

Identification of a GDP-L-fucose:polypeptide fucosyltransferase and enzymatic addition of *O*-linked fucose to EGF domains

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An assay for GDP-fucose:polypeptide fucosyltransferase has been established. The enzyme catalyzes the reaction that attaches fucose through an *O*-glycosidic linkage to a conserved serine or threonine residue in EGF domains. The assay uses recombinant human factor VII EGF-1 domain as acceptor substrate and GDP-fucose as donor substrate. Synthetic peptides with sequences taken from five proteins previously shown to contain *O*-linked fucose (Harris and Spellman, 1993; *Glycobiology* 3, 219–224) did not serve as efficient acceptor substrates. These synthetic peptides did not comprise complete EGF domains and did not contain all six cysteine residues that define the EGF structure. Therefore, the enzyme appears to require more than just a consensus primary sequence and likely requires that the EGF domain disulfide bonds be properly formed. The enzymatic reaction showed linear dependency of its activity on time, amount of enzyme, and substrates. Although the enzyme did not exhibit an absolute requirement for Mn²⁺, enzymatic activity did increase ten fold in the presence of 50 mM MnCl₂. The *in vitro* glycosylation reaction resulted in complete conversion of the acceptor substrate to glycosylated product, and characterization of the purified product by electrospray mass spectrometry revealed that one fucose was added onto the polypeptide. Most of the enzymatic activity was found to be in the soluble fraction of CHO cell homogenates. However, when enzyme was prepared from rat liver in the presence of protease inhibitors, 37% of the activity was recovered by Triton X-100 extraction of the membrane particles after extensive aqueous washes. The result suggests that the enzyme is probably a membrane protein and, by analogy with other glycosyltransferases, probably has a 'stem' region that is very susceptible to proteolysis.

Key words: fucosyltransferase/*O*-linked fucose/EGF domain/glycosylation

Introduction

The first evidence of fucose moieties in glycosidic linkages to serine or threonine was described by Hallgren *et al.* (1975) and Klinger *et al.* (1981), who reported the identification of compounds such as Glcβ1→3Fucα1→O-Ser/Thr and Fucα1→O-Ser/Thr in human urine and rat tissue. The latter group also demonstrated the incorporation of metabolically labeled fucose

into glycoproteins, and the label was released by alkaline borohydride treatment as glucosylβ1→3fucitol (Klinger *et al.*, 1981). The identification of *O*-linked fucose attached to a specific protein was first made by Kentzer *et al.* (1990) who found a residue of fucose covalently linked to a peptide derived from the EGF domain of recombinant urokinase. The same modification was later identified to be on tissue plasminogen activator (Harris *et al.*, 1991), human factor VII (Bjoern *et al.*, 1991), and human factor XII (Harris *et al.*, 1992). Similar fucosylation was also shown to occur on the EGF domain of human factor IX (Nishimura *et al.*, 1992), but in this case the fucose residue was at the reducing end of a tetrasaccharide: NeuAcα2→6Galβ1→4GlcNAcβ1→3Fucα1→O-Ser61 (Harris *et al.*, 1993). In all cases, the attachment of *O*-linked fucose was found to occur within the sequence Cys-Xaa-Xaa-Gly-Gly-Ser/Thr-Cys (Harris and Spellman, 1993).

Other studies also showed that this type of modification is not limited to the five proteins characterized above. Stults and Cummings (1993) reported the incorporation of ¹⁴C-labeled fucose into proteins expressed by the mutant CHO cell line lec1, which does not synthesize complex type N-linked oligosaccharides. Using a similar approach, Moloney *et al.* (1995) reported the presence of *O*-linked glucose-fucose glycans in the same mutant cells.

Structural characterization of a protease inhibitor PMP-C from insect cells also revealed an *O*-linked fucose attached to a threonine residue (Nakakura *et al.*, 1992) although the flanking sequence was not represented by the consensus sequence mentioned above. Detailed NMR study (Mer *et al.*, 1996) showed that the *O*-fucosylation of the peptide was responsible for an overall decrease of the dynamic fluctuations of the molecule and an increase in stability as monitored by thermal denaturation. It is not yet clear whether the biosynthesis of this type of *O*-fucosylation is carried out by the same enzyme that modifies the EGF domains described above.

