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MINI REVIEW

Fucosyltransferases: structure/function studies

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α3-fucosyltransferases (α3-FucTs) catalyze the final step in the synthesis of a range of important glycoconjugates that function in cell adhesion and lymphocyte recirculation. Six members of this family of enzymes have been cloned from the human genome, and their expression pattern has been shown to be highly regulated. Each enzyme has a unique acceptor substrate binding pattern, and each generates a unique range of fucosylated products. Results from a range of studies have provided information on amino acids in the FucT sequence that contribute to the differential acceptor specificity for the FucTs, and to the binding of the nucleotide sugar donor GDP-fucose. These results, in conjunction with results obtained from the analysis of the disulfide bond pattern, have provided useful clues about the spatial distribution of amino acids that influence or directly contribute to substrate binding. This information is reviewed here, and a molecular fold prediction is presented which has been constructed based on the available information and current modeling methodology.

Key words: fucosyltransferase/structure/function/modeling/ disulfide linkages

Introduction

Fucosyltransferases (FucTs) have been reviewed on a number of occasions, focusing on occurrence and specificities (Macher *et al.*, 1991; De Vries and van den Eijnden, 1992), evolution of genes (Mollicone *et al.*, 1995; Costache *et al.*, 1997b; Oriol *et al.*, 1999), or their role in leukocyte trafficking (Lowe, 1997). The focus of this review is on structure/function relationships of α3(4)-FucTs. This article will specifically review only those enzymes (FucTs) that catalyze the transfer of a fucose residue from the donor substrate, GDP-fucose, to an acceptor substrate in an α3- or α4- linkage to GlcNAc in Gal-GlcNAc- sequences (FucTs III–VII and IX). Additional FucTs that catalyze fucose transfer from GDP-fucose in an α2-linkage to terminal Gal residues in N- or O-glycans (FucTs I and II) or in an α6-linkage to the innermost asparagine-linked (core) GlcNAc in N-glycans (FucT VIII) have been reviewed elsewhere (see Costache *et al.*, 1997b; Oriol *et al.*, 1999).

The FUT gene family encodes a group of proteins (FucTs) that shows a complex tissue- and cell type–specific expression pattern. This expression pattern varies during development and malignant transformation. FucTs display different but sometimes overlapping enzymatic properties, making it possible to discriminate among the various enzyme activities by assessing their substrate specificity patterns. Many tissues contain multiple FucTs. In general, they catalyze the final step in the synthesis of a range of fucosylated glycoconjugates, some of which have been shown to be essential for normal biological function. Six genes encoding FucTs have been identified to date. They include FUT3, encoding the Lewis α3/4-FucT, FucT III (Kukowska-Latallo *et al.*, 1990); FUT4, encoding the myeloid-type enzyme FucT IV (Goelz *et al.*, 1990; Lowe *et al.*, 1991; Kumar *et al.*, 1991); FUT5, which encodes the enzyme FucT V (Weston *et al.*, 1992a); FUT6, encoding the plasma-type FucT VI (Koszdin and Bowen, 1992; Weston *et al.*, 1992b); FUT7, responsible for the leukocyte expressed FucT VII (Natsuka *et al.*, 1994; Sasaki *et al.*, 1994); and finally, the recently identified FUT9, which encodes FucT IX (Kaneko *et al.*, 1999). Three (FucTs III, V, and VI) have a high degree of sequence similarity, whereas the other three (FucTs IV, VII, and IX) are less similar both to each other as well as to the former group. FucTs III, V, and VI have evolved relatively recently and are found only in humans and chimpanzees (Costache *et al.*, 1997a). They do not appear to have an essential biological role because not all humans have functional forms of these enzymes. However, they have proven to be very useful in defining the importance of amino acids in binding to and discriminating among various acceptor substrates, in GDP-fucose binding and enzyme activity. In contrast, gene knockout studies of FucTs IV and VII have demonstrated that these enzymes are essential for normal leukocyte trafficking and function (Weninger *et al.*, 2000). Because of their more divergent sequences, they have been less useful for identifying which of their amino acids are essential for substrate binding, specificity, or activity.

A current emphasis in the glycosyltransferase field is solving the x-ray crystal structure of these proteins and using the information to identify conserved folds present in these enzymes, and their substrate binding sites. To date, the crystal structures of three mammalian glycosyltransferases have been determined (Ünligil and Rini, 2000; and references therein).

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No crystal structure has yet been solved for any member of the FucT family. However, a significant amount of biochemical information has been accumulated in a number of studies to define residues that are critical for substrate binding and catalysis. These results provide information on residues that are involved in substrate binding and/or those that are close together in space. The biochemical results are summarized in this review and the information is used, together with a method of homology modeling and threading, to present a fold prediction for the α3-FucTs. An introductory section also presents a summary of the available information regarding the chromosomal localization of the FucT genes, and the limited information available on the regulation of FucT gene expression.

