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A highly conserved His-His motif present in α1→3/4fucosyltransferases is required for optimal activity and functions in acceptor binding

Anne L. Sherwood, David A. Upchurch, Mark R. Stroud, William C. Davis, and Eric H. Holmes[1]

Northwest Hospital, Molecular Medicine, Department of Cell Surface Biochemistry, 21720 23rd Drive SE, Suite 101, Bothell, WA 98021, USA

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α1→3/4Fucosyltransferases (FucTs) from several species contain a highly conserved His-His motif adjacent to an enzyme region correlating with the ability to catalyze fucose transfer to type 1 chain acceptors. Site-directed mutagenesis has been employed to analyze structure–function relationships of this His-His motif in human FucT-IV. The results indicate that most changes of His^{113} and His^{114} and nearby residues of FucT-IV reduced the specific activity of the enzymes. Analysis of acceptor properties demonstrated close similarity of most mutants with wild-type FucT-IV, whereas an apparent preference for the H-type II acceptor was observed for the His^{114} mutants. Kinetic studies demonstrated that mutants of His^{114} had a substantially increased K_m for acceptor compared to other enzymes tested. The dramatic increase in acceptor K_m for the His^{114} mutants, particularly for the nonfucosylated acceptor, suggests that this His-His motif is involved in acceptor binding and perhaps interacts with GlcNAc residues of type 2 acceptors. The presence of fucose in acceptor substrates may promote more efficient substrate binding and presumably partially overcomes the weaker interaction with GlcNAc caused by the mutation.

Key words: carbohydrate binding site/fucosyltransferase/His motif/structure–function relationships

Introduction

α1→3/4Fucosyltransferases (FucTs) catalyze fucose transfer on GlcNAc residues in lacto- or neolacto-series cell surface carbohydrates of glycolipids or glycoproteins. These structures, representing the Le^a- and Le^x-related antigens, accumulate in many human cancers (Hakomori, 1989; Alhadeff, 1989), undergo developmental regulation (Hakomori, 1989; Alhadeff, 1989; Sanders and Kerr, 1999; Listinsky *et al.*, 1998), or function as natural ligands for leukocyte adhesion during inflammation (Sanders and Kerr, 1999; Listinsky *et al.*, 1998; Renkonen *et al.*, 1997; McEver, 1997). A large number of distinct α1→3/4FucTs have been identified and cloned in multiple organisms. For example, in humans, six distinct enzymes, designated FucT-III, -IV, -V, -VI, -VII, and -IX, have been cloned (Kukowska-Latallo *et al.*, 1990; Goelz *et al.*, 1990; Weston *et al.*, 1992a,b; Koszdin and Bowen, 1992; Sasaki *et al.*, 1994; Natsuka *et al.*, 1994; Kaneko *et al.*, 1999). Although each enzyme has the common property of binding the GDP-fucose donor substrate, subtle differences in acceptor specificity and tissue distribution distinguish members of this family of enzymes. Additionally, α1→3/4FucTs have been identified and cloned from other mammalian species (Nishihara *et al.*, 1999; Costache *et al.*, 1997; Gersten *et al.*, 1995; Smith *et al.*, 1996; Ozawa and Muramatsu, 1996; Kudo *et al.*, 1998; Sajdel-Sulkowska *et al.*, 1997; Zhang *et al.*, 1999; Oulmouden *et al.*, 1997), chicken (Lee *et al.*, 1996), fish (Kageyama *et al.*, 1999), invertebrates (Trottein *et al.*, 2000; DeBose-Boyd *et al.*, 1998), plants (Leiter *et al.*, 1999), and bacteria (Rasko *et al.*, 2000). Many of these enzymes share significant sequence homology and enzymatic properties with one or more human counterparts.

Most sequence homology among FucTs occurs in the C-terminal catalytic domain. Structure–function studies of amino acids in this region have identified residues involved in GDP-fucose binding (Holmes *et al.*, 1995; Sherwood *et al.*, 2000), catalysis (Sherwood *et al.*, 1998; Vo *et al.*, 1998; Britten and Bird, 1997), and disulfide bonding (Holmes *et al.*, 2000). Greater sequence heterogeneity is found in the N-terminal cytoplasmic and transmembrane domains, and the stem region. Residues involved in aspects of acceptor binding have been reported to occur in more N-terminal portions of the protein (Legault *et al.*, 1995; Dupuy *et al.*, 1999; Nguyen *et al.*, 1998). For example, Legault *et al.* (1995) reported that amino acids present in a segment of FucT-III spanning residues 105 through 151 were associated with the enzyme's ability to transfer to type 1 chain structures. Substitution of residues in this region unique to FucT-VI disabled transfer to type 1 chain acceptors. Among these residues is R^{110} of FucT-VI (W^{111} in FucT-III). In a recent report, Dupuy *et al.* (1999) confirmed that residues in this position contribute in defining type 1 and 2 chain acceptor specificity. In another report, Nguyen *et al.* (1998) demonstrated that amino acids from a more N-terminal region of the protein were also involved in defining type 1 versus type 2 chain specificity. Modification of as few as two amino acids of FucT-V to the corresponding residues of FucT-III (Asn^{86} to His and Thr^{87} to Ile) increased α1→4FucT activity with both oligosaccharide and glycolipid acceptors. Despite the ability of these amino acid changes to elicit α1→4fucosyl transfer to type 1 acceptors, kinetic parameters clearly showed that other residues present in FucT-III must also be required for optimal transfer (Nguyen *et al.*, 1998).

