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Modification of Epidermal Growth Factor-like Repeats with *O*-Fucose

MOLECULAR CLONING AND EXPRESSION OF A NOVEL GDP-FUCOSE PROTEIN *O*-FUCOSYLTRANSFERASE*

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The *O*-fucose modification is found on epidermal growth factor-like repeats of a number of cell surface and secreted proteins. *O*-Fucose glycans play important roles in ligand-induced receptor signaling. For example, elongation of *O*-fucose on Notch by the β1,3-*N*-acetylglucosaminyltransferase Fringe modulates the ability of Notch to respond to its ligands. The enzyme that adds *O*-fucose to epidermal growth factor-like repeats, GDP-fucose protein *O*-fucosyltransferase (*O*-FucT-1), was purified previously from Chinese hamster ovary (CHO) cells. Here we report the isolation of a cDNA that encodes human *O*-FucT-1. A probe deduced from N-terminal sequence analysis of purified CHO *O*-FucT-1 was used to screen a human heart cDNA library and expressed sequence tag and genomic data bases. The cDNA contains an open reading frame encoding a protein of 388 amino acids with a predicted N-terminal transmembrane sequence typical of a type II membrane orientation. Likewise, the mouse homolog obtained from an expressed sequence tag and 5′-rapid amplification of cDNA ends of a mouse liver cDNA library encodes a type II transmembrane protein of 393 amino acids with 90.4% identity to human *O*-FucT-1. Homologs were also found in *Drosophila* and *Caenorhabditis elegans* with 41.2 and 29.4% identity to human *O*-FucT-1, respectively. The human gene (*POFUT1*) is on chromosome 20 between *PLAGL2* and *KIF3B*, near the centromere at 20p11. The mouse gene (*Pofut1*) maps near *Plagl2* on a homologous region of mouse chromosome 2. *POFUT1* gene transcripts were expressed in all tissues examined, consistent with the widespread localization of the modification. Expression of a soluble form of human *O*-FucT-1 in insect cells yielded a protein of the predicted molecular weight with *O*-FucT-1 kinetic and enzymatic properties similar to those of *O*-FucT-1 purified from CHO cells. The identification of the gene encoding protein *O*-fucosyltransferase I now makes possible mutational strategies to examine the functions of the unusual *O*-fucose post-translational modification.

The extent and variety of carbohydrate modifications found on proteins are enormous. Although historically a major focus has been on determining the structure and function of *N*-glycans attached to asparagine residues, our knowledge of the scope and variety of serine/threonine-linked *O*-glycans has expanded greatly in the last decade. In addition to the well known mucin-type *O*-GalNAc and glycosaminoglycan *O*-xylose classes of glycans, several others have been described, including *O*-GlcNAc modification of nuclear and cytoplasmic proteins (1), *O*-mannose modification of brain glycoproteins (2), and *O*-glucose and *O*-fucose modifications of epidermal growth factor-like (EGF)[1] repeats (3). Several recent publications demonstrate that these novel forms of protein *O*-glycosylation play interesting and significant roles in the biological functions of the proteins they modify (4, 5).

Fucose in *O*-linkage to serine or threonine was originally identified in amino acid fucosides isolated from human urine (6). The first protein reported to bear an *O*-fucose modification on an EGF repeat was urinary-type plasminogen activator (uPA) (7). Several other proteins were subsequently found to have *O*-fucose modifications, and comparison of the sequences surrounding modified serines or threonines led to the proposal of a consensus sequence for *O*-fucose modification: C^2XXGGS/TC^3, where C^2 and C^3 are the second and third conserved cysteine residues of the EGF repeat, *X* is any amino acid, and S/T is the modified serine or threonine (3). Many proteins containing this sequence in the context of an EGF repeat have been identified in data base searches, and several of these proteins have subsequently been demonstrated to contain *O*-fucose residues (8–10). *O*-Fucose glycans exist on glycoproteins as either a monosaccharide (Fuc-*O*-Ser/Thr), a tetrasaccharide (NeuAcα2, 3/6Galβ1, 4GlcNAcβ1, 3Fuc-*O*-Ser/Thr), or a di- or trisaccharide intermediate in tetrasaccharide biosynthesis (3, 5).

Recent work from several laboratories has demonstrated a functional role for *O*-fucose glycans on the Notch protein. Fringe, a modulator of Notch signaling (11), is an *O*-fucose β1,3-*N*-acetylglucosaminyltransferase that extends the *O*-fucose moieties on Notch (5, 12). Elongation of *O*-fucose residues on EGF repeats of Notch by Fringe modulates Notch activation by the ligands Delta and Serrate/Jagged. Fringe potentiates signaling from Delta but inhibits signaling from Serrate/

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF375884 and AF375885.

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[1] The abbreviations used are: EGF, epidermal growth factor-like; uPA, urinary-type plasminogen activator; *O*-FucT-1, protein *O*-fucosyltransferase 1; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; kb, kilobase(s); EST, expressed sequence tag; Ni^{2+}-NTA, nickel-nitrilotriacetic acid; HPLC, high performance liquid chromatography; TSR, thrombospondin type 1 repeat.

Jagged (13, 14). How the elongation of *O*-fucose residues on Notch mediates altered signaling is not yet understood, but the β1,3-*N*-acetylglucosaminyltransferase activity is essential for Fringe to exert its effects on Notch (5, 12, 15). These results demonstrate that the *O*-fucose modification plays a key role in regulating an important signal transduction event: activation of the Notch receptor. Another signaling reaction influenced by *O*-fucose is that of uPA and its receptor (16). Because *O*-fucose exists on a number of other biologically important proteins, it may also influence their functions.

The enzyme responsible for adding *O*-fucose to EGF repeats (GDP-fucose protein *O*-fucosyltransferase 1 or *O*-FucT-1) was originally identified and purified from Chinese hamster ovary (CHO) cells (17, 18). Interestingly, *O*-FucT-1 does not fucosylate synthetic peptides containing the C^2XXGGS/TC^3 consensus sequence, but it does require a properly folded EGF repeat with the consensus sequence as acceptor (18). A significant fraction of *O*-FucT-1 activity in CHO cells is soluble, but it appears to be a membrane-bound protein released by proteolysis as observed with many other Golgi glycosyltransferases (19). As with most glycosyltransferases, *O*-FucT-1 is strongly activated by manganese. It was purified more than 5,000-fold using affinity chromatography on columns made with acceptor substrate (recombinant EGF repeat from factor VII) and donor substrate (GDP-hexanolamine-Sepharose) (18). In this paper we present the N-terminal sequence for the CHO *O*-FucT-1 and its use in identifying a cDNA for human *O*-FucT-1. Transcripts of the gene (HGNC-approved symbol: *POFUT1*) are expressed in all human tissues examined, and homologous genes exist in mice, *Drosophila*, and *Caenorhabditis elegans*. These results are consistent with the widespread occurrence of the *O*-fucose modification on proteins.