Chromosomal localization and expression of human FucTs

Chromosomal localization studies have demonstrated that the FUT4 gene is found at 11q21 (Reguigne *et al.*, 1994), the FUT7 gene at 9q34.3 (Reguigne-Arnould *et al.*, 1996), and the FUT9 gene at 6q16 (Cailleau-Thomas *et al.*, 2000). The FUT3, 5, and 6 genes form a cluster (within 35 kb) on human chromosome 19 (19p13.3) (McCurley *et al.*, 1995). In addition, their protein products (i.e., FucTs III, V, and VI) have a high degree of sequence similarity (~90% identity), which suggests that the human FUT3-FUT5-FUT6 cluster was generated by duplication events. The sequences of FUT3, FUT5, and FUT6 are highly polymorphic, that is, several inactivating mutations have been described explaining the frequent occurrence of negative phenotypes for these enzymes (see section on naturally occurring mutations). The open reading frames (ORFs) of FUT3 to FUT6 as well as FUT9 are encoded by single exons, whereas the ORF of FUT7 is assembled from two exons (Britten *et al.*, 1998). FucT VI is abundantly expressed in epithelial cells and in liver, kidney, and gastrointestinal tissues, specifically, stomach, jejunum, and colon, and minimally expressed in spleen, lung, and cervix uteri. FucT VI is not detected in brain, adrenal cortex or peripheral blood leukocytes. The tissue distribution of FucT III is similar to that of FucT VI, except that FucT III is not expressed in liver and is less abundant in kidney. FucT V is only minimally expressed in spleen and in restricted quantities in liver, colon, and testes (Cameron *et al.*, 1995; Kaneko *et al.*, 1999). FucT IV is widely expressed in various cells and tissues (Gersten *et al.*, 1995). FucT VII is expressed at high levels in hematopoietic cells, such as leukocytes, and in high endothelial cells of the venule (Natsuka *et al.*, 1994; Sasaki *et al.*, 1994). FucT IV and FucT VII complimentarily direct the synthesis of fucosylated cell surface epitopes that are recognized by selectins (Niemela *et al.*, 1998). Transcripts for FucT IX are abundantly found in brain, stomach, spleen, and peripheral blood cells (Kaneko *et al.*, 1999). Interestingly, FucT IX is among the more conserved FucTs, because the difference between human, rat, and mouse FucT IX is only 1% (Kaneko *et al.*, 1999).

FUT gene expression is complex and regulated in a tissue- and stage-specific manner. For example, FUT4 and FUT9 are transcribed early in human embryogenesis, whereas FUT3 and FUT6 transcripts do not appear until after the 10th week of development (Cailleau-Thomas *et al.*, 2000). The expression pattern suggests control at both the transcriptional and post-transcriptional levels. However, few studies on FUT gene regulation have been published. Recently, Taniguchi *et al.* (2000) described the expression and transcriptional regulation of the human FUT4 gene in myeloid and colon adenocarcinoma cell lines. FUT4 appeared to have two different transcription initiation sites that result in the production of a long and a short form of FUT4 mRNA. Two cell line–specific enhancers were identified at different locations in the FUT4 genomic DNA: a colon adenocarcinoma cell line–specific enhancer and a myeloid cell line–specific one. Withers and Hakomori (2000) demonstrated that a fragment with high FUT4 promoter activity in U937 and HL-60 cell lines contains a consensus binding site for the Ets-1 transcription factor family member Elk-1. Elk-1 is important in both RAS-dependent and RAS-independent mitogen-activated protein kinase signaling pathways in numerous cell types (Whitmarsh *et al.*, 1995). Both growth factor–regulated kinases and stress-activated kinases c-Jun and p38 have been found to activate Elk-1 (Whitmarsh *et al.*, 1995). Upon activation of HUVEC cells with tumor necrosis factor and IL-1, FucT VI (but not FucT III) activity was increased (Schnyder-Candrian *et al.*, 2000). Furthermore, it has been shown that IL-4 and IL-12 control the expression of FucT VII in human Th1 and Th2 cells (Wagers *et al.*, 1998). When naive T cells are activated to differentiate into Th1 effector cells in the presence of the cytokine IL-12, expression of FucT VII is enhanced. Expression of FucT VII in leukocytes is essential for their homing to sites of inflammation (Maly *et al.*, 1996). Conversely, in the presence of IL-4, which promotes Th2 cell development, FucT VII expression is inhibited (Wagers *et al.*, 1998). Among an array of other cytokines, only TGF-β1 was able to up regulate FucT VII expression (Wagers and Kansas, 2000). Surprisingly, induction of FucT VII by TGF-β1 appeared to be selectively dependent on p38 mitogen-activated protein kinase.

Structure/function studies

The family of human α3(4)-FucTs is composed of six proteins that share a significant level of amino acid sequence homology yet differ in enzymatic properties, thus providing an opportunity to define protein regions and amino acids that are responsible for specific properties. Several studies have focused on identifying regions in these enzymes that are responsible for their acceptor binding specificities. The results from these studies have lead to the identification of amino acid residues that are critical for acceptor substrate discrimination (e.g., α4- and/or α3-transferase specificity) and residues that affect GDP-fucose binding.