[1]To whom correspondence should be addressed; E-mail: eholmes@nwbio.com

A highly conserved His-His motif is present in this N-terminal hypervariable region of most presently cloned FucTs (see Figure 1). This is adjacent to amino acids already discussed, which have been shown to influence acceptor binding properties (Legault *et al.*, 1995; Dupuy *et al.*, 1999; Nguyen *et al.*, 1998). We conducted site-directed mutagenesis studies to change these His residues (as well as other nearby residues) present in FucT-IV to determine their impact on enzyme activity and acceptor binding properties. The results demonstrate that the inherent enzymatic activity is reduced by many of these changes, and, in particular, changes in His^{114} results in altered acceptor affinities.

Results

Structure–function studies among human α1→3/4FucTs have led to the identification of amino acids involved in defining carbohydrate acceptor binding specificity (Legault *et al.*, 1995; Dupuy *et al.*, 1999; Nguyen *et al.*, 1998). In general, these residues are found in the N-terminal portions of the enzyme's catalytic domain. A productive strategy to define functional characteristics of specific amino acids has proven to be analysis of aligned protein sequences of α1→3/4FucTs to identify highly conserved amino acids, production of site-directed mutants at these positions, and analysis of the impact of these modifications on enzymatic properties. One highly conserved motif among cloned α1→3/4 FucTs is a His-His sequence corresponding, for example, to His^{113} and His^{114} of FucT-IV (see Figure 1). Although there is generally considerable sequence heterogeneity in this region among FucTs, this His-His motif is found in most enzyme forms, including all human FucTs. To analyze the functional properties of this motif, site-directed mutagenesis was used to change each of these sites to an Asn residue individually and together, and the effect of these mutations on acceptor properties was analyzed.

Site-directed mutagenesis and analysis of expressed enzymes

Site-directed mutagenesis was performed by replacing the sequence for codons within FucT-IV as summarized in Table I. No mutation resulted in the introduction of a new potential N-linked glycosylation site into the enzyme. Sequencing on both strands of all mutants confirmed that there were no other nucleotide modifications present compared to the wild-type FucT-IV sequence (data not shown). The plasmid containing both the wild-type FucT-IV sequence and that of the mutant enzymes was composed of a truncated form of the FucT-IV sequence missing the coding sequence for the first 57 amino acids of the FucT-IV enzyme in the pPROTA vector. The expressed protein was thus a fusion protein containing the Protein A Ig binding domain fused to the FucT-IV sequences. This aided in the isolation of the expressed protein by allowing direct binding to Ig-Agarose beads in a single-step purification. The presence of the Protein A Ig binding domain in the fusion protein could also be used to visualize and quantitate the protein in a western blot. No difference in enzymatic properties has been observed between pPROTA expressed FucTs and their native, full-length parent enzymes (De Vries *et al.*, 1995).

Figure 2 shows results from a western blot analysis of the pPROTA expressed wild-type FucT-IV and the mutant enzymes. Bands migrating at approximately 70–80 kDa corresponding to expressed wild-type FucT-IV and mutant fusion proteins are observed. In some cases these bands are weaker due to differences in expression levels. The majority of the expressed protein for enzymes containing the H113N mutation (both the single and double mutant) correspond to protein bands migrating between 40 and 45 kDa. This was a consistent finding because similar results were observed with expressed enzymes containing the H113N mutation from multiple expression experiments. These faster-migrating bands most likely result from degradation of the expressed fusion protein through protein turnover, possibly related to improper protein folding caused by the H113N mutation. For specific activity determinations, protein concentrations were based