EXPERIMENTAL PROCEDURES

Determination of N-terminal Peptide Sequence of CHO O-FucT-1—CHO *O*-FucT-1 was purified as described (18), and 2 μg of purified enzyme was subjected to N-terminal peptide sequence analysis using a Hewlett-Packard model G1000A protein sequencer.

O-FucT-1 cDNA Sequence of Human and Mouse—Based on the peptide sequence of CHO *O*-FucT-1, a blast search was performed in GenBank (Unigene), and a human cDNA sequence, KIAA0180 (accession number D80002) was obtained which contains an open reading frame encoding a protein of 366 amino acids. PCR primers were designed based on sequences from KIAA0180: kiaa 16–55 (5′-CTTCTTGGGCTCTCTGGCATTTGCAAAGCTGCTAAACCGT-3′) and kiaa 1110–1071 (5′-GCCCTGGGATATGGAGCGTCTCCCTCCTTGAGGGGTCCCT-3′). Using a human heart cDNA library (CLONTECH) as template, a 1.1-kb PCR product was obtained. A radiolabeled probe was generated from the PCR product by random priming, and the probe was used to screen the human heart cDNA library according to the manufacturer's instructions. Recombinant λ DNA from purified positive clones was digested with *Eco*RI and subjected to Southern analysis. The probe for the Southern analysis was made from two partially overlapping oligonucleotides from the 5′-end of the KIAA0180 sequence (kiaa 16–55, see above, and kiaa 80–41, 5′-CAATCCAAGGAGGGACAGCCAAGGTACGGTTTAGCAGCTT-3′). The probe was filled in using Klenow fragment and ^{32}P-labeled dNTPs. *Eco*RI fragments reacting with the probe were purified on agarose gels and subcloned into pBluescript II SK+ plasmid (Stratagene), and the resulting plasmids were sequenced. A clone containing the coding region of the *POFUT1* gene was used for all subsequent experiments (pBS-hOFT). Additional 3′- and 5′-untranslated regions were determined by assembling overlapping *POFUT1* ESTs from dbEST and from the human genomic DNA sequence (accession number AL121897).

To obtain the mouse sequence, KIAA0180 was used to search the mouse EST data base, and one EST (accession number AI664300, 460 base pairs) was found. To obtain 5′ sequence two primers (PS513, 5′-CCGTCCTCACCATCTCATCTGA-3′; and PS511, 5′-ACATGTCTTCTTATCAGGACTTCG-3′) based on this EST were used with Marathon-ready cDNA (CLONTECH) from mouse liver. From a 0.5-kb 5′-rapid amplification of cDNA ends product and the EST a 760-base pair sequence was obtained. The 3′-region was derived from mouse genomic DNA sequence (accession number AC078911) and was confirmed by reverse transcription-PCR using primers, PS598 (5′-GGGACCAGTTTCATGTGAGTTTCAATAAGTCAGA-3′) and PS599 (5′-CCACCTCTGGCAGAAAAGAAAAGGGATGTGTAAT-3′) with Marathon-ready cDNA from liver (CLONTECH) as the template.

Northern Blot Analysis—The *POFUT1* coding sequence was excised from pBS-hOFT using *Eco*RI and labeled by random priming using the Ready-to-Go kit from Amersham Pharmacia Biotech according to the manufacturer's instructions to a specific activity of 1×10^9 cpm/μg. A human multitissue RNA blot was purchased from CLONTECH, probed, and washed under high stringency conditions according to the manufacturer's instruction for cDNA probes.

Overexpression of POFUT1 in Insect Cells—Baculovirus-mediated insect cell expression was used to express a soluble form of human *O*-FucT-1 (amino acids 24–388) with an N-terminal His_6 tag. A baculovirus transfer vector with a signal peptide was generated from pVL1392HAX (20). Two complementary oligonucleotides (RS1, 5′-CATGGCCAAGTTCCTGGTCAACGTGGCCCTGCTGCTGCTGCTGCTGCTGTCCGGAGCCTGGGCCCA-3′; and RS2, 5′-TATGGGCCCAGGCTCCGGACAGCAGCAGCAGCAGCAGCAGGGCCACGTTGACCAGGAACTTGGC-3′) encoding the signal sequence from honeybee melittin were generated and subcloned into the *Nco*I/*Nde*I site of pVL1392HAX. The resulting transfer vector was called pbSP. The coding sequence for the luminal domain of human *POFUT1* was excised from pBS-hOFT using *Sac*II and *Xba*I. Two complementary oligonucleotides (YW1, 5′-TGGGCCCATATGAGATCCCATCACCATCACCATCACATGCCCGCGGGCTCC-3′; and YW2, 5′-GGAGCCCGCGGGCATGTGATGGTGATGGTGATGGGATCTCATATGGGCCCA-3′) encoding *Nde*I and *Sac*II sites and the His_6 sequence was used as linker for the cDNA and vector. The baculovirus transfer vector (pbSP) was cut with *Nde*I and *Spe*I (compatible with *Xba*I). A triple ligation was performed with pbSP, the excised cDNA, and the His_6-encoding linker, and the resulting plasmid was transfected into Sf9 cells using the BaculoGold expression kit (Pharmingen). Recombinant viral clones were plaque purified three times. Viral stocks of 10^8 plaque-forming units/ml were prepared by repeated amplification. The protein was expressed by infecting 2×10^7 Sf9 cells with 5×10^8 plaque-forming units of recombinant virus. Medium was collected 72 h after infection, clarified by centrifugation, dialyzed against 20 mM Tris-HCl, pH 8.0, and loaded onto a 0.5-ml Ni^{2+}-NTA-agarose column (Qiagen). The column was washed with 4 ml of 0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl and eluted sequentially with 2 ml of 25 mM imidazole HCl, pH 7.0, 0.5 M NaCl followed by 3 ml of 0.5 M imidazole HCl, pH 7.0. Each fraction was subjected to SDS-polyacrylamide gel electrophoresis, stained with silver and assayed for enzyme activity. *O*-FucT-1 activity was assayed using GDP-[^{3}H]fucose and recombinant His_6-tagged factor VII EGF domain as described (17). All assays were performed as duplicates. N-terminal sequence analysis was performed on the purified protein as described above. Product analysis (reverse phase HPLC, β-elimination of *O*-linked sugars, gel filtration using a Superdex column, and high pH anion exchange chromatographic analysis on a Dionex MA1 column) of fucosylated EGF repeat was performed as described (10).