The FucT catalytic domain

FucTs are type II transmembrane glycoproteins residing in the Golgi vaccules. They are composed of a small N-terminal cytoplasmic tail, a membrane-spanning region, and a catalytic domain oriented lumenally in the Golgi apparatus. Between the membrane-spanning region and the catalytic domain is a region called the stem (Paulson and Colley, 1989). The N-terminal regions (cytoplasmic, transmembrane, and stem regions) of the FucTs contain the highest sequence heterogeneity. Soluble forms of FucTs, produced by naturally occurring proteases, have been found in serum, milk, amniotic fluid, semen, and other body fluids (Mollicone *et al.*, 1990; and references

therein). This suggests that a portion of the N-terminus of FucTs is not required for enzyme activity, but does not establish exactly which portion of the amino acid sequence is required for enzyme activity. Results from protein truncation studies have demonstrated that a significant portion (50 and 70 amino acids, depending on enzyme form) of the N-terminal region can be deleted without affecting catalytic activity, whereas removing only a few amino acids from the C-terminus results in a complete loss of enzyme activity (Xu *et al.*, 1996). For example, the catalytic domain of FucT III is composed of amino acids 62–361 (see Table I).

Amino acid residues involved in acceptor substrate binding

Comparison of the amino acid sequences of the catalytic domains of FucTs III and V show that they differ at less than 25 residues, yet these enzymes have significantly different acceptor substrate specificities. Thus the variation in acceptor specificity can be attributed to this limited set of variable amino acid residues. Amino acids involved in acceptor specificity properties have been mapped to amino acids present in the N-terminal, hypervariable region (spanning amino acids 73 through 151) of FucT III (Legault *et al.*, 1995; Xu *et al.*, 1996; Nguyen *et al.*, 1998; Dupuy *et al.*, 1999). This region was identified using a series of chimeric enzymes, created by swapping various portions of FucTs III, V, and VI and analyzing the ability of each to catalyze fucose transfer to either type-1-chain (Galβ1-3GlcNAc-R)–based acceptor substrate structures or type-2-chain (Galβ1-4GlcNAc-R)–based acceptors. Legault *et al.* (1995) demonstrated that as few as 11 amino acid residues differing among FucTs III, V, and VI, and contained in two subdomains spanning aa100 and aa160 of FucT III, determine the ability to transfer fucose in an α4-linkage to type-1-chain–based acceptors. A second report (Xu *et al.*, 1996) demonstrated that amino acid differences between FucT III and FucT V could be divided into two regions comprised of either 8 or 12 amino acid differences. The results demonstrated that substitution of either region from FucT III into a recombinant enzyme, composed otherwise of FucT V sequences, produced an enzyme capable of transferring fucose in an α4-linkage to type 1 acceptors. Substitution of region 1 (with 8 amino acid differences between aa73 and aa105) from FucT III gave rise to an enzyme with dual specificity for type 1 and type 2 acceptors, whereas substitution with region 2 (with 12 amino acid differences between aa115 and aa151) of FucT III resulted in an enzyme with only type 1 acceptor specificity. A subsequent report (Nguyen *et al.*, 1998) demonstrated that of the eight amino acid differences in region 1, His^{73} and Ile^{74} of FucT III correlated with the ability to catalyze transfer to type 1 acceptors. More recently, Dupuy *et al.* (1999) demonstrated that Trp^{111} of FucT III was also associated with transfer specificity to type 1 acceptors.

An additional amino acid residue that affects the acceptor substrate specificity of FucT III was identified near the C-terminus of the catalytic domain. Substitution of an Ala residue (the residue found in the homologous site in FucT V) for Asp^{336} of FucT III produced an enzyme that had reduced affinity for a range of acceptor substrates, including a 40-fold reduction in its affinity for an H-type 1 acceptor substrate (Vo *et al.*, 1998). When the Ala residue (Ala^{349}) of FucT V that is homologous to Asp^{336} of FucT III was substituted with an Asp residue the resulting protein had a higher affinity for a range of acceptor substrates.

Amino acid residues involved in GDP-fucose binding

One means to discriminate FucTs is based on their degree of sensitivity to the sulfhydryl-group reactive reagent N-ethylmaleimide (NEM) (Mollicone *et al.*, 1990). Some FucTs, such as the Lewis (FucT III) and the plasma-type (FucT VI) enzymes

Table I. Characteristics of human α3/4-FucTs

Enzyme	aa	N-sites[a]	Chrom. Loc.	Product(s)	-SH Groups[b]	Disulfides	Cys[c]	Acc. No.[d]	Reference(s)
FucT III	361	2	9p13.3	Le^b, (S)Le^a	Yes	C^{81}–C^{338}	5	U27328	Kukowska-Latallo *et al.*, 1990
				Le^Y		C^{91}–C^{341}			
FucT IV	405	2	11q21	Le^Y, Le^X	No	unknown	4	M58597	Goelz *et al.*, 1990
									Lowe *et al.*, 1991
									Kumar *et al.*, 1991
FucT V	374	5	19p13.3	Le^Y, (S)Le^X	Yes	unknown	5	U27330	Weston *et al.*, 1992a
				Le^b, (S)Le^a					
FucT VI	359	5	19p13.3	Le^Y, (S)Le^X	Yes	unknown	5	U27333	Koszdin and Bowen, 1992
									Weston *et al.*, 1992b
FucT VII	342	2	19p13.3	SLe^X	No	C^{68}–C^{78}	6	X78031	Natsuka *et al.*, 1994
						C^{211}–C^{214}			Sasaki *et al.*, 1994
						C^{318}–C^{321}			
FucT IX	359	3	9q34.3	Le^Y, Le^X	No	unknown	6	AJ238701	Kaneko *et al.*, 1999