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human FucT-III          1 MDPLGAAKPQWPWRRCLAALLFQLLVAVCFFSYLRVSRDDATGSPRAPSGSSRQDTTPTRPTLLILLWIWPFHIPVALSR  80
                       81 CSEMVPGIADCHITADRKVYPQADTVIVHHWDIMSNPKSRLPPSPRPQSQRWIWFNLEPPPNCQHLEALDRYFNLIMSYR 160

chimpanzee FucT-III    91 CSEMVPGAADCHITADRKVYPQADAVIVHHWDIMYNPKSRLPPSPRPQSQRWIWFNLEPPPNCQHLEALDRYFNLIMSYR 170
human FucT-IV          86 CP-LRFNISGCRLLITDRASYGEAQAVLFHHRDLVKGPPDWPPPSPRPPGQRWVWMNFESPSHSPGLRSLSNLFNWTLSYR 196
rat FucT-IV           112 CS-LRFNISGCRLLITDRAAYGEAQAVLFHHRVLVKGPPDWPPPSS-RPPGQRWVWMNFESPSHTPGLRGLKDLFNWTLSYR 224
mouse FucT-IV         112 CS-LRFNISGCRLLITDRAAYGEAQAVLFHHRDLVKELHDWPPPSS-RPPGQRWVWMNFESPSHTPGLRGLKDLFNWTLSYR 224
human FucT-V           94 CSEMVPGAADCNITADSSVYPQADAVIVHHWDIMYNPSANLPPPTRPQSQRWIWFSMESPSNCRHLEALDGYFNLIMSYR 173
chimpanzee FucT-V      94 CSEMVPGAADCNITADSNVYPQADAVIVHHWDIMYNPSANLPPPTRPQSQRWIWFSMESPSNCRHLEALDGYFNLIMSYR 173
human FucT-VI          80 CSEMVPGIADCNITADRKVYPQADAVIVHHREVMYNPSAQLPRSPRRQSQRWIWFSMESPSHCWQLKAMDGYFNLIMSYR 159
chimpanzee FucT-VI     80 CSEMVPGIADCNITADRKVYPQADAVIVHHREVMYNPSAQLPRSPRRQSQRWIWFSMESPSNCRHLEALDGYFNLIMSYR 159
hamster FUT6           82 CSKMLPGIADCQMTVNRSLYPQADAVIPHHREISPNPRSLLPSQPRPPGQRWVWFSLESPSHCSRLSALDGYFNLIMSYR 161
human FucT-VII         66 DTCTRYGIARCHLSANRSLLASADAVVPHHRE-LQTRRSHLPLAQRPRGQPWVWASMESPSHTHGLSHLRGIFNWVLSYR 144
mouse FucT-VII         66 DTCTRYGMASCRLSANRSLLASADAVVPHHRE-LQTRQSLLPLDQRPHGQPWVWASMESPSNTHGLHRFRGIFNWVLSYR 144
human FucT-IX          81 SCQAMFNIQGCHLTIDRSLYNKSHAVLIHHRDISWDLTNLPQQARPP-FQKWIWMNLESPTHTPQKSGIEHLFNLTLTYR 159
mouse FucT-IX          81 SCQAMFNIQGCHLTIDRSLYNKSHAVLIHHRDISWDLTNLPQQARPP-FQKWIWMNLESPTHTPQKSGIEHLFNLTLTYR 159
bovine futb            85 CSELWPGIADCQLIVNRSEYPQADAVFVHHREVSHRPKMQLPPSPRPADQRWVWFSMESPSNCLKLKDLDGYFNLIMSYR 164
chicken CFT1           74 DCRRRYNITGCLLSADRGRYGEARAVLFHHRDLALHGRQGLPRGPRPPRQRWVWMNFESPSHSPGLRGLAGLFNWIMSYR 155
zebrafish FucT-1      116 VCGSQFNIHGCHLTDDRSMIQKAHGVMFHHRDL----SADLFQPPRPAFQKWIWMNMESPSNSHPNSLINDGFNLTSSFR 191
zebrafish FucT-2      116 DCGLEYNIHGCQLTYDRSLIQKAHGVMFHHRDM----SIDFPQPPRPDFQKWIWMNMESPTHCNQNDFLNDRFNVTSSYN 191
S. mansoni SmFuct     95 ---------GCLVSNCVLHKT---KWRSYYAEMVI-ITNGPKVKNHQ-NQAWLAYHYEAPPHSRLNKLLGEKINFTATYR 162
mung bean FucT        133 CS--VGCKFGFSG--DRKP----DAAFGL----------------PQPSGIASILRSMESAENNIAMARRRGY-NIVMITS 192
C. elegans FucT        93 VPKKENPVGKEVLTSSDIKLEERMTATVIPWNAGHSQDQGCPPDPKP-NQYVVYPSQESPANSGIQIPRPDYINMTLGFR 218
H. pylori FucT         65 ESDLVFGNPLGSARKILSYQNAKRVFYTGENESPNFN------LELDFNDRYLRMPL---YYDRLHHKAESVNDTTAPYK 144