Attempts have been made by several laboratories to determine the biological function of *O*-linked fucose. Hajjar and Reynolds (1993) reported that this modification was involved in binding of tissue plasminogen activator to HepG2 cells and, hence, that the *O*-linked fucose played a role in clearance of tPA by hepatocytes. On the other hand, Camani and Kruithof (1995) demonstrated that the nonfucosylated finger-EGF domain of tPA inhibited the binding of tPA to hepatocytes, while free fucose did not. From this result, they concluded that the *O*-linked fucose on tPA did not play a role in the binding of tPA to hepatocytes.

Although much effort has been devoted to the study of this novel posttranslation modification, little is known about its biosynthesis and the enzymes involved in the process. In the present study, we report for the first time the identification of an *O*-fucosyltransferase activity in CHO cells and rat liver. This work includes the establishment of an enzyme assay for GDP-fucose:polypeptide fucosyltransferase, initial character-

ization of this activity, and structural analysis of the *in vitro* glycosylation product.

Results and discussion

Development of an assay for *O*-linked fucosyltransferase

Our initial attempts to develop an assay for *O*-fucosyltransferase focused on the use of synthetic peptides based on sequences previously reported (Harris and Spellman, 1993) to be modified by *O*-linked fucose (Table I). In no case did the synthetic peptides correspond to an entire EGF domain, and none of the peptides contained more than two of the six cysteine residues that define the structure of EGF domains (Savage *et al.*, 1973). None of these peptides was found to function as a significant acceptor substrate in an enzyme assay utilizing CHO cells and rat liver extracts as the enzyme source and ¹⁴C-labeled GDP-fucose as the donor substrate. In the best case, using peptide 6 (Table I), acceptor substrate-dependent counts were only about 20% above background (data not shown). The results indicated that the acceptor substrate requirements of the enzyme exceeded a simple consensus primary sequence and that the enzyme likely recognized three-dimensional structural elements of EGF domains.

Because synthetic peptides were not found to be acceptable substrates, we turned to the recombinant expression of complete EGF domains in an attempt to generate properly folded acceptor substrates. Our first attempts involved production of a fusion protein of the tPA EGF domain and glutathione-S-transferase (GST-EGF), using a commercially available expression system (Pharmacia). The GST-EGF fusion protein was found to serve as an acceptor substrate for the *O*-fucosyltransferase, in that acceptor substrate-dependent counts were 500% above background in the assay described in *Materials and methods* (data not shown). The GST-EGF fusion protein was not pursued, however, mainly because of difficulties in obtaining efficient proteolytic release of the EGF domain from the fusion protein.

A recombinant form of the first EGF domain of human

factor VII was constructed with the sequence from human factor VII (residue 45 to 87) including all six conserved cysteine residues. A hexahistidine tag was also engineered onto the amino terminus to facilitate purification using metal chelating affinity chromatography. The protein was expressed in *E. coli* and purified to homogeneity. It migrated as a single peak on reversed phase HPLC, and preliminary NMR data confirmed that its conformation was similar to that of the native EGF-1 module (G.F.Lee and R.F.Kelley, unpublished observation).

Experiments using factor VII EGF-1 as acceptor substrate showed *O*-fucosyltransferase activity present in rat and hamster liver and CHO cell homogenates. CHO cell paste was used as the source of enzyme in all subsequent experiments unless otherwise noted, because it had relatively higher activity than the liver, and the majority of the activity was recovered in aqueous supernatants. The enzyme activity was further enriched and partially purified to obtain a preparation with high activity and relative stability. Two chromatographic steps were used in the procedure as described in *Materials and methods*. First, a DE-52 anion exchange column was used, and all of the activity was recovered in the flow-through fractions. The fractions containing activity were directly loaded onto the next column, Affi-Gel Blue. All activity was retained on the column and then eluted with 1 M NaCl. The fractions containing activity were then pooled, dialyzed, and adjusted to contain 50% glycerol. The enzyme solution was stable for at least a month when stored at -20°C. The total purification was about eight-fold, and the yield was approximately 40%.