[a]N-sites, Number of potential N-linked glycosylation sites.
[b]–SH Groups, presence of catalytically essential –SH groups.
[c]Cys, number of Cys residues (free or disulfide-linked) in catalytic domain.
[d]Acc. No. are for either the GenBank or Swiss-Prot databank.

are inhibited by alkylating reagents (i.e., NEM sensitive), whereas others such as the myeloid-type enzyme (FucT IV) are insensitive to NEM treatment. Alignment of the amino acid sequences of the FucTs demonstrated the presence of several highly conserved Cys residues in the catalytic domain of each enzyme. Those enzymes that were observed to be NEM-insensitive contained either a Ser or Thr residue in place of one of the Cys residues. A detailed analysis of this site, employing site-directed mutagenesis and enzyme kinetic studies, revealed that the site corresponding to Ser^{178} of FucT IV (i.e., Cys^{143}, Cys^{156}, Cys^{142}, Thr^{127}, and Thr^{142} of FucT III, FucT V, FucT VI, FucT VII, and FucT IX, respectively) was responsible for NEM inactivation, and more importantly that GDP-fucose but not other acceptor nucleotide sugars could protect the enzyme from NEM inactivation (Holmes *et al.*, 1995).

Pyridoxal-5′-phosphate (PLP) has been shown to be a competitive inhibitor of FucTs with respect to GDP-fucose. Treatment of the FucTs with PLP/ $NaBH_4$ caused enzyme inactivation that could be prevented by GDP-fucose (Sherwood *et al.*, 1998), indicating the involvement of a catalytically essential Lys residue in the protein. Sequence alignment of the human FucTs demonstrated that three Lys residues are highly conserved. Site-directed mutagenesis studies revealed that sites corresponding to Lys^{228} and Lys^{283} of FucT IV were required for activity, but not substrate binding, and that the Lys residue corresponding to Lys^{300} of FucT IV was associated with GDP-fucose protectable PLP/$NaBH_4$ inactivation (Sherwood *et al.*, 1998, 2000). Both Lys^{283} and Lys^{300} of FucT IV are located within the "α3-FucT motif" (discussed later), which is highly conserved among α3-FucTs from human and other sources (Martin *et al.*, 1997).

Mutation studies of FucT III have also implicated Asp^{336} in the binding of GDP-fucose. Substitution of Ala for Asp^{336} produced a protein that had a fourfold reduction in its affinity for GDP-fucose when compared with FucT III (Vo *et al.*, 1998).

Considered together, the results from studies of NEM and PLP inactivation suggest that the GDP-fucose binding site of the enzyme is composed of amino acid residues from at least three distinct regions of the protein. Presumably, protein folding places these portions of the molecule in close proximity of each other in 3D space to comprise elements of the GDP-fucose binding site. The extent to which other amino acids from perhaps other portions of the enzyme contribute to the GDP-fucose binding site remains to be determined. Additionally, structural similarities or differences of GDP-fucose binding sites between the three different families of α2-, α3-, and α6-FucTs is an intriguing area for future study.

Disulfide linkages

Sequence alignments of human FucTs demonstrate that they contain four conserved Cys residues in their catalytic domains. Based on the results described previously demonstrating that amino acids at the N- and C-terminus of the catalytic domain of FucTs III and V, we proposed that one or two disulfide bonds would occur between the highly conserved Cys residues at the two ends of the catalytic domain. Our studies of the disulfide bonding pattern of FucT III demonstrated that the four conserved Cys residues present in the catalytic domain are involved in disulfide bonds (Cys^{81} to Cys^{338} and Cys^{91} to Cys^{341}) that bring the two ends of the catalytic domain close in space (Holmes *et al.*, 2000). FucTs III, V, and VI are believed to arise via gene duplication of an ancestral gene (Oriol *et al.*, 1999) and have a relatively high sequence homology (more than 90%). Thus it is expected that the same pattern of disulfide bonds occurs in FucT V and VI. In contrast, similar studies of the disulfide bonding pattern of FucT VII provided a completely different result. In addition to the four highly conserved Cys residues present in FucTs III, V, and VI, this protein contains two additional closely spaced Cys residues at positions 211 and 214. The results of the disulfide analysis demonstrated that three disulfide bonds were formed (Cys^{68} to Cys^{76}, Cys^{211} to Cys^{214}, and Cys^{38} to Cys^{321}) (De Vries *et al.*, 2001). Thus disulfide bonds occur between each closely spaced pair as opposed to disulfide bridges occurring between Cys residues at opposite ends of the catalytic domain. FucT VII shares approximately 40% amino acid sequence homology with each of the other enzyme forms. FucT IV and FucT IX also each share approximately 40% amino acid sequence homology with the other FucTs. FucT IV contains four and FucT IX contains six Cys residues in their respective catalytic domains. Given the sequence heterogeneity of these FucTs, it is difficult to predict whether FucT IV and FucT IX will have a disulfide bonding pattern similar to that of FucT III or FucT VII.