human FucT-III        161 SDSDIFTPYGWLEPWSGQPAHPPLNLSAKTELVAWAVSNWKPDSARVRYYQSLQAHLKVDVYGRSHKPLPKGTMMETLSR 240
                      241 YKFYLAFENSLHPDYITEKLWRNALEAWAVPVVLGPSRSNYERFLPPDAFIHVDDFQSPKDLARYLQELDKDHARYLSYF 320
                      321 RWRETLRPRSFSWALDFCKACWKLQQESRYQTVRSIAAWFT                                        361
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Fig. 1. Aligned amino acid sequences of cloned α1→3/4FucTs around a His-His motif (shown in bold) distributed in the N-terminal region. The aligned sequences are shown where they occur in the sequence compared to human FucT-III. Amino acid deletions are shown by dashes where they occur in the alignments. For clarity and ease of sequence comparison, underscored amino acids define flanking residues of amino acid insertions of variable size from 1 to 26 amino acids. The sequences of the inserted residues are not shown. The alignments shown for the enzymes from *S. mansoni* (Trottein *et al.*, 2000), mung bean (Leiter *et al.*, 1999) *C. elegans* (DeBose-Boyd *et al.*, 1998), and *H. pylori* (Rasko *et al.*, 2000) are based on those given in the indicated references.

Table I. Site-directed mutant enzymes used in this study

Position	Native codon	Native amino acid	Mutant codon	Mutant amino acid	Mutant designation
110	GTG	Val	GCA	Ala	V110A
113	CAC	His	CTC	Leu	H113L
113	CAC	His	AAC	Asn	H113N
113/114	CAC/CAC	His/His	AAC/AAC	Asn/Asn	H113N/H114N
114	CAC	His	GCA	Ala	H114A
114	CAC	His	CAA	Gln	H114Q
114	CAC	His	AAC	Asn	H114N
119	AAG	Lys	TAT	Tyr	K119Y

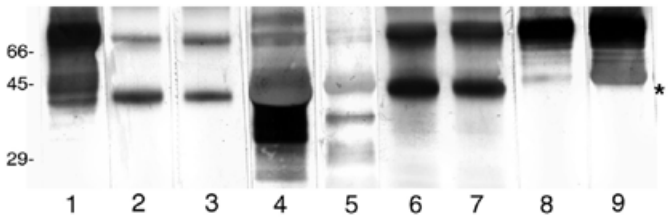

Fig. 2. Western blot analysis of pPROTA expressed enzymes. Lane 1, wild-type FucT-IV (30 ng); lane 2, V110A mutant (4 ng); lane 3, H113L mutant (7 ng); lane 4, H113N mutant (7 ng); lane 5, H113N/H114N double mutant (3 ng); lane 6, H114A mutant (18 ng); lane 7, H114Q mutant (15 ng); lane 8, H114N mutant (30 ng); and lane 9, K119Y mutant (40 ng). The conditions of the experiment were as described under *Materials and methods* using a standard volume of beaded enzyme containing the amount of pPROTA expressed enzyme shown in parentheses. The asterisk on the right indicates the position of a band corresponding to antibody heavy chain derived from the IgG-Agarose beads used to capture the pPROTA expressed protein. The migration of protein molecular weight standards (in kDa) is shown to the left.

upon the amount of the 70–80-kDa protein present for each enzyme.

Effect on enzyme specific activity and acceptor substrate specificity

Table II shows the effect of the mutations on enzyme-specific activity utilizing the disaccharide LacNAc as the acceptor. Although the specific activity for the K119Y mutant was very similar to wild-type FucT-IV, other mutants had reduced specific activities of from 3- to 3500-fold compared with wild-type FucT-IV. In particular, broad changes in specific activities for His^{114} mutants were observed with the H114Q, H114A, and H114N mutants having values 3-, 17-, and 80-fold lower, respectively, compared with wild-type FucT-IV. The H113L mutant and the H113N/H114N double mutant were found to be inactive with LacNAc as the acceptor substrate.

To further characterize the active mutants, a range of acceptor substrates were used. This analysis utilized synthetic 8-methoxycarbonyl derivatives of acceptor oligosaccharides because many of these free carbohydrates are not commercially available. The results are shown in Table III and are reported relative to the activity observed with the 8-methoxycarbonyl derivative of LacNAc. A standard acceptor concentration was used in each case based on optimized concentrations for wild-type FucT-IV. In these experiments, wild-type FucT-IV and mutants at positions 110, 113, and 119 behaved very similarly for each acceptor substrate tested. Similar results in comparison to wild-type FucT-IV were also observed for the His^{114} mutants with all acceptor oligosaccharides tested except for the H-type 2 acceptor. Relative transfer to the H-type 2 acceptor was increased approximately two- to threefold compared to wild-type FucT-IV for these mutants, indicating an inherent preference for an acceptor with a terminal α1→2-linked fucose for the His^{114} mutant enzyme. Wild-type FucT-IV is known to transfer fucose to sialylated acceptors poorly, and no difference was observed in the relative transfer to the sialylated acceptor by either His mutant. No significant fucose transfer to type 1 chain acceptors occurred with any enzyme tested.

