a single concentration of the simple acceptor substrate Galβ1,3GlcNAc and Galβ1,4GlcNAc, have shown that they have an acceptor substrate specificity similar to the corresponding parental enzyme (13). Thus, III/V1 and FucT III are essentially α1,4-FucTs with disaccharide acceptors, whereas III/V1 and FucT V have higher activity with type 2 than with type 1 acceptor disaccharides. An interesting trend was noted when these chimeric enzymes were further characterized with a range of acceptor substrates as illustrated in Fig. 2. III/V1 consistently has a lower activity than FucT III...
for three different type 1 acceptor substrates (Fig. 2, A–C). In contrast, the V/III¹ chimera consistently showed a higher activity than FucT V with a set of type 2 acceptor substrates (Fig. 2, D–F). These results suggest that the single amino acid site (Asp³³⁶ of FucT III and Ala³⁴⁹ of FucT V), which constitutes the only difference in the sequence of FucT III and V over the final 210 COOH-terminal amino acid residues, impacts the acceptor substrate profiles of FucT III and V. To further characterize the effects of this amino acid substitution, substrate saturation curves with the favored acceptor substrates (H-type 1 for FucT III and III/V¹, and H-type 2 for FucT V and V/III¹) were carried out.

As shown in Fig. 3, A and B, III/V¹ has a lower \( V_o \) than FucT III at all concentrations of the H-type 1 acceptor substrate tested. The \( V_m (\text{app}) \) (46 versus 120 nmol/min mg) and \( K_{cat} \) (see Table I) for III/V¹ are approximately 2-fold lower than that for FucT III. An even larger difference (~40-fold) was determined for the apparent affinity constant of FucT III versus III/V¹ for the acceptor substrate. Overall, the catalytic efficiency (\( K_{cat}/K_m \)) was reduced nearly 100-fold for III/V¹ compared with that of FucT III. In contrast, a comparison of kinetic parameters for FucT III and a chimeric FucT resulting from the substitution of a portion of the NH₂ terminus of the catalytic domain of FucT V for the corresponding region of FucT III (i.e., V⁸/III; see Fig. 1) shows similar analyses were carried out with FucT V and V/III¹ using an H-type 2 acceptor substrate (Fig. 4, A and B). The \( V_m (\text{app}) \) (54 versus 26 nmol/min mg) and \( K_{cat} \) (see Table I) for III/V¹ are approximately 2-fold higher than that for FucT V. A slightly larger difference (~4-fold) was observed for the apparent affinity constant of V/III¹ versus FucT V for the acceptor substrate. Overall, the catalytic efficiency was increased approximately 8-fold for V/III¹ compared with that of FucT V. In contrast, a comparison of kinetic parameters for FucT V and a chimeric FucT resulting from the substitution of a portion of the NH₂ terminus of the catalytic domain of FucT III for the corresponding region of FucT V (i.e. III⁸/V; see Fig. 1) shows
that these two proteins have similar kinetic parameters and essentially identical catalytic efficiency constants.

Substrate saturation curves for GDP-fucose were carried out for FucT III and V and the domain swap FucTs, and the $K_m$ for each is presented in Table II. The results demonstrate that all but one (III/V1) had a similar affinity for the nucleotide sugar donor substrate. The $K_m$ for III/V1 was 4–5 times as large as that found for the FucTs, indicating that the single amino acid substitution near the COOH terminus of this protein (compared with FucT III) significantly reduces the protein’s affinity for GDP-fucose.

A comparison of amino acid sequences of the COOH-terminal half of the catalytic domains of FucTs III, V, and VI shows that these enzymes have a remarkable degree of homology within a given species and between species. Among the few amino acids that have not been conserved in the COOH-terminal half of the catalytic domain of FucTs III, V, and VI is one that exists as either an Ala or an Asp residue, depending on the enzyme. Thus, both human and chimpanzee FucT III enzymes have an Asp residue at this amino acid position (amino acid 336 and 347, respectively). Human FucT V has an Ala residue at the equivalent position (i.e. amino acid 349), whereas the chimpanzee FucT V contains an Asp residue. Both the human and chimpanzee FucT VI proteins have an Ala residue at the corresponding position (amino acid 335). Thus, all primate forms of FucT III, V, and VI contain either an Ala or Asp residue, depending on the enzyme.