Effect of glycosylation on enzyme activity

The six human FucT members cloned thus far contain between two and five potential N-linked glycosylation sites. The extent to which this type of posttranslational modification occurs and is significant for enzyme activity has been the subject of some studies. Recent evidence demonstrates that both N-linked sites of FucT III are glycosylated (Holmes *et al.*, 2000). These sites are conserved among FucTs III, V, and VI. In addition, FucTs V and VI have two extra sites, one of which (Asn^{105}) is not glycosylated on FucT V when expressed in COS cells (Nguyen *et al.*, 1998). FucT VII has two potential sites (Asn^{80} and Asn^{291}), which were both found to be glycosylated (De Vries *et al.*, 2001).

Some studies have addressed the question whether glycosylated N-linked sites are necessary for enzyme activity. A study of human FucTs III, V, and VI was conducted focusing on the two conserved N-linked sites present in FucT III (Christensen *et al.*, 2000). Two lines of investigation were used involving the effect of the glycosylation inhibitors tunicamycin and castanospermine and the effect of site-directed mutagenesis on enzyme activity. The results obtained demonstrated that tunicamycin abolished FucT III activity and castanospermine treatment resulted in an enzyme with 40% of the native activity. Site-directed mutagenesis experiments wherein N-linked Asn residues were changed to Gln residues resulted in a series of mutants each with lower enzymatic activity than the wild type enzyme. Elimination of individual sites had different effects on activity; however, protein expression in the cell was not affected and the expected reduction in molecular mass was observed. A kinetic analysis of the N185Q mutant of FucT III showed that the K_m for donor and acceptor substrates did not change. A similar study considered the effect of glycosylation on rat FucT IV (Baboval *et al.*, 2000). The two potential N-linked sites present in this enzyme were mutated singly and together, and the effect on enzyme activity was determined. The results demonstrated that the protein required

glycosylation for activity but that intracellular localization of the protein was not affected. Decreased activity occurred at a level of 64%, 5%, and 1% of the wild-type enzyme for mutations at Asn^{117}, Asn^{218}, or both, respectively (Baboval *et al.*, 2000). Considered together, these studies show that N-glycosylation is required for optimal FucT activity, although whether its significance is to ensure or maintain proper protein folding of the enzyme or through an alternate mechanism is presently unclear.

Effect of naturally occurring mutations on enzyme activity

Additional clues about the functional importance of specific amino acids have been obtained from a variety of studies linking population genetics data with phenotype expression. Genes that are found to be polymorphic so far are FUT3, FUT5, FUT6, and FUT7. For example, the genetic bases for the Lewis negative phenotype has been identified in African, Caucasian, and Indonesian populations (Nishihara *et al.*, 1993; Koda *et al.*, 1993; Elmgren *et al.*, 1993, 1997; Mollicone *et al.*, 1994b; Ørntoft *et al.*, 1996; Pang *et al.*, 1998a,b) as shown in Table II. Many of these mutations occur in the catalytic domain of FucT III, leading to a substantial reduction in enzymatic acitivity. Inspection of these inactivating mutations indicates that these include nonconservative amino acid changes from, for example, neutral or hydrophobic amino acids to charged residues (such as W68R or I356K), as well as more subtle changes, such as L149M or V270M. Another mutation, D162N, has been found that gives rise to less than complete inactivation (20% of wild-type activity). Furthermore, an interesting mutation, L20R (mutation of a nonconserved amino acid) has been identified in the membrane-spanning region of FucT III, which has no effect on enzyme activity or substrate affinities. However, it appears that this mutation alters Golgi membrane anchoring or targeting of FucT III.

When the locations of these amino acid mutations in FucT III are compared with regions of the FucT III catalytic domain that contain amino acids affecting substrate binding, an interesting correlation is found. Mutant W68R occurs in a region of the protein thought to have something to do with acceptor binding. Similarly, mutants L149M, D162N, and V270M are in close proximity to amino acids involved in GDP-fucose binding. The extent to which other inactivating mutations of FucT III (e.g., G223R or I356K) correlate with critical changes in substrate binding or catalytic functions remains to be determined.

Table II. Effect of naturally occurring polymorphisms in FucT genes on enzyme activity

Mutation site	Effect on activity	Reference
FucT III		
L20R	Wild-type activity and substrate affinities	Nishihara *et al.*, 1993
	May have altered Golgi membrane anchoring	Koda *et al.*, 1993; Mollicone *et al.*, 1994b
W68R	May have 1% of wild-type activity	Elmgren *et al.*, 1997
Q102K	Functional enzyme	Pang *et al.*. 1998a
T105M	Active	Elmgren *et al.*, 1997
S124A	No apparent activity	Pang *et al.*. 1998a
L149M	No apparent activity	Ørntoft *et al.*, 1996
D162N	20% of wild-type activity	Pang *et al.*, 1998a,b
G170S	No apparent activity	Nishihara *et al.*, 1993; Koda *et al.*, 1993
G223R	No apparent activity	Pang *et al.*, 1998a,b
V270M	No apparent activity	Pang *et al.*, 1998a,b
I356K	No apparent activity	Mollicone *et al.*, 1994b; Nishihara *et al.*, 1994
FucT V		
R173C	Wild-type activity	Mollicone *et al.*, 1994a
P187L	Wild-type activity	Mollicone *et al.*, 1994a
T338M	Wild-type activity	Mollicone *et al.*, 1994a
FucT VI		
P124S	Wild-type activity	Mollicone *et al.*, 1994a
L244V	1/3 wild-type activity	Elmgren *et al.*, 2000
E247K	No apparent activity	Mollicone *et al.*, 1994a
Y315stop	Inactive, enzyme missing 45 amino acids from the C-terminal of the catalytic domain	Mollicone *et al.*, 1994a
R303G	2/3 wild-type activity	Elmgren *et al.*, 2000
FucT VII		
R110Q	No apparent activity	Bengtson *et al.*, 2001