Analysis of enzyme kinetic parameters

The apparent K_m values for wild-type FucT-IV and mutant enzymes for GDP-fucose donor and various acceptor oligosaccharides were determined as shown in Table II. The results demonstrate that none of the mutant enzymes had a significantly altered K_m for GDP-fucose compared to wild-type FucT-IV. In contrast, mutants of His^{114} had significantly higher K_m values for both LacNAc and H-type 2 acceptors when compared with wild-type FucT-IV, as well as mutants at other nearby sites. Interestingly, the increase in K_m was not of the same magnitude for both acceptor substrates. Wild-type FucT-IV and mutants at positions 110, 113, and 119 had K_m values for LacNAc in generally the 1 mM range, which was reduced to approximately 0.25 mM for the H-type 2 acceptor. A much larger proportionate difference was observed with the His^{114} mutants tested. K_m values for LacNAc varied from 5 to 11.6 mM and 0.6 to 1.1 mM for H-type 2. Thus the His^{114} mutant enzymes have an increased K_m for both acceptor substrates compared to wild-type FucT-IV, but the relative (fucosylated versus nonfucosylated substrate) effect on acceptor substrate binding is different. This suggests that mutations of His^{114} differentially alter the acceptor substrate binding site. Therefore, this amino acid may be closer to the portion of the enzyme that binds the GlcNAc rather than the Gal residue of type 2 acceptors. In contrast to the alterations in acceptor substrate K_m values, no significant differences were found in V_{max} when either LacNAc or H-type 2 was used as an acceptor. Due to the very low activity obtained with the H113N enzyme, limited kinetic studies were possible. The results obtained indicate that the H113N mutant has a K_m for LacNAc similar to that observed for the wild-type FucT-IV enzyme. The results obtained with

Table II. Specific activities and K_m values for wild-type FucT-IV and site-directed mutants

Enzyme	Specific activity (nmol/h/mg protein)	K_m (GDP-fucose; μM)	K_m (LacNAc; mM)	K_m (H-Type 2; mM)
FucT-IV	20.82	28 ± 4	1.06 ± 0.12	0.25 ± 0.01
V110A	0.42	19 ± 3	1.8 ± 0.3	0.23 ± 0.1
H113N	0.006	—	0.8 ± 0.1	—
H113L	ND	—	—	—
H114N	0.26	35 ± 2	11.6 ± 2.0	1.0 ± 0.2
H114A	1.22	33 ± 2	5.0 ± 0.1	1.1 ± 0.1
H114Q	6.50	27 ± 2	10.4 ± 0.2	0.57 ± 0.03
H113N/H114N	ND	—	—	—
K119Y	23.60	27 ± 2	1.4 ± 0.4	0.3 ± 0.1

Assays were conducted as described under *Materials and methods* using free oligosaccharides as acceptors. Specific activities were defined using free LacNAc as the acceptor.
ND = none detected.

Table III. Fucose transfer to oligosaccharide acceptors catalyzed by wild-type FucT-IV and site-directed mutants

Acceptor[a]	Wild-type FucT-IV	V110A	H113N	H114N	H114A	H114Q	K119Y
Galβ1→3GlcNAc-R_1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
H(Type I)-R1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
H(Type II)-R_1	1.14 ± 0.22	1.2 ± 0.22	1.52 ± 0.18	3.31 ± 0.21	1.87 ± 0.04	1.9 ± 0.04	1.33 ± 0.27
LacNAc-R_1	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Sulfo-LacNAc-R_1	0.83 ± 0.04	1.13 ± 0.15	1.27 ± 0.38	1.48 ± 0.5	0.72 ± 0.01	0.72 ± 0.21	1.18 ± 0.32
Sialyl-LacNAc-R_1	0.15 ± 0.05	0.35 ± 0.08	0.21 ± 0.05	0.08 ± 0.01	0.07 ± 0.06	0.07 ± 0.02	0.32 ± 0.18

Relative fucose transfer normalized to activity found with LacNAc-R1 as the acceptor is shown.
[a]R_1 = O-$(CH_2)_8COOCH_3$.

the two His mutants were different even though the two amino acids are adjacent to each other in the linear sequence.