RESULTS

Amino Acid Sequence of CHO O-FucT-1—O-FucT-1 was purified from CHO cells as described (18) and subjected to N-terminal sequencing. The N-terminal peptide sequence of 61 amino acids was obtained: RLAGSWDLAGYLLYXPXMGRFGNQADHFLGSLAFAKLXVRTLAVPPWIEYQHHKPPFTNLH, where cycles that yielded uncertain residues are marked as *X*.

Identification of the POFUT1 Gene in Human, Mouse, Drosophila, and C. elegans—Blast searches with the sequence above revealed a cDNA, KIAA0180 (accession number D80002), coding for a 366-amino acid protein of unknown function from the human myeloblast cell line KG-1. Mammalian glycosyltransferases are typically type II transmembrane proteins (19, 21), but computer analysis failed to find a transmembrane domain near the N terminus of the predicted human protein. To obtain a clone with a complete open reading frame, a human heart cDNA library was screened. Initially, a PCR fragment was obtained from the library using kiaa 16–55 and kiaa 1110–1071 as primers, and a probe was made from the PCR fragment using the random priming method. The human heart cDNA library was screened using this probe, and a num-

ber of clones were identified. DNA sequencing confirmed that they contained the KIAA0180 sequence with varying 5′-extensions. One clone extended the open reading frame upstream to an "ATG" that results in a protein with a classic type II transmembrane domain (accession number for cDNA AF375884). This 5′-sequence is present in the human genomic DNA sequence (accession number AL121897). Additional cDNA sequence including 3′- and 5′-untranslated regions were determined by assembling overlapping *POFUT1* ESTs from dbEST and from the human genome sequence.

The human *POFUT1* cDNA encodes a protein of 388 amino acids with a predicted type II transmembrane organization (Fig. 1, accession number AF375884). The N-terminal sequence obtained from CHO *O*-FucT-1 begins at the end of the predicted transmembrane domain, suggesting that during purification, CHO *O*-FucT-1 was proteolyzed, as is typical of many glycosyltransferases, and consistent with the solubility properties of CHO *O*-FucT-1 (17, 18). The cDNA also contains an extensive 3′-untranslated region, typical of many glycosyltransferases (22). Two potential polyadenylation signals were found, providing an explanation for the existence of a doublet by Northern analysis (see below).

To obtain a mouse *POFUT1* cDNA sequence, KIAA0180 was used to search the mouse EST data base, and one EST (accession number AI664300, 460 base pairs) was found. Two primers based on this EST sequence, PS513 and PS511, were used to amplify the 5′-region with Marathon-ready cDNA from mouse liver. By assembling the 0.5-kb PCR product and the EST, a 760-base pair sequence that encodes the N-terminal region of a protein with a typical type II transmembrane domain was obtained. The remaining 3′-coding sequence of mouse *POFUT1* was derived from mouse genomic DNA sequence (accession number AC078911) and was confirmed by PCR using PS598 and PS599 with Marathon-ready cDNA from mouse liver. 15 nucleotide discrepancies between the cDNA sequence and the genomic DNA sequence included 7 that give amino acid changes. All 7 amino acid residues in the mouse cDNA are identical to the equivalent residue in the human *POFUT1* sequence. The mouse *POFUT1* cDNA encodes a protein of 393 amino acids with a type II transmembrane domain (accession number AF375885).

The full-length amino acid sequence of human *O*-FucT-1 was used to search the *Drosophila* and *C. elegans* data bases, and a related gene is present in both organisms (accession number AAF58290.1 for *Drosophila* and T15511 for *C. elegans*). The *C. elegans POFUT1* gene includes 109 extra amino acids at the N terminus, which derives from three 5′-exons predicted by computer algorithm. These 109 amino acids represent an unusually long cytoplasmic domain for a glycosyltransferase and may or may not be present in the *C. elegans O*-FucT-1. No *POFUT1* gene homologs were found in yeast or prokaryotes.

Human and mouse *O*-FucT-1 are both type II transmembrane proteins and share a high level of identity (90.4%) (Fig. 2), whereas the *Drosophila* and *C. elegans* homologs show 41.2 and 29.4% identity with human *O*-FucT-1, respectively. Six cysteines and one potential *N*-glycan consensus site are conserved, as well as the majority of aromatic residues. The CHO *O*-FucT-1 is known to be *N*-glycosylated (18), suggesting that valine in the CHO sequence at the predicted *N*-glycosylation site (Fig. 2) is probably the result of a sequencing error. A conserved D*X*D-like motif (ERD, see Fig. 2), found in many classes of glycosyltransferase (23), is located near the C terminus.

Organization of the Human POFUT1 Gene—Comparison of the human *POFUT1* cDNA coding sequence with the genomic DNA sequence (accession number AL121897) revealed that human *POFUT1* is organized into 7 exons (Fig. 3*A*). The boundary sequences of the six exon/intron junctions are shown in Fig. 3*B*. Previous analysis of the KIAA0180 sequence mapped it to human chromosome 20 ((24), www.kazusa.or.jp/huge/gfpage/KIAA0180/), and analysis of the BAC clone RP11-392M18 (accession number AL121897) shows that *POFUT1* lies between *PLAGL2* and *KIF3B*, close to the centromere of chromosome 20 at 20q11. The mouse *Pofut1* gene has the same exon/intron organization as the human gene (data not shown), whereas the *Drosophila* and *C. elegans Pofut1*-related genes are organized differently (data not shown). Analysis of the BAC clone RP23-111A22 (accession number AC078911) from mouse chromosome 2 reveals that *Pofut1* lies adjacent to *Plagl2* in a region homologous with human chromosome 20. The mouse BAC clone terminates before the *Kif3B* gene predicted to follow *Pofut1*.

The POFUT1 Gene Is Widely Expressed in Human Tissues—Northern analysis showed major transcripts from the *POFUT1* gene of ~5 kb expressed at high levels in all human tissues examined (Fig. 4). Several other weaker bands also hybridized, suggesting that there may be other transcripts expressed in some tissues. Previous analysis of the expression pattern of KIAA0180 ((22), www.kazusa.or.jp/huge/gfpage/KIAA0180/) showed a similar pattern of expression in spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocytes. They also showed resolution of the major transcripts into two species of ~4.8 and 5.4 kb, consistent with the sizes predicted from the cDNA sequence using the two polyadenylation signals identified in the 3′-untranslated region (Fig. 1).