Substrate saturation curves for GDP-fucose were carried out for FucT III and V and the domain swap FucTs, and the $K_m$ for each is presented in Table II. The results demonstrate that all but one (III/V1) had a similar affinity for the nucleotide sugar donor substrate. The $K_m$ for III/V1 was 4–5 times as large as that found for the FucTs, indicating that the single amino acid substitution near the COOH terminus of this protein (compared with FucT III) significantly reduces the protein’s affinity for GDP-fucose.

### Table I

| Enzyme | $K_m$ (app) | $K_{cat}$ | $K_{cat}/K_m$ |
|--------|-------------|-----------|---------------|
| H-type 1 | 0.1 ± 0.01 | 8.02 ± 0.63 | 80.20 |
| III/V1 | 3.8 ± 0.4  | 3.02 ± 0.26 | 0.82 |
| V/III  | 0.4 ± 0.3  | 4.44 ± 0.28 | 11.10 |
| H-type 2 | 3.9 ± 0.4  | 1.70 ± 0.15 | 0.44 |
| FucT V | 1.1 ± 0.1  | 3.60 ± 0.16 | 3.28 |
| V/III  | 2.6 ± 0.4  | 1.08 ± 0.12 | 0.42 |

### Table II

| Enzyme | $K_m$ (μM) |
|--------|-------------|
| FucT III | 33.6 |
| III/V1  | 114.5 |
| V/III  | 23.3 |
| FucT V  | 18.8 |
| V/III  | 23.3 |
| III/V   | 21.8 |

### DISCUSSION

Among the five human FucTs that have been cloned, three (FucTs III, V, and VI) have a high degree of sequence homology, yet they have unique acceptor substrate specificities. Homologs of these enzymes have been recently cloned from chimpanzee (17) and share a similar substrate specificity with their human counterparts (1–12) (Fig. 5). Interestingly, it appears that non-primate mammals do not have the same complement of FucTs but have a single form of FucT, which shares considerable sequence homology (Fig. 5) with primate FucTs III, V, and VI (18). This enzyme has a specificity that is most like that of human and chimpanzee FucT VI.

A comparison of amino acid sequences of the COOH-terminal half of the catalytic domains of FucTs III, V, and VI shows that these enzymes have a remarkable degree of homology within a given species and between species. Among the few amino acids that have not been conserved in the COOH-terminal half of the catalytic domain of FucTs III, V, and VI is one that exists as either an Ala or an Asp residue, depending on the enzyme. Thus, both human and chimpanzee FucT III enzymes have an Asp residue at this amino acid position (amino acid 336 and 347, respectively). Human FucT V has an Ala residue at the equivalent position (i.e. amino acid 349), whereas the chimpanzee FucT V contains an Asp residue. Both the human and chimpanzee FucT VI proteins have an Ala residue at the corresponding position (amino acid 335). Thus, all primate forms of FucT III, V, and VI contain either an Ala or Asp residue at this position.

The results we present here demonstrate that this single amino acid can significantly affect the substrate binding and catalytic efficiency of the FucTs. Specifically, the substitution of Ala for Asp in FucT III produces an enzyme that is less active than the wild-type enzyme when tested with three different acceptor substrates. Furthermore, this substitution produces an enzyme with reduced apparent affinity for both of its substrates (i.e. H-type 1 and GDP-fucose) and with an overall reduction in catalytic efficiency. Interestingly, the complementary substitution in FucT V (Asp for Ala) in the corresponding position of the amino acid sequence generates an enzyme that is more active than FucT V and has a higher apparent affinity for the acceptor substrate. Based on the results we have obtained, it can be concluded that the substitution of a nonpolar amino acid for a negatively charged residue at amino acid 336 of the FucT III sequence significantly affects the enzyme’s substrate binding properties. Thus, the presence of an Asp residue in FucT III and V produces an inherently more active enzyme. It is not clear whether a more conservative substitution (e.g. Asp to Glu, to maintain a similar charge; or Asp to Asn, to preserve a similar function group) would produce a protein with properties similar to those of the wild-type enzyme or if there is a high specificity for the side chain of Asp. Further mutational analyses are currently in progress to address these issues.