Discussion

Glycosyltransferases catalyze the biosynthesis of cell surface carbohydrates composed of multiple sugar moieties in discrete sequences and linkages. A surprising observation is that families of enzymes capable of catalyzing the same reaction are commonly found in a single organism. Examples include families of β1→3- and β1→4galactosyltransferases (Amado *et al.*, 1999), α2→3sialyltransferases (Natsuka and Lowe, 1994; Kapitonov and Yu, 1999), and α1→3/4FucTs (Oriol *et al.*, 1999). Although we do not understand why there are so many forms of these enzymes expressed in one organism, the availability of the multiple forms provides opportunities to obtain useful information about how the substrate specificity is encoded into the protein.

To date, six human α1→3/4fucosyltransferases have been cloned (Kukowska-Latallo *et al.*, 1990; Goelz *et al.*, 1990; Weston *et al.*, 1992a,b; Koszdin and Bowen, 1992; Sasaki *et al.*, 1994; Natsuka *et al.*, 1994; Kaneko *et al.*, 1999). These enzymes have relatively high sequence homology, particularly in the C-terminal catalytic domain. They differ in their tissue distribution and acceptor specificity properties. Previous reports have mapped amino acids involved in acceptor specificity properties, particularly the ability to catalyze fucose transfer to type 1 chain acceptors to amino acids present in the N-terminal hypervariable region (spanning amino acids 76 through 151) of FucT-III (Legault *et al.*, 1995; Dupuy *et al.*, 1999; Nguyen *et al.*, 1998). In particular, these reports identified His^{76}, Ile^{77} (Nguyen *et al.*, 1998), and Trp^{111} (Dupuy *et al.*, 1999) as being associated with transfer to type 1 chain acceptors. In addition to variable amino acids that correlate with a specific catalytic property, highly conserved amino acids are also found in this region of the protein and likely also play a critical role in acceptor binding.

One example is a highly conserved His-His motif that is immediately proximal to the W/R position, which correlates with type 1/type 2 chain transfer specificity (Dupuy *et al.*, 1999). This His-His motif is conserved among FucTs from mammalian (Nishihara *et al.*, 1999; Costache *et al.*, 1997; Gersten *et al.*, 1995; Smith *et al.*, 1996; Ozawa and Muramatsu, 1996; Kudo *et al.*, 1998; Sajdel-Sulkowska *et al.*, 1997; Zhang *et al.*, 1999; Oulmouden *et al.*, 1997), chicken (Lee *et al.*, 1996), and fish (Kageyama *et al.*, 1999) species. However,

FucTs from evolutionarily more distant organisms (i.e., plant [Leiter *et al.*, 1999], bacteria [Rasko *et al.*, 2000], and invertebrate species [Trottein *et al.*, 2000; DeBose-Boyd *et al.*, 1998]) do not have this motif. Studies of the acceptor specificities of enzymes that do not contain the His-His motif are incomplete; however, the evidence that is available indicates that at least some of these enzymes catalyze fucose transfer to either different (Leiter *et al.*, 1999) or a wider variety (DeBose-Boyd *et al.*, 1998) of acceptor carbohydrate structures than lacto- or neolacto-series structures.

The results presented demonstrate these His residues in human FucT-IV are important for optimal activity. Modification of His^{113} resulted in either inactive or weakly active enzymes; in some cases the mutant enzyme was partially degraded, possibly due to improper protein folding. Mutants of His^{114} also had reduced specific activity compared to wild-type FucT-IV, although to a lesser extent of 3–80-fold. A double mutant H113N/H114N was catalytically inactive with LacNAc. Other mutants at surrounding positions had varying effects on specific activity but had kinetic parameters very similar to the wild-type enzyme. Interestingly, analysis of acceptor specificity properties of the active mutants revealed that when normalized to the activity with LacNAc as the acceptor, all had very similar acceptor properties to the wild-type enzyme except for mutants of His^{114}, which displayed a two- to threefold increased preference for the H-type 2 acceptor compared to the wild-type and other enzymes. Kinetic analyses of these mutant enzymes revealed that all mutant enzymes had a K_m for GDP-fucose similar to the wild-type enzyme. Apparent K_m values for LacNAc for mutants other than His^{114} were determined to be equal to that of wild-type FucT-IV. In contrast, significant differences in the K_m for acceptors were found between wild-type FucT-IV and the His^{114} mutants.