Expression of POFUT1 cDNA in Insect Cells—To demonstrate that the cloned *POFUT1* cDNA encodes a protein with *O*-FucT-1 activity, a partial *POFUT1* cDNA encoding the predicted *O*-FucT-1 luminal domain (amino acids 24–388, lacking the transmembrane domain) was cloned into a baculoviral expression plasmid containing an N-terminal signal sequence and His_6 tag for expression in insect cells (Fig. 5*A*). Sf9 cells were infected with recombinant virus, and the His_6-tagged human *O*-FucT-1 was purified from the medium using Ni^{2+}-NTA-agarose. Fractions from the purification were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 5*B*) and for *O*-FucT-1 enzymatic activity (Fig. 5*C*). The activity bound to the Ni^{2+}-NTA-agarose column and eluted with imidazole. A 43-kDa protein coeluted with the activity (Fig. 5*B*), in agreement with the predicted size of truncated *O*-FucT-1. Amino terminal sequence analysis of the purified protein gave: *XX*RSHHHHH-HMPAGSWDPAGYLLY*X*P*X*MGR, confirming that the expressed protein was the His_6-tagged recombinant human *O*-FucT-1.

Product characterization demonstrated that the secreted *O*-fucosyltransferase generated the correct product. Reverse phase HPLC analysis of ^{3}H-fucosylated product showed that the radioactivity migrated exclusively with the factor VII EGF repeat (Fig. 6*A*), demonstrating that the [^{3}H]fucose was covalently associated with the EGF repeat. The [^{3}H]fucose was released from the EGF repeat by alkali-induced β-elimination forming the monosaccharide [^{3}H]fucitol (Fig. 6, *B* and *C*), the expected product from the β-elimination of *O*-fucose in the presence of sodium borohydride. These results demonstrate that recombinant *O*-FucT-1 adds *O*-fucose to factor VII EGF repeat.

To compare recombinant human *O*-FucT-1 with the purified CHO *O*-FucT-1 further, the dependence of activity on protein and substrate concentrations was examined (Fig. 7).