The partially purified *O*-fucosyltransferase activity showed linear dependency on time, amount of enzyme preparation and amount of donor and acceptor substrates (Figure 1). The substrate dependency experiments were done using limited amounts of one of the two substrates to observe the change of incorporation of counts; therefore, these are not kinetic measurements of initial reaction rate. Experiments using the recombinant GST-EGF fusion protein also showed similar substrate dependency. Since the fucosylated residue in the factor VII sequence is a serine whereas in the tPA sequence it is a threonine, the results suggested that the partially purified enzyme preparation contained *O*-fucosyltransferase activity(ies) which glycosylates both serine and threonine residues.

Most of the glycosyltransferases studied to date require divalent cations as activators (Sadler *et al.*, 1982). The metal ion requirements of *O*-fucosyltransferase were also examined, and the results are shown in Table II, where Mn²⁺ showed the highest degree of activation. The enzyme apparently does not have an absolute requirement for any divalent metal ion for its activity, as it was active when assayed with no added divalent metal ions or in the presence of EDTA. Ca²⁺ was included in the study because of its role in some EGF domains' interactions with other proteins. Ca²⁺ served as an activator for the enzyme, but to a lesser extent than Mn²⁺. It is unlikely that calcium is involved in the binding between the *O*-fucosyltransferase and its substrate, because assays carried out in the presence of both Mn²⁺ and Ca²⁺ showed no difference from those with Mn²⁺ alone. Ni²⁺ was used to assess the influence of the poly-histidine tag at the amino terminus of the substrate on the enzyme activity. As shown in the same table, there is apparently no adverse effect of Ni²⁺ on the enzyme activity. A Mn²⁺ dependency study was done to determine the optimal concentration for the assay (Figure 2). The enzyme showed

Table I. Synthetic peptides evaluated as substrates for the *O*-fucosyltransferase

Peptide	Sequence ^a	Source
1	RCFNGG T CQQALY	tPA 55-67
2	RNGG T CQQALY	tPA 57-67
3	SEPRCFNGG T CQQALYF	tPA 52-68
4	Ac-VKSCSEPR S FNGG T CQQA-NH ₂ ^b	tPA 48-65
5	DCLNGG T CVS	Urokinase 12-21
6	PCQNGG S CKDQL	Factor VII 54-65
7	NPCLNGG S CKDDI	Factor IX 54-66
8	HSPCQGG T CVNMPSG	Factor XII 82-97
9	LNGG T CVSA ^c	

Peptides were synthesized with automated solid phase synthesizer using *t*-Boc chemistry. The cleaved peptides were then purified by reverse phase HPLC and their molecular weights assessed using mass spectrometry. The assay used was similar to that described in *Materials and methods*, except 1-4 mM peptide was present in each reaction.

^aThe consensus sequence for *O*-fucosylation is in bold face and the potential glycosylation sites are underlined.

^bThe N- and C-termini of Peptide 4 were modified with acetyl and amide groups respectively. The two cysteine residues of Peptide 4 were oxidized and formed a disulfide bond.

^cThe sequence of Peptide 9 was taken from urokinase except for the alanine at the C-terminus