Although a contribution of His^{114} to maintaining proper protein structure cannot be ruled out, changes in this site dramatically altered the ratio of the K_ms for the two acceptors, leading to the likelihood that this position is directly involved in acceptor binding. Saturation of wild-type FucT-IV, as well as mutants in positions 110, 113, and 119, with LacNAc resulted in an apparent K_m for the acceptor of approximately 1 mM, similar to other published reports (Sherwood *et al.*, 1998, 2000; De Vries *et al.*, 1995). An approximately fourfold reduced K_m was observed when the H-type 2 acceptor was used (see also De Vries *et al.*, 1995). Increased K_m values were observed for both acceptors with the His^{114} mutants. The apparent K_m for LacNAc varied between 5 and 11.6 mM for the His^{114} mutants versus values between 0.6 and 1.1 mM for the H-type 2 acceptor. Although both K_ms are increased, the significantly lower K_m for H-type 2 gives rise to the increased relative activity with this acceptor for the His^{114} mutant enzymes. The altered relative binding of a fucosylated versus a nonfucosylated acceptor suggests that this region may be an element of an acceptor binding pocket that interacts with GlcNAc residues of type 2 acceptors. Presumably, the presence of a fucose residue on the terminal Gal of the acceptor partially overcomes the reduced acceptor binding caused by weaker interactions with the GlcNAc residue.

Human α1→3/4FucTs contain five highly conserved His residues. The data presented in this report demonstrate an involvement in acceptor binding for certain of these residues. Other studies we are conducting address the involvement of His residue(s) in GDP-fucose binding. Future reports will address these results to provide a broader perspective of structure–function relationships within human FucTs.

Materials and methods

Materials

COS-7 cells were obtained from the American Type Cell Collection (Rockville, MD). N-acetyllactosamine (LacNAc), 2′-fucosyl-N-acetyllactosamine (H-type 2 trisaccharide), GDP-fucose, rabbit IgG-agarose beads, and DEAE-Dextran were obtained from Sigma (St. Louis, MO). Plasmid pCR2.1 TOPO was from Invitrogen (San Diego, CA), and pPROTA and pPROTA-FucT-IV (long form, amino acids 58–405) plasmids were received from Dr. Bruce A. Macher (San Francisco State University, San Francisco, CA). GDP-[^{14}C]fucose (283 mCi/mmol) and [^{35}S]dATP were obtained from Dupont NEN (Boston, MA). DNA sequencing of initial constructs was done using the Sequenase Version 2.0 DNA sequencing kit or the Sequenase polymerase chain reaction (PCR) product sequencing kit obtained from U.S. Biochemical (Cleveland, OH). Automated DNA sequencing of final pPROTA constructs was performed at the San Francisco State University Sequencing Facility, by Thetagen (Seattle, WA), or by GeneMed Synthesis (San Francisco, CA). PCR primers were obtained from Oligos, Etc. (Wilsonville, OR), Integrated DNA Technologies (Coralville, IA), or were made on a Beckman Oligo 1000 Synthesizer. All other reagents were of the highest quality commercially available.

Cell culture

COS-7 cells were grown in tissue culture plates in Dulbecco's modified Eagle's medium (DME), supplemented with 10% fetal calf serum. These were passed 1:4 every 5–6 days.

Transfection of FucT constructs into COS-7 cells for enzyme expression

COS-7 cells were transfected by the DEAE dextran technique (Ausubel *et al.*, 1993) with the pPROTA Fuc T-IV constructs: chimeric, truncated wild-type FucT-IV, V110A, H113L, H113N, H113N/H114N, H114A, H114Q, H114N, and K119Y. Three to five days posttransfection, secreted fusion proteins were adsorbed to rabbit IgG-Sepharose beads (Sigma), washed extensively with phosphate buffered saline (PBS), and stored in PBS containing 0.02% NaN_3 for use in these experiments.

Site-directed mutagenesis

Parental FucT coding sequences for site directed mutagenesis were truncated catalytic domain forms of the native FucT-IV in the pPROTA vector (Henion *et al.*, 1994). Properties of pPROTA expressed enzymes have been shown to be very similar to those of full-length enzymes (De Vries *et al.*, 1995). Site-directed mutant enzymes used in this study are shown in Table I. The flanking and mutagenic primers used in forming the FucT-IV V110A, H113L, H113N, H113N/H114N, H114A, H114Q, H114N, and K119Y mutants described in this study are shown in Table IV. Site-directed mutagenesis of FucT-IV in pPROTA was conducted as follows via recombinant PCR (Higuchi, 1990). The forward flanking primers used for the three FucT-IV mutants contained an EcoR1 site