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1                                                   cttccctccccgactgtgcgccgcggctggctcgggttcccgggccgacatgggc
1                                                                                                       M  G
56    gccgccgcgtgggcacggccgctgagcgtgtctttcctgctgctgcttctgccgctcccggggatgcccgcgggctcctgggacccggcc
3     A  A  A  W  A  R  P  L  S  V  S  F  L  L  L  L  L  P  L  P  G  M  P  A  G  S  W  D  P  A
146   ggttacctgctctactgcccctgcatgggcgctttgggaaccaggccgatcacttcttgggctctctggcatttgcaaagctgctaaac
33    G  Y  L  L  Y  C  P  C  M  G  R  F  G  N  Q  A  D  H  F  L  G  S  L  A  F  A  K  L  L  N
236   cgtaccttggctgtccctccttggattgagtaccagcatcacaagcctcctttcaccaacctccatgtgtcctaccagaagtacttcaag
63    R  T  L  A  V  P  P  W  I  E  Y  Q  H  H  K  P  P  F  T  N  L  H  V  S  Y  Q  K  Y  F  K
326   ctggagcccctccaggcttaccatcgggtcatcagcttggaggatttcatggagaagctggcacccacccactggcccccctgagaagcgg
93    L  E  P  L  Q  A  Y  H  R  V  I  S  L  E  D  F  M  E  K  L  A  P  T  H  W  P  P  E  K  R
416   gtggcatactgctttgaggtggcagcccagcgaagcccagataagaagacgtgccccatgaaggaaggaaacccctttggcccattctgg
123   V  A  Y  C  F  E  V  A  A  Q  R  S  P  D  K  K  T  C  P  M  K  E  G  N  P  F  G  P  F  W
506   gatcagtttcatgtgagtttcaacaagtcggagcttttacaggcatttccttcagtgcttcctacagagaacaatggagccagagattt
153   D  Q  F  H  V  S  F  N  K  S  E  L  F  T  G  I  S  F  S  A  S  Y  R  E  Q  W  S  Q  R  F
596   tctccaaaggaacatccggtgcttgccctgccaggagccccagcccagttccccgtcctagaggaacacaggccactacagaagtacatg
183   S  P  K  E  H  P  V  L  A  L  P  G  A  P  A  Q  F  P  V  L  E  E  H  R  P  L  Q  K  Y  M
686   gtatggtcagacgaaatggtgaagacgggagaggcccagattcatgcccaccttgtccggccctatgtgggcattcatctgcgcattggc
213   V  W  S  D  E  M  V  K  T  G  E  A  Q  I  H  A  H  L  V  R  P  Y  V  G  I  H  L  R  I  G
776   tctgactggaagaacgcctgtgccatgctgaaggacgggactgcaggctcgcacttcatggcctctccgcagtgtgtgggctacagccgc
243   S  D  W  K  N  A  C  A  M  L  K  D  G  T  A  G  S  H  F  M  A  S  P  Q  C  V  G  Y  S  R
866   agcacagcggccccccctcacgatgactatgtgcctgcctgacctgaaggagatccagagggctgtgaagctctgggtgaggtcgctggat
273   S  T  A  A  P  L  T  M  T  M  C  L  P  D  L  K  E  I  Q  R  A  V  K  L  W  V  R  S  L  D
956   gcccagtcggtctacgttgctactgattccgagagttatgtgcctgagctccaacagctcttcaaagggaaggtgaaggtggtgagcctg
303   A  Q  S  V  Y  V  A  T  D  S  E  S  Y  V  P  E  L  Q  Q  L  F  K  G  K  V  K  V  V  S  L
1046  aagcctgaggtggcccaggtcgacctgtacatcctcggccaagccgaccactttattggcaactgtgtctcctccttcactgcctttgtg
333   K  P  E  V  A  Q  V  D  L  Y  I  L  G  Q  A  D  H  F  I  G  N  C  V  S  S  F  T  A  F  V
1136  aagcgggagcgggacctccaggggaggccgtcttctttcttcggcatggacaggcccccctaagctgcgggacgagttctgattctggccg
363   K  R  E  R  D  L  Q  G  R  P  S  S  F  F  G  M  D  R  P  P  K  L  R  D  E  F  *
1226  gagcaccagaccctctgatcctggagggaccagagtctgagctggtccttccagccaggcctggcagccagaggtgctccgggattgcaa
1316  actcctcttctcacctgccaaagatggagaagagtgccagggacccctcaaggagggagacgctccatatcccagggcataggacttgca
1406  ggttcctaggagcaggagcatctcccatcgcacgtgctttctgctcttctgggaatttctcacactggcaaagcagtccagcctccgtct
1496  tctggtccactctgctctgagcagcctgggatgctgaactcttcagagagatttttttatagagagatttctataattttgatacaaggt
1586  catgactatcctagaactctctgtggtttttgaaaatcattgaattctattaatgtaggtacctaaagtgaccttaactgaatgtggatg
1676  aggctggggctggtgtgggtcttttggctgcttttcaaggtgtcccccaatgtggccctcaagagccatccccactgcctggccagagcc
1766  attgttgtcccctacttcctaggccatttctggggcttgggggatgaatgctgtcctgtgctgtaaacactatgcaaatggaagttatcg
1856  gttgtggtgctgtgcagcgctctgtgggcgactaagtgccactcacgcagcatgttcctggcaaggagcacataccatcaagccacacta
1946  tcatggtattgttctcacagtcttttggtggttgatggccactgcaaacctggcaccatcagatctcttctgatctcttgccccagtggg
2036  gcctggttggtagaatgttggcattcggttgatatccaaagcctgttctcccagccgtcctcctgcagctggagccttcaggccgtattc
2126  tcacgagggaacgtttgccaaggctctgacctcacagaagatgcccagggcccagaagccatcagaattatcagtggagaagcaccttttt
2216  gactcttcccttccaatgtaatctctgccaacaccatgaggcttaaggtgctctaagtcatgagtgttttggtctcaaatgctgcagttt
2306  taataatctgtgactcctgagagcccatggtttttttgaccttgtggttctaaaattccttgtctgacccctgtagatcttttccttgcca
2396  tgtcacctcccttggcctttgatcctggaaaggtggcagagcctccactgagccaggcccagagctccttgcagtgccttcttccttgtt
2486  tacctgtgggaggaaacactttttttgtcaggggcagcctggttcagagctcagaggtcacactgtatcaaagatctcaaacagcaaagt
2576  cagcatttgctgtatagagctgccacccaactctaagcaggagaaactgtacagaaagggctttgctatttttccctttgggaaaacaa
2666  tgaagtgttttaagtcctgggtggactgagagatggtttgcctgtccagacttgctctcaagcctcatccagagaaggagctgcagatga
2756  gggagcccgtacactccctgccaccactaggttgtaagcctgtagctggctggctgatttcattttggaattcatttgccatccacagcc
2846  ttacactaggcacacactttagagtctggggctccagtggggcccgcctaatttttttcccccccaagacagggccttgctctgtctccc
2936  aggctggagtgcagtggcatgatcatggcttactgcagccttgatctcccaggctcaagcgatccttctgcctcagcctctctggtagct
3026  gagactgcatgcccagctccaaatcaccttgattcatatcagcagtaataatcacttgtgttctgaaagaaagggcaccagaagttctag
3116  caaaattcagttgtgttctgtgagctagcactttttcctctgacccaatttcttacctataaaatggtgataaaaaccgacaggttgtt
3206  caaaggcccagatcagctaaagcatgtatataagagcacgttgtaaacttgaaagagacaaaggcacaaatgtggctgttgattaatttg
3296  actgcttctcgttgctcgtcacctccatgccaggcactgtgcttgctaattgctttatgggggcattctcttatttattccccagccctg
3386  ggaaataggagctgtcattatccttctctttctgcacaaggaaaaattaatgccctgagaattgtcataattttcccaaggctgcccagc
3476  tggtggtgttaagccagaatttgacctcccagagccagtttccattagctgccatgctctgctgcctctaattcacagaatgcactttct
3566  accctgtgtgccatggagacctcctatggaaaaatgatcagccaccttaccttctactgggtacctgctgtgagtctgcctatgccagaa
3656  ggattaaggaggggaggttacccaagaaacaaagcctacatgccgcttacagcccccgttggatggttgctcagtacaacagtcttgcat
3746  tcagcaggtgtttgttcatcacctactatgtgtcaggctctatgctaggtactggggatacaggagagaatcaagcgtaaagtctttgtt
3836  ctcaaggaatttgcattctagaaagtagaagatgtaataaatgtactgtgggacatgttaataagtgctataaagaaatataaagggttt
3926  gggagcaaaaagagggagtggatctatttagatgagcccaggtaagacctctctgaagagctgtcatgaaggagggagggagcacattc
4016  ctggcagagaaaacagcacgtgcaaaggccccgagactggagtgtgttcctgaagagcagccaggaggccagcatggctggagaggcagg
4106  cataggcagggaaccgagcagcaggtcagagcaggcgagctgacattctgcagcctggacggccatggcaggaagcttttagttggagag
4196  atacaggaagcctcctagggttctgagcagaagaggggcatgagctgattcacattctgaaggacctctctagctggccagtgctgagga
4286  ggttggagagagaaagggtgaaagcagagagaccagtgcagggctgttaacagggttgcaggcgagagactggggtgctgggctcccctа
4376  gactaggactccagtgccctcctctcccaagagacaaaggccattgcattgaaggaggtgggaaatgattagattctgaacatatgtaat
4466  tatttttcagtctttttcaaagatacaaatatttacatagttttaatcatgtaatatatacaatttaatgtcctagtgttttacttaata
4556  gtgtatcatgtttttccctgttggtatgtagcctggataaatgctcttaattataaaaaattctgtcGaGgagtgttccatagtttattgt
4646  tttcctattatgagaatttaggccaagtgtggtggctcatgcctgtaatcccagcactttgcgaggccgaggtgggcagatcacttgagg
4736  tgaggagttcaagaccagcctggccaacatggtgaattatctctactaaaaatacaaaaaaataataataatagccaggcgtggtggcac
4826  atgcctgtattcccagctgcttgggaggctgaggcaggagaatggcttgaacctgggaggtggaggttgcagtgagccgagatggtgcca
4916  ctgcattccagcctgggcaacagagcgagactccatctcaaaaaaaaggagacttcatgtgcccccaatttttcactattgttatttgaa
5006  aaaatatttttatttgtaagagtttttctttatttaaaatgttcattaataaagttgttggacgggaagcaaaaaaaaaaagttgtttaa
5096  gataaattcccagaagtgaatttgttagatcaaacacttaaaactttttgttatggaagaattcaaatataaataaaaaattgtgagtaa
5186  taaaatgaactcacagtttcaacaatggcccacaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa      5249
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FIG. 1. **Full-length cDNA sequence of human *POFUT1* with translation of the coding sequence.** The cDNA sequence consists of 5249 nucleotides with an open reading frame encoding 388 amino acids. The amino acid sequence *underlined* is the predicted transmembrane domain. The nucleotide sequence *underlined* is an *Alu* repetitive sequence. The two polyadenylation signals, ***ataaa*** (4591–4595) and ***aataaa*** (5174–5179), were determined by comparing several groups of ESTs. 20 of the ESTs generated from different human tissues have a uniform end at the first **G** in *bold* (nucleotide 4622), followed by a poly(A) tail. Another 10 ESTs were found to end at the second **G** in *bold* (nucleotide 4624). For these transcripts, the ***ataaa*** at 4591–4595 is believed to be the polyadenylation signal, corresponding to the 4.8-kb transcripts as shown by Northern analysis. The second polyadenylation signal and the poly(A) tail were derived from a different EST (accession number AI828591) and may explain the 5.4-kb band on the Northern blot. The accession number for the human heart cDNA sequence is AF375884.