Enzyme-inactivating mutations have also been found in individuals deficient in the plasma FucT VI enzyme (Mollicone *et al.*, 1994a; Elmgren *et al.*, 2000). Although one reported mutation is a mutation at Y^{315} of FucT VI resulting in a stop codon, other mutations lead to full-length enzymes that are inactive (E247K), or have reduced activity (L244V and R303G, which have 1/3 and 2/3 the activity of wild-type FucT VI, respectively [Mollicone *et al.*, 1994a; Elmgren *et al.*, 2000]). Another FucT VI mutation, P124S, appears in conjunction with other alterations but, by itself, has wild-type activity. Once again, inspection of the distribution of these mutations demonstrates close association with elements of the GDP-fucose binding site for both L244V and E247K mutants. Both of these residues lie within the "α3-FucT motif" of the enzyme (see discussion later). However, the mutation at L244 did not affect binding to either GDP-fucose or acceptor substrate (Elmgren *et al.*, 2000).

Interestingly, naturally occurring mutations of FucT V have been found in the Indonesian population (Mollicone *et al.*, 1994a) that result in enzymes with normal activity. These mutations occur in regions of the protein that do not correspond to those presently known to affect substrate binding (e.g., R173C, P187L, and T338M).

Three individuals, suffering from chronic inflammatory diseases, were found to carry a polymorphism of the FUT7 gene. Only one mutation was found, leading to an amino acid substitution of R to Q at position 110. Screening of 106 Caucasian plasma donors showed a frequency of 1% for this mutation. The mutation resulted in an enzyme that lacked FucT VII activity (Bengtson *et al.*, 2001).

α3-FucT motif

Sequence alignment of α3-FucTs from species ranging from bacteria to humans reveals a highly conserved stretch of 17 amino acids, FxL/VxFENS/TxxxxYxTEK, generally referred to as the α3-FucT motif (Martin *et al.*, 1997). Because all enzymes in the FucT family bind GDP-fucose, it has been speculated that this consensus sequence is involved in donor substrate binding. As mentioned in an earlier section, two of the amino acids (e.g., Lys^{283} and Lys^{300} of FucT IV) found in the motif have been shown to be essential for activity, but only one (Lys^{300}) appears to be involved in GDP-Fuc binding.

Structural modeling

To date, no experimentally determined 3D structures have been reported for the FucTs. In addition, the protein databank (PDB) (Berman *et al.*, 2000) contains no structures with sufficient sequence similarity to the FucTs to be used for direct homology modeling. Fold recognition or threading software allows for the identification of structural homologies based on 3D packing requirements in cases of low sequence identity between a protein sequence and a template 3D protein fold (Fischer *et al.*, 1996). This technique was used by Breton *et al.* (1996) to propose the first structural model for a member of the FucT family. They identified the crystal structure of β-glucosyltransferase (Vrielink *et al.*, 1994) (PDB code 2BGU; Berman *et al.*, 2000) as a potential structural template to model the spatial fold of FucT IV. In a recent contribution (De Vries *et al.*, 2001), the experimentally determined disulfide bridge cross-linking in FucT VII was used as a constraint for identifying suitable 3D folds for this enzyme. It was shown that the β-glucosyltransferase fold was unable to accomodate the observed cysteine pairing and a new fold was proposed similar to the crystal structure of dihydrodipiconilate synthase (Mirwaldt *et al.*, 1995; PDB entry 1dhp). This structure belongs to the $(\alpha/\beta)_8$- or TIM-barrel family of 3D folds, which encompasses a number of carbohydrate processing enzymes. This TIM- or $(\alpha/\beta)_8$-barrel fold consists of a cylindrical arrangement of eight parallel β-strands, linked together by α-helical segments positioned on the outside of the resulting barrel. Although the proposed model did not completely agree with predicted positions of secondary structure elements, it did provide a feasible structural template for the cysteine pairings in FucT VII.

Recent experiments (Holmes *et al.*, 2000) probing the topology of the cysteine bridges in FucT III have elucidated a strikingly different pairing of cysteines in this enzyme. In FucT III, four cysteines were found to covalently connect regions relatively far apart in the FucT III sequence, in contrast to the observed pairing of adjacent cysteines in the amino acid sequence of FucT VII (De Vries *et al.*, 2001). Alignment of the FucT III sequence on our previously proposed model for FucT VII indicated that this fold would not be able to accommodate both topologically different types of cysteine pairings observed in FucT III and FucT VII. Therefore, we set out to identify a fold that would be in agreement with the current available experimental data on both of these FucTs.