Table IV. Primers used for recombinant PCR mutagenesis

Single mutations	
Flanking primers	
	Forward:5′ TGgaattcGCCAACCCCGTCGCGACCG 3′
	Reverse:5′ AGgtcgacTTCACCGCTCGAACCAGCTGGCCAA 3′
Mutagenic primers	
V110A	Forward: 5′ GCTCAGGCCGCACTTTTCCACCAC 3′
	Reverse: 5′ GTGGTGGAAAAGTGCGGCCTGAGC 3′
H113L	Forward: 5′ GCCGTGCTTTTCCTCCACCGCGAC 3′
	Reverse: 5′ GTCGCGGTGGAGGAAAAGCACGGC 3′
H113N	Forward: 5′ CTTTTCAACCACCGCGACCTCGTG 3′
	Reverse: 5′ CACGAGGTCGCGGTGGTTGAAAAG 3′
H114A	Forward: 5′ CTTTTCCACGCACGCGACCTCGTG 3′
	Reverse: 5′ CACGAGGTCGCGTGCGTGGAAAAG 3′
H114Q	Forward: 5′ CTTTTCCACCAACGCGACCTCGTG 3′
	Reverse: 5′ CACGAGGTCGCGTTGGTGGAAAAG 3′
H114N	Forward: 5′ CTTTTCCACAACCGCGACCTCGTG 3′
	Reverse: 5′ CACGAGGTCGCGGTTGTGGAAAAG 3′
K119Y	Forward: 5′ CCACCGCGACCTCGTGTATGGGCC 3′
	Reverse: 5′ GGCCCATACACGAGGTCGCGGTGG 3′
Double mutations	
Flanking primers	
	Forward: 5′ GgaattcACCAACCCCGTCGCGAC 3′
	Reverse: 5′ CgaattcTCACCGCTCGAACCAGC 3′
Mutagenic primers	
H113N/H114N	Forward: 5′ CTTTTCAACAACCGCGACCTCGTG 3′
	Reverse: 5′ CACGAGGTCGCGGTTGTTGAAAAG 3′

and nucleotides flanking the truncated form of each enzyme. The reverse flanking primers contained nucleotides flanking the C-terminal end of each enzyme, a STOP codon, and an EcoR1 site for the H113N/H114N double mutant, or a SalI site for the other single mutants. The SalI site was included in the single-mutant constructs to provide flexibility in cloning into an alternate pPROTA expression vector with a more diverse polylinker, if needed. This site was not used.

In the first step, a specific mutation was introduced by using a pair of mutagenic primers and a pair of opposite end flanking primers. For the H114N mutant, for example, one PCR mixture included the forward flanking primer and H114N reverse primer to generate the N-terminal half of the mutated FucT-IV; another PCR mixture included H114N forward and reverse flanking primers to generate the C-terminal half of the mutated FucT-IV. The PCR products were gel purified and used together with only the flanking primers for the second-step PCR mixture. The resulting PCR product for each FucT-IV mutant was a 1053-bp DNA fragment encompassing the truncated FucT-IV (Pro^{58} to Arg^{405}) catalytic domain. These PCR products were ligated into Invitrogen pCR2.1 TOPO vector for amplification. The 1053-bp DNA fragments were excised with EcoR1 and subcloned into pPROTA. The correct orientation for these constructs was established by either HindIII/MluI or HindIII/EcoNI double digestion. Each mutation was confirmed by DNA sequencing using an Applied Biosystems Model 373A DNA sequencing system.

Enzyme assays

FucT-IV enzyme activities utilizing oligosaccharide acceptors were determined in standard reaction mixtures composed of 1 µmol HEPES buffer, pH 7.2, 6 nmol GDP-[^{14}C]fucose (15,000 cpm/nmol), 0.4 µmol LacNAc or 0.072 µmol of 8-methoxycarbonyloctyl glycoside derivatives, 2 µmol NaCl, 0.125 µmol $MnCl_2$, 10 µg bovine serum albumin, 0.01 µmol ATP, and chimeric enzyme bound to IgG-Agarose beads in a total volume of 0.02 ml. The reaction mixture was incubated for 1 h at room temperature and stopped and quantitated as described previously utilizing Dowex-1 for oligosaccharide acceptors (De Vries *et al.*, 1995) or C18 columns for 8-methoxycarbonyl glycoside derivatives as acceptors (Palcic *et al.*, 1988).

Assays conducted with the mutant enzymes were modified to increase sensitivity of the assay. This was accomplished by increased specific activity of GDP-[^{14}C]fucose (30,000 cpm/nmol), longer assay times up to 4 h, and increased amounts of beaded enzyme in the 0.02 ml reaction mixture. These modifications resulted in assays with increased sensitivity of detection to allow activity determinations for enzymes with low inherent activity. All assays were linear with respect to both time and protein concentration over the assay times used.

Western blot analysis of pPROTA expressed enzymes

The pPROTA expressed recombinant FucT enzymes were separated on 12% Tris-glycine polyacrylamide gels, transferred to nitrocellulose membranes, and probed as described previously (Nguyen *et al.*, 1998) to determine protein concentration of the expressed enzyme. In all cases, protein quantitation from western blot data for the purposes of specific activity determinations was based on the amount of intact fusion protein migrating between 70 and 80 kDa present in the expressed protein bound to IgG-Agarose beads.

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Abbreviations

FucT, fucosyltransferase; PBS, phosphate buffered saline; PCR, polymerase chain reaction.

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