The kinetic parameters of human *O*-FucT-1 for the substrates factor VII EGF repeat and GDP-fucose compare favorably with the published values for purified CHO *O*-FucT-1. The K_m obtained for factor VII EGF was 6 μM compared with 11 μM for CHO *O*-FucT-1 (18), and for GDP-fucose, the K_m was 4 μM compared with 5.9–6.4 μM for CHO

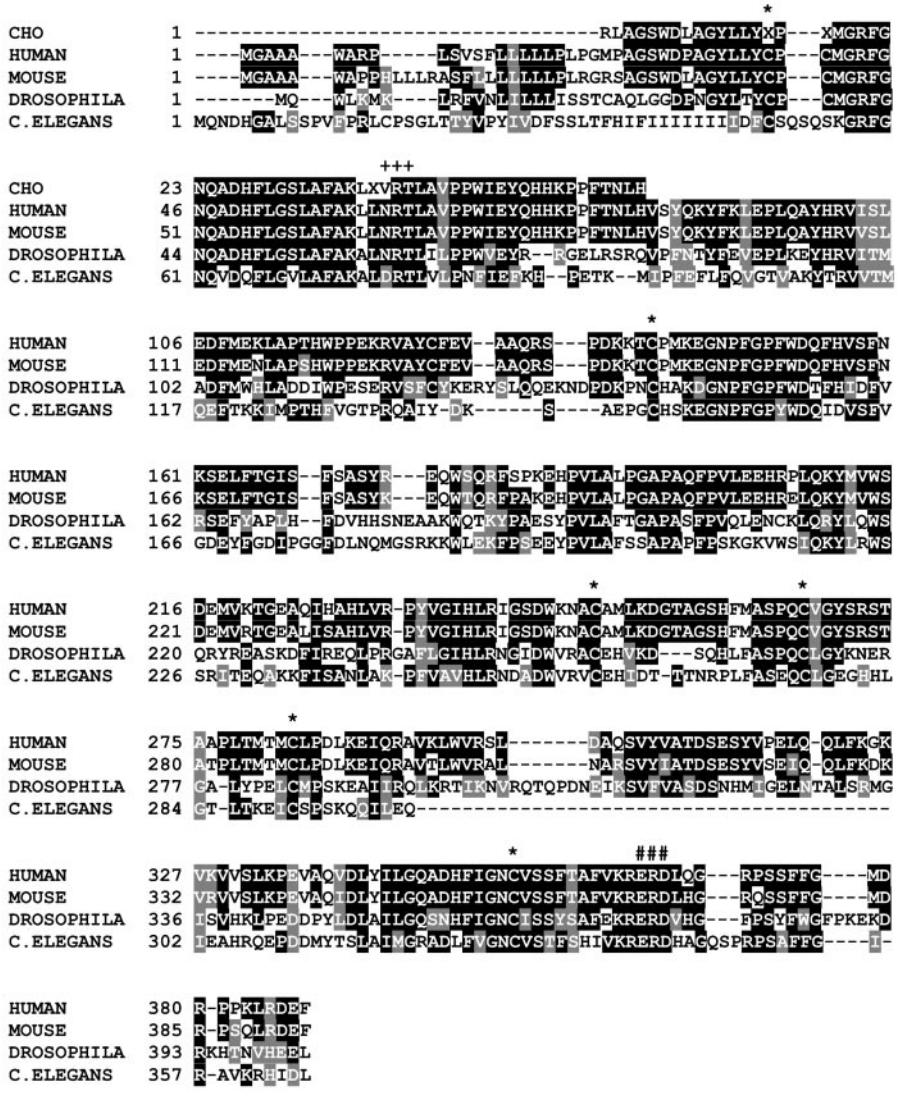
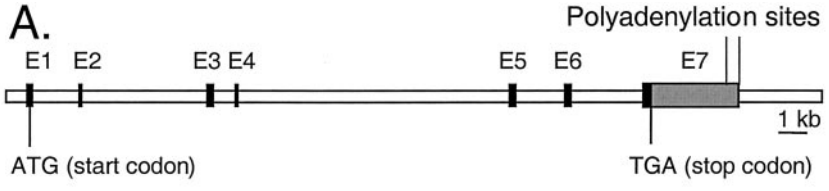


FIG. 2. **Clustal W alignment of *POFUT1* from CHO (partial), human, mouse, *Drosophila*, and *C. elegans*.** The amino acid identity, strong similarity, and weak similarity were determined between human and mouse (90.4, 4.3, and 2.0%), human and *Drosophila* (41.2, 20.5, and 9%), and human and *C. elegans* (29.4, 17.7, and 11.2%). At the nucleotide level, identity was determined between human and mouse (85.6%), human and *Drosophila* (54.3%), and human and *C. elegans* (47.3%). There are 6 conserved Cys residues marked by an *asterisk* (*). A conserved DXD-like motif, ERD, is marked by ###. One potential *N*-glycosylation site, NRT (marked by +++), was found in human, mouse, and *Drosophila*, but not in CHO (probably because of a sequencing error) or *C. elegans*. The accession numbers of these four sequences are: AF375884 (human), AF375885 (mouse), AAF58290.1 (*Drosophila*), and T15511 (*C. elegans*).



B.

Exon	3′ Splice Acceptor	Exon size	5′ Splice Donor	Intron size
1		173	----CCCTGCATGGgtaaggcctc----	2006
2	----ccatttccagGGCGCTTTGG----	122	----TTTCACCAACgtgagtactt----	5077
3	----tttcctgtagCTCCATGTGT----	183	----CCCCATGAAGgtgggtcctg----	1158
4	----ctttctgcagGAAGGAAACC----	113	----GGAGCCAGAGgtacttggag----	11542
5	----tcctgtctagATTTTCTCCA----	193	----CTCTGACTGGgtaacttccc----	2364
6	----ctctccgtagAAGAACGCCT----	243	----CAAAGGGAAGgtatgtgtgg----	3412
7	----cttcctgcagGTGAAGGTGG----	3597 or 4191		

FIG. 3. **Organization of the human *POFUT1* gene.** *Panel A*, schematic representation of the genomic DNA organization of the human *POFUT1* gene, which was determined by comparing the human *POFUT1* cDNA (Fig. 1) with a *POFUT1* genomic DNA sequence (accession number AL121897). *Panel B*, sequence of each exon and intron junction.