Three different computational threading approaches were employed to attempt to identify 3D folds for FucT III and FucT VII among published protein structures. The threading programs used in this study were Molecular Simulations Inc. (San Leandro, CA) SeqFold program (Fischer and Eisenberg, 1996) as implemented in InsightII version 1998, the Bioinbgu threading Internet server (Fischer, 2000) and the TopLign threading server (Alexandrov *et al.*, 1996). For fold recognition searches, only the nonmembrane-bound portions of FucT III and FucT VII, comprising residues 61–361 and 41–342, respectively, were used. Folds that ranked among the top 10 scorers identified by the various threading methods and of which the sequence in the obtained alignments was of comparable length as the FucTs were downloaded from the PDB and visually inspected. Besides the requirement that the proposed fold should be able to accommodate the experimentally determined cysteine-pairings of both FucT III and FucT VII, a second constraint was applied that required the N- and C-termini of the resulting model to be in close spatial proximity. The latter restraint has its foundation in mutagenesis studies (Vo *et al.*, 1998) that showed residues from both the N- and C-terminus to be involved in acceptor binding, indicating their proximity in space.

Only a single fold was identified, using the TopLign threading program run with contact capacity scoring using the Dayhoff matrix (Zimmer *et al.*, 1998), that fulfilled all of the aforementioned requirements. This fold corresponded to the α-subunit of the tryptophan synthase enzyme from *Salmonella typhimurium* (Rhee *et al.*, 1996, PDB entry 1UBS) and represents another TIM-barrel type fold. It ranked sixth in fold recognition searches performed with both the FucT III and FucT VII sequences and had a sequence identity with these enzymes in the range of 7.8% and 8.6%, respectively.

Using the Modeller program (Sali and Blundell, 1993), implemented in the MSI Quanta 98 package, 3D homology models were built and energy minimized using Charmm (Brooks *et al.*, 1983) to ensure correct geometries for the disulfide bridges. Figure 1 shows the revised models for FucT III and FucT VII side by side. The experimentally determined disulfide bonding patterns can be accommodated because the N-terminal cysteines 81 and 91 in FucT III (68 and 78 in FucT VII) are located in a loop region devoid of predicted secondary structure. Though the C-terminal cysteines (338 and 341 in FucT III, 318 and 321 in FucT VII) are relatively fixed by the α-helix in which they are embedded, the extended loop region provides sufficient flexibility to allow for disulfide bridges between adjacent cysteines in FucT VII and between the N- and C-terminus in FucT III. Although the modeling software represents the loop region in FucT VII in a completely extended conformation (Figure 1b) this loop could in reality be folded to a more compact structure similar to that in FucT III. As shown in Figure 1A–B, the N- and C-terminus of the catalytic domains are in close spatial proximity in these models and the FucT motif (in red) is located on the same side of the protein as the N- and C-termini.

The region separating the FucT motif and Cys^{143} in FucT III (residues His^{145} to Lys^{201}) is considered to have the highest probability of being in error. This is due to two factors: First, the insertion of a large, unstructured loop (running from Arg^{160} to Ala^{188}) of which the true fold cannot be predicted due to lack of corresponding amino acids in the template structure. Second, a mismatch between predicted α-helical structure for the region from His^{189} to Trp^{195}, which is extended or β-sheet in the original template structure (and therefore also in the derived model). A different fold and/or positioning of the His^{145} to Lys^{201} region could bring the FucT motif and Cys^{143} in closer proximity than is possible on the basis of models derived from currently available protein structures. Although the current model is in agreement with observed cysteine pairings and spatial proximity of the N- and C-terminus, the mutual positioning of the FucT motif and Cys^{143} is expected to be influenced by regions in the FucT III sequence for which no suitable template is available in the PDB.

The topologies of recently solved crystal structures of glycosyltransferases were discussed in a review by Ünligil and Rini (2000). These authors showed a diverse set of glycosyltransferases to share a common, novel topology. The $(\alpha/\beta)_8$- or TIM-barrel topology that is consistently identified for the FucTs by various threading programs is markedly different from the topology described by Ünligil and Rini (see Figure 2). In addition, alignment of FucT VII with the crystal structure of one of these glycosyltransferases, bovine β4-galactosyltransferase (Gastinel *et al.*, 1999) showed that this fold was not consistent with the observed cysteine pairing of FucT VII (De Vries *et al.*, 2001). Thus, although the models presented here portray only an approximate picture of the 3D fold of the FucTs, it appears likely that this enzyme family represents a novel fold with an overall barrel-like character.