Other single amino acid substitutions in the coding region of FucTs have been identified in the tissues and serum of indi-
individuals who are classified as Lewis- or plasma FucT-negative (19–26). These mutations have been identified by sequencing the coding region of the FucT on the alleles of negative individuals and, in some cases, by evaluating the consequence of such mutations on the in vitro activity (enzyme assay and/or cell surface expression of Lewis antigens) of the FucT. Several mutations have been identified in FucT III. Among these mutations is one at amino acid residue 20 that appears to result in the mistargeting (secreted instead of Golgi retention) of the enzyme (26). Mutations at amino acid 105, 170, or 356 have been shown to lead to a loss of enzyme activity. Elmgren et al. (24) have recently demonstrated that a Trp 68 → Arg mutation dramatically reduces FucT activity (reported to have less than 1% of wild-type activity) but does not result in a completely inactive enzyme. Another amino acid mutation in FucT III (Thr105 → Met), which has always been found in conjunction with the mistargeting (secreted instead of Golgi retention) of the enzyme (26). Mutations at amino acid 105, 170, or 356 have been shown to lead to a loss of enzyme activity. Elmgren et al. (24) have recently demonstrated that a Trp 68 → Arg mutation dramatically reduces FucT activity (reported to have less than 1% of wild-type activity) but does not result in a completely inactive enzyme. Another amino acid mutation in FucT III (Thr105 → Met), which has always been found in conjunction

Fig. 5. Comparison of the predicted amino acid sequences of FucT III, V, and VI from humans and chimpanzees and the bovine FucT sequence. The residues near the NH₂ (residues 86 and 87 in the FucT III sequence) and COOH terminus (residue 336 in the FucT III sequence) of the catalytic domains that affect substrate specificity and affinity are underlined. Conserved Cys residues are shown with a double underline (residues 94, 104, 338, and 341 in the FucT III sequence).
with the Trp\textsuperscript{68} → Arg mutation, does not lead to a reduction in enzyme activity compared with wild-type FucT III when expressed alone. However, Elmgren et al. (24) report that the double mutant has some FT activity but that it is even lower than that of the single, Trp\textsuperscript{68} → Arg mutant. The Trp\textsuperscript{68} → Arg mutant was found to dramatically decrease the enzyme’s V\textsubscript{max} but it did not affect the enzyme’s affinity for either the acceptor or nucleotide sugar donor substrates.

Some individuals who lack plasma FucT activity have been shown to have a mutation that results in the substitution of Lys for Glu at amino acid 247 of FucT VI (26), which abolishes enzyme activity. Another mutation (Pro\textsuperscript{124} → Ser) has been found in the FT VI sequence (26). In vitro studies demonstrate that this mutation results in an enzyme with higher activity (2–3-fold) than wild-type enzyme. The mutations that result in such in vitro increases of the protein that are more than 100 amino acids apart have been established, and it has been shown that among the mutations all lead to enzyme inactivation. Studies of other glycosyltransferases have also demonstrated that single amino acid changes can affect substrate binding. For example, Datta and Paulson (27) established that the binding of CMP-sialic acid can be altered significantly by the mutation of single amino acid residues within the “sialylmotif.” Yamamoto and McNeill (28) and Seto et al. (29) have shown that single amino acid modifications in the coding sequence of the human blood group A and B transferases can alter the K\textsubscript{m} for the nucleotide sugar donor as well as the catalytic efficiency. Further studies of this type, along with protein chemical studies, are needed to establish the nature of the substrate binding sites in glycosyltransferases.

The results we report here and in the accompanying papers (15, 31) demonstrate that amino acids at the two extremes of the catalytic domain affect substrate specificity and affinity, suggesting the possibility that the native enzymes must fold in a manner that would bring these two segments of the protein into close proximity. Although little is known about the disulfide bond pattern of glycosyltransferases, this possibility is consistent with what has been previously published. Thus, the disulfide bonding pattern of β1,4-galactosyltransferase (5, 30) has been established, and it has been shown that among the five Cys residues found in human β1,4-galactosyltransferase sequence, only two (Cys\textsuperscript{314} and Cys\textsuperscript{322}; amino acid numbering based on the sequence of the human enzyme) are involved in a disulfide bond. This disulfide bond brings together two segments of the protein that are more than 100 amino acids apart in the linear sequence. As shown in Fig. 5, two sets of highly conserved Cys residues are found near the NH\textsubscript{2} and COOH termini of the catalytic domain of FucTs III, V, and VI. If the NH\textsubscript{2} and COOH termini of FucTs are located near one another, it is possible that this arrangement is stabilized by a disulfide bond(s) between Cys residues at the ends of the catalytic domain of FucTs. We have initiated studies to determine if this hypothesis is true by creating Cys to Ser mutants at each conserved Cys residue.

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