O-FucT-1 (18). The V_{max} for recombinant human *O*-FucT-1 was 3 μmol/min·mg compared with 2.5 μmol/min·mg for CHO *O*-FucT-1 (18). As noted for purified CHO *O*-FucT-1 (18), high concentrations of EGF repeat caused inhibition of fucose transfer by human *O*-FucT-1 (data not shown). In addition, human *O*-FucT-1 was inhibited by EDTA (data not shown), consistent with the manganese requirement of CHO *O*-FucT-1 activity (18). These results demonstrate that the cloned *POFUT1* cDNA encodes the *O*-FucT-1 glycosyltransferase.

DISCUSSION

The *O*-fucose modification has been found on proteins from a wide variety of species, ranging from humans to fruit flies (3,

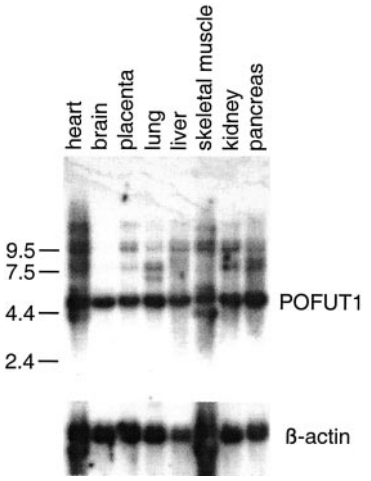


FIG. 4. ***POFUT1* is widely expressed in human tissues.** Northern analysis of *POFUT1* expression was performed as described under "Experimental Procedures." Similar results were obtained for the human cDNA KIAA0180 and can be viewed at www.kazusa.or.jp/huge/gfpage/KIAA0180/.

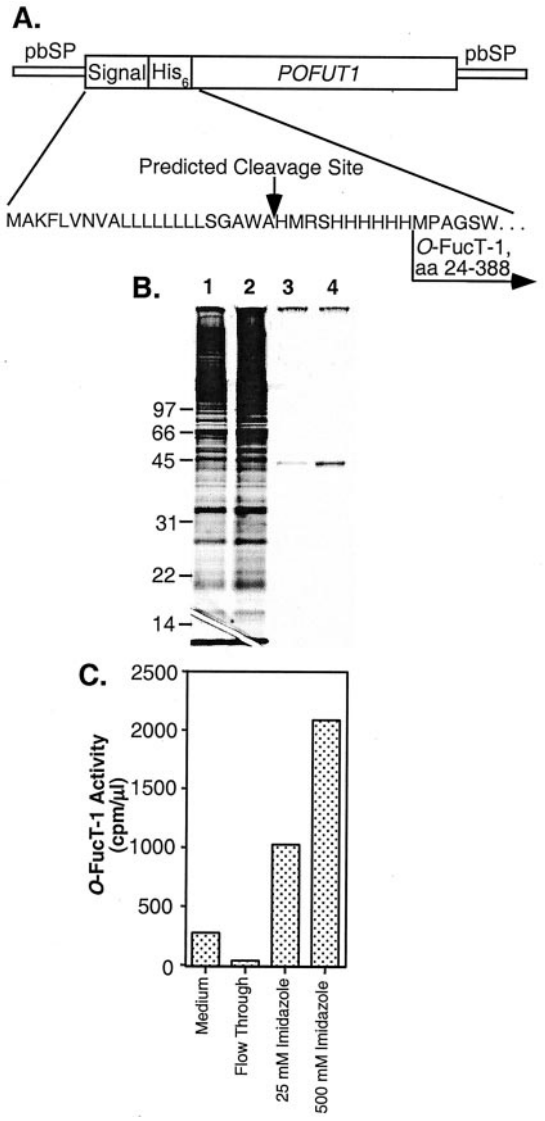


FIG. 5. **Expression of *POFUT1* cDNA in insect cells yields enzymatically active *O*-FucT-1.** *Panel A*, the coding sequence for the lumenal domain of *O*-FucT-1 (amino acids 24–388) was cloned in-frame with a signal sequence and His_6 tag in the insect cell transfer vector, pbSP. *Panel B*, the recombinant His_6-tagged human *O*-FucT-1 was purified from medium using Ni^{2+}-NTA-agarose and analyzed by SDS-polyacrylamide gel electrophoresis followed by silver staining. *Lane 1*, culture medium; *lane 2*, Ni^{2+}-NTA-agarose flowthrough; *lane 3*, 25 mM imidazole elution; *lane 4*, 0.5 M imidazole elution. *Panel C*, fractions from the Ni^{2+}-NTA-agarose column were assayed for *O*-FucT-1 activity as described under "Experimental Procedures."

5), and a protein *O*-fucosyltransferase that generates the modification has been purified from CHO cells (18). In this paper we report the sequences of human and mouse cDNAs that encode *O*-FucT-1 and the organization of the human *POFUT1* gene. The *POFUT1* gene sequence is highly conserved in mammals and is also present in *Drosophila* and *C. elegans*. The gene is expressed in all mammalian tissues examined, suggesting that the *O*-fucose modification may play biological roles in different contexts. *O*-FucT-1 has a predicted type II transmembrane structure, consistent with the domain organization of nearly all known mammalian Golgi glycosyltransferases (19, 21) and the membrane association data of the enzyme (17). *O*-FucT-1 does not share any obvious sequence similarity with any other class of glycosyltransferases, including the α1,2, α1,3/4, or α1,6-fucosyltransferases (22). Six highly conserved cysteines are present in *O*-FucT-1 as well as a D*X*D-like motif (ERD), conserved in mammals, *Drosophila*, and *C. elegans*. Both features are characteristic of several glycosyltransferase families (22, 23). Only one homologous gene has been identified for each species.

The presence of *O*-fucose on EGF repeats is known to play a critical role in signal transduction through two distinct pathways. Binding of uPA to the uPA receptor is mediated through the EGF repeat of uPA (16, 25). Removal of *O*-fucose from the EGF repeat has no effect on binding to the uPA receptor, but bound, unfucosylated EGF repeat does not activate the receptor. Thus, the *O*-fucose modification is not required for binding, but it plays an essential role in activation of the uPA receptor. The details of how *O*-fucose mediates this effect are not understood, but it is known that the addition of *O*-fucose to an EGF repeat does not significantly alter the conformation of the polypeptide chain. Using NMR, Kao and co-workers (26) examined the structure of the factor VII EGF repeat with and without an *O*-fucose. The presence of the *O*-fucose had very little effect on the tertiary structure of the EGF repeat, but it did form a significant knob-like feature on one face of the module. Thus, modification of the protein with *O*-fucose would not be predicted to cause a conformational change in the protein, but it could certainly affect the interactions of the EGF repeat with other molecules.