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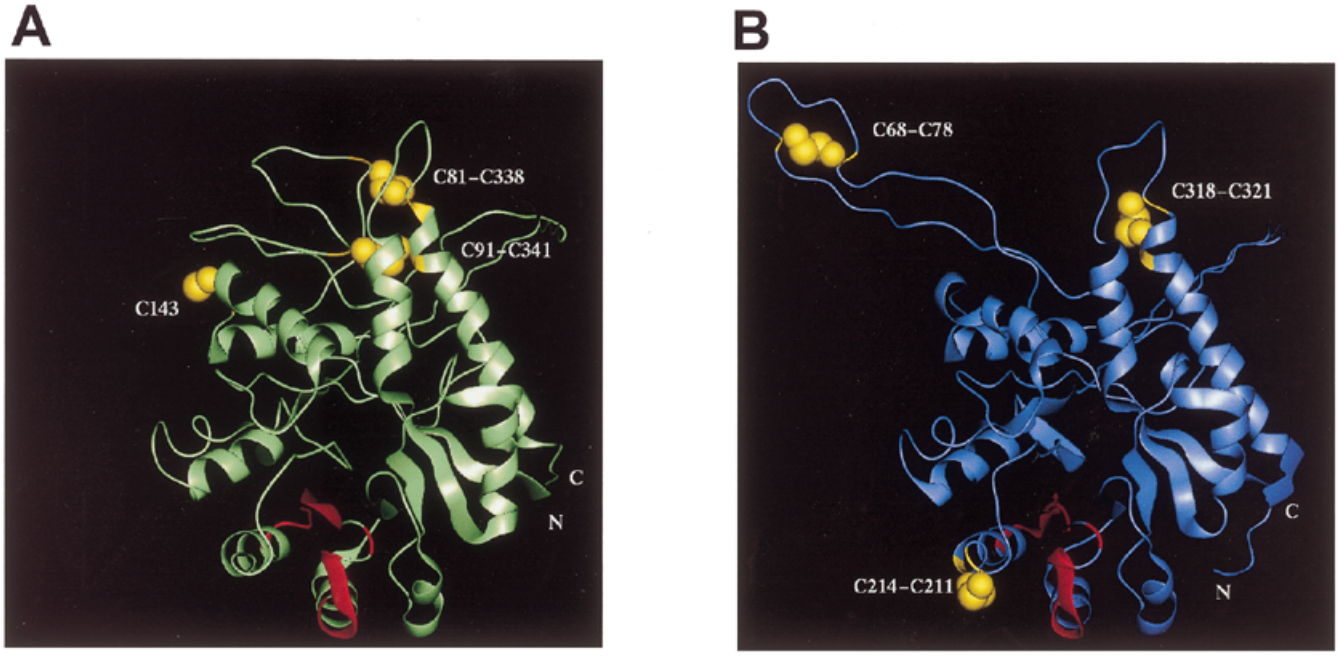

Fig. 1. (**A**) depicts a homology model for FucT III based on the crystal structure of the α-subunit of tryptophan synthase from *Salmonella typhimurium* (Rhee *et al.*, 1996, PDB entry 1UBS). The protein main chain is shown as a green ribbon with the FucT domain colored red. Cysteines are denoted by their one-letter amino acid code and residue number and are shown as space-filling van der Waals spheres. The N- and C-termini are indicated by the letters N and C, respectively. (**B**) shows the corresponding homology model for FucT VII with the protein main chain as a blue ribbon. Color coding for the FucT domain and cysteines is as in panel **A**. The long loop containing cysteines 68 and 78 may in reality be folded to form a more compact domain comparable to that in FucT III.

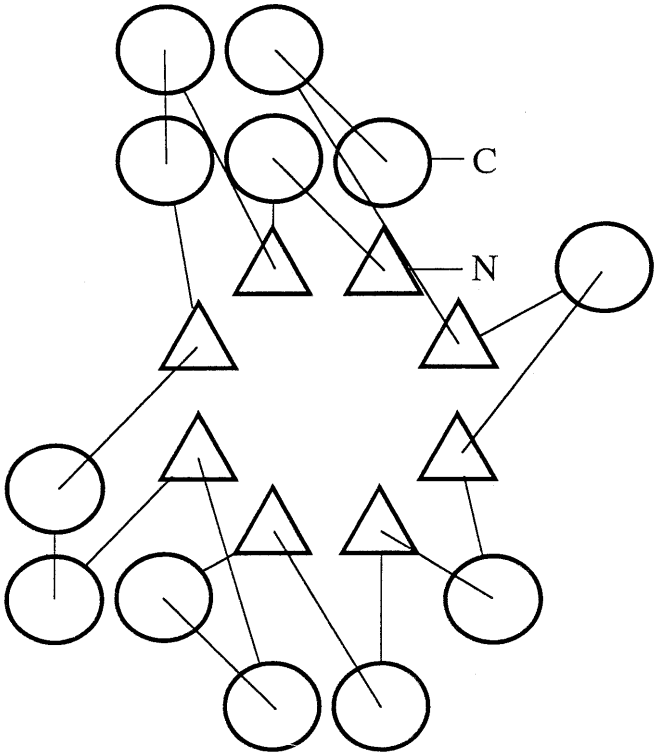

Fig. 2. Simplified topology diagram (Westhead *et al.*, 1998) of the TIM-barrel fold of tryptophan synthase (Rhee *et al.*, 1996, PDB entry 1UBS) consisting of a cylindrical arrangement of eight parallel β-strands, linked together by α-helical segments positioned on the outside of the resulting barrel. α-Helices are shown as circles and β-strands as triangles.

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Abbreviations

FucT, fucosyltransferase; GalT, galactosyltransferase; NEM, N-ethylmaleimide; ORF, open reading frame; PDB, protein databank; PLP, pyridoxal-5′-phosphate.

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