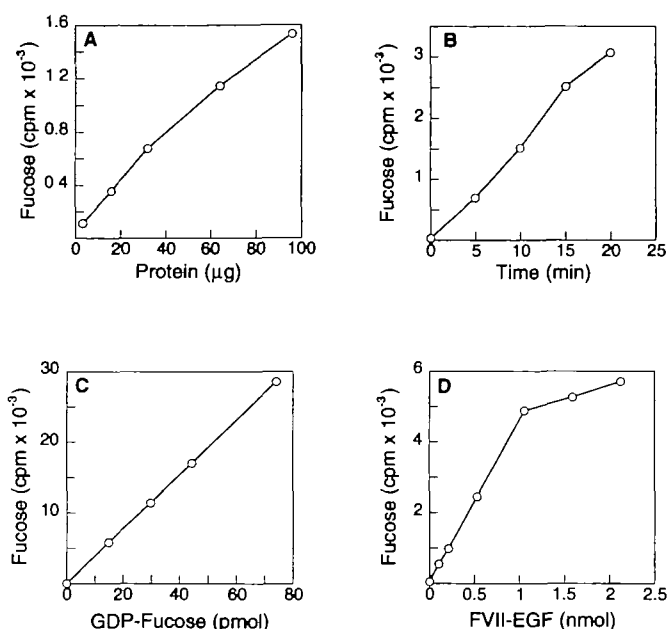


Fig. 1. *O*-fucosyltransferase activity as function of: (A) enzyme concentration; (B) time; (C) amount of donor substrate, GDP-fucose; and (D) amount of acceptor substrate, Factor VII EGF-1 domain. When not varied, the conditions of the assay were as described in *Materials and methods*. In (A), since partially purified preparation was used, enzyme concentration was represented by the amount of protein used in each reaction. The specific activity of GDP-¹⁴C-fucose used in (C) was 270 mCi/mmol and the amount was ~100-fold less than the regular assay. All data points represent the average of duplicate assay reactions.

highest activity in the presence of 50 mM of MnCl₂; the activity under this condition was 10 times that observed in the absence of Mn²⁺.

Characterization of the enzyme product

In order to purify and characterize the *O*-fucosylated product, reaction mixtures were directly loaded onto a reversed phase HPLC column and eluted with a TFA:acetonitrile:H₂O solvent system. As shown in Figure 3, the fucosylated factor VII EGF-1 domain exhibited shorter retention time on HPLC than the nonglycosylated substrate. This would be expected because of the hydrophilic nature of the fucose residue. The reaction apparently reached completion under the condition described in *Materials and methods*, and scintillation counting of the collected fractions demonstrated that the elution position of the radioactivity matched that of the glycosylated polypeptide peak (data not shown).

The fractions with radioactivity were subjected to electro-spray mass spectrometry to determine the molecular weight of the polypeptide (Figure 4). When the mass spectrum was acquired with the orifice potential set to 50 V (Figure 4A), four

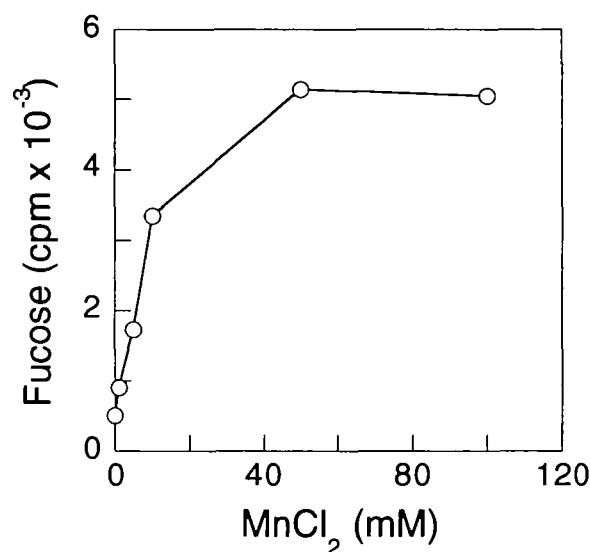


Fig. 2. Activation of *O*-fucosyltransferase activity by Mn²⁺. Other than MnCl₂ concentration, all the conditions were the same as described in *Materials and methods* for *O*-fucosyltransferase assay

major ion species were observed (*m/z* 1947.3, *m/z* 1460.7, *m/z* 1169.0, and *m/z* 974.3), corresponding to a single peptide with 3, 4, 5, and 6 charges, respectively. The molecular weight calculated from these ions was 5838.8, which compares well with the theoretical molecular weight (5837.2) of the factor VII EGF peptide plus one residue of fucose. The slight difference (<2 mass unit) between the observed versus theoretical masses may reflect some deamidation of the peptide. Upon increase of