In the Notch signaling pathway, *O*-fucose also plays a key role in receptor activation. *O*-Fucose serves as an acceptor for Fringe, a β1,3-*N*-acetylglucosaminyltransferase, resulting in the elongation of the *O*-fucose monosaccharide into a tetrasaccharide on EGF repeats of Notch (5, 12). This alteration in the carbohydrate structure somehow modulates receptor activation. Interestingly, the activation of Notch can be either potentiated or inhibited, depending on the ligand (13, 14). Again, it is unlikely that the change in sugar structure alters the conformation of the individual EGF repeat, but such a change could certainly affect the ability of the modified EGF repeat to interact with ligands, other proteins, or even other portions of the Notch protein itself.

Recently the presence of *O*-fucose in a different protein context was reported (27). Hofsteenge and co-workers showed the presence of the disaccharide Glc-Fuc *O*-linked to serines and threonines within the three thrombospondin type 1 repeats (TSR) of human thrombospondin-1. By comparing the sequence contexts surrounding the modified residues in each TSR they proposed the consensus sequence, CS*X*S/TCG, where S/T is the

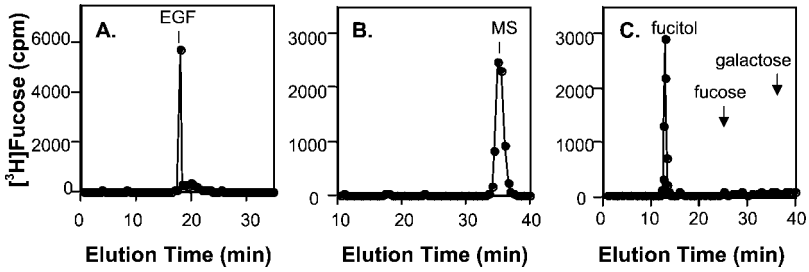


FIG. 6. **Product characterization of ^{3}H-fucosylated factor VII EGF repeat confirms that the *POFUT1* cDNA encodes a protein *O*-fucosyltransferase.** *Panel A*, product from a reaction like that shown in Fig. 5*C* was subjected to reverse phase HPLC analysis. The *arrow* shows the elution position of the factor VII EGF repeat. *Panel B*, the [^{3}H]fucose-labeled EGF repeat purified in *panel A* was subjected to alkali-induced β-elimination, and the products were analyzed by gel filtration chromatography as described under "Experimental Procedures." *MS* (monosaccharide) indicates the elution position of authentic fucitol. *Panel C*, the monosaccharide peak from *panel B* was subjected to high pH anion exchange chromatographic analysis on an MA1 column under conditions that separate fucitol from fucose and other alditols as described by Moloney and co-workers (10). The elution positions of fucitol, fucose, and galactose are shown.

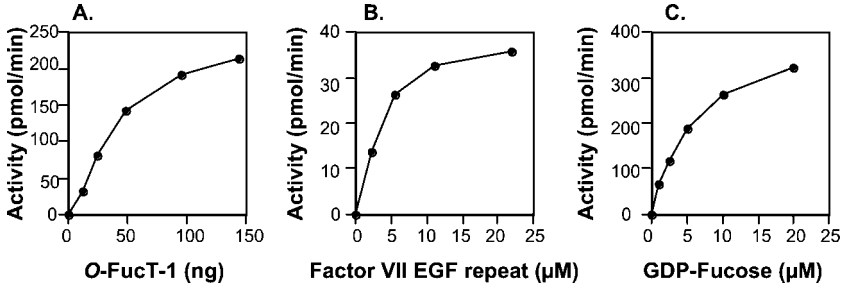


FIG. 7. **Baculovirus-overexpressed *O*-FucT-1 has kinetic parameters very similar to the purified CHO *O*-FucT-1.** Dependence of recombinant baculovirus *O*-FucT-1 activity on enzyme concentration (*panel A*), GDP-fucose concentration (*panel B*), and factor VII EGF repeat concentration (*panel C*) is shown.

modified residue. An *O*-linked Glcβ1,3Fuc modification of proteins in CHO cells has also been described (28), although CHO proteins with this modification were not identified. The results of Hofsteenge and co-workers suggest that two separate *O*-fucose pathways exist: one modifying EGF repeats, and the other modifying TSRs. Elongation of *O*-fucose occurs in both pathways, but the elongation does not appear to overlap. *O*-Fucose on EGF repeats can be modified by a β1,3-linked GlcNAc, catalyzed by Fringe (5, 12), whereas *O*-fucose on TSRs appears to be elongated by a β1,3-linked Glc (27). An enzymatic activity capable of addition of Glc in a β1,3-linkage to fucose has been described (9). Interestingly, the β1,3-glucosyltransferase would not use *O*-fucose attached to an EGF repeat as a substrate under various conditions tested,[2] suggesting that the addition of the Glc to fucose occurs only when the fucose is in a particular protein context such as a TSR. The region of thrombospondin-1 modified with the Glc-Fuc disaccharide binds to heparin and appears to be involved in a number of biologically important interactions (29), raising the interesting possibility that this class of *O*-fucose modifications may modulate biological events in a manner similar to *O*-fucose on the Notch receptor.

The importance of fucose modifications on proteins has been highlighted recently by the description of the disease leukocyte adhesion deficiency type II (30). Patients with this disorder have reduced levels of fucose on proteins. The underfucosylation is caused by defects in the GDP-fucose transporter resulting in inefficient transport of GDP-fucose into the Golgi apparatus (31, 32). The patients suffer from a wide variety of developmental and pathological problems (30). As the name of the disorder implies, they have a defect in recruitment of leukocytes to sites of inflammation, resulting in severe recurring bacterial infections. Leukocytes are recruited to sites of inflammation by recognition of specific fucose-containing oligosaccharide structures on their surfaces, and reduced levels of fucose impair efficient recruitment (33). In addition to recurrent infections, these patients suffer from developmental abnormalities. They are short in stature, have unusual facial and skeletal abnormalities, and are severely mentally retarded. Because Notch receptors play key roles in numerous developmental events (34), it may be that several of the other features of leukocyte adhesion deficiency type II can be explained by defects in Notch function.

[2] D. J. Moloney and R. S. Haltiwanger, unpublished observation.

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Modification of Epidermal Growth Factor-like Repeats with*O*-Fucose: MOLECULAR CLONING AND EXPRESSION OF A NOVEL GDP-FUCOSE PROTEINO-FUCOSYLTRANSFERASE

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