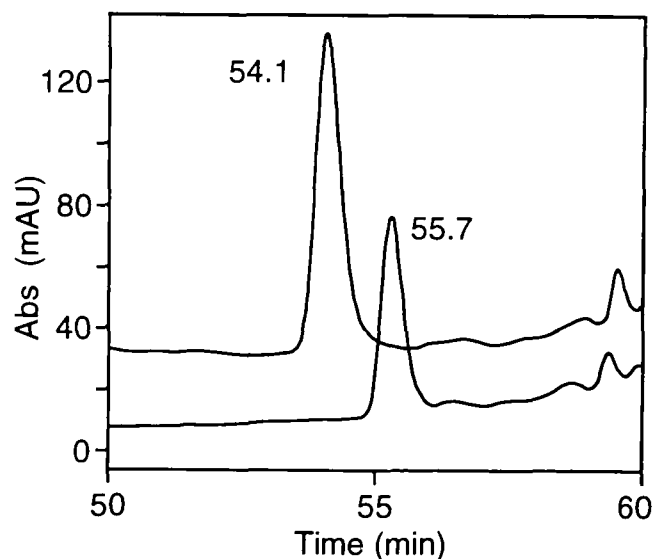


Fig. 3. Purification and analysis of fucosylated and nonfucosylated Factor VII EGF-1 domain by HPLC. The procedure was outlined in *Materials and methods*. The upper trace is the chromatogram of the *O*-fucosylated factor VII EGF-1 domain. The lower trace is from control reaction (in the absence of GDP-¹⁴C-fucose), the nonfucosylated factor VII EGF-1 domain. Fractions were collected for both runs and the ones from the first chromatography were counted in a scintillation counter. Peak 54.1 min was radioactive. The H₂O/acetonitrile/TFA gradient was as described by Stone and Williams (1993). Briefly, Buffer A = 0.06% TFA, H₂O, Buffer B = 0.052% TFA, 80% acetonitrile. Gradient: 0–60 min, 2.0–37.5% B; 60–90 min, 37.5–75% B; 90–105 min, 75–98% B.

Table II. Metal ion requirements

Metal ions (10 mM)	Activity (cpm)	Activity vs. no metal ions (%)
None	1159	100
Mn ²⁺	4829	417
Ca ²⁺	2270	196
Ni ²⁺	2469	213
EDTA	1132	98

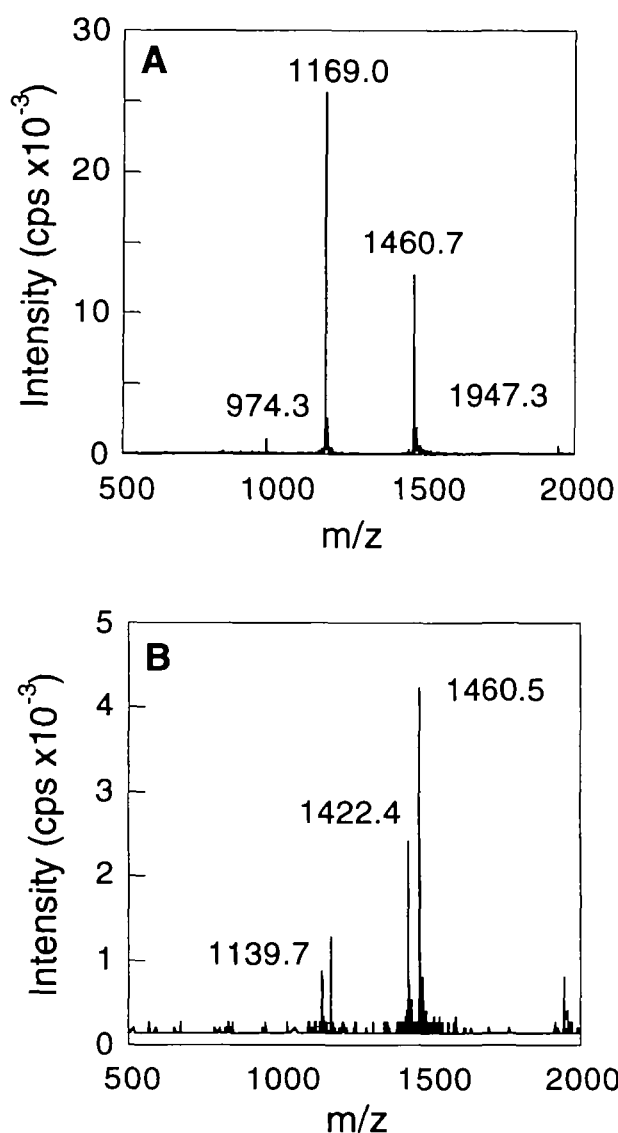


Fig. 4. Product identification using electrospray mass spectrometry. (A) The mass spectrum of Peak 54.1 min of Figure 3 with orifice potential set at 50 V. (B) The mass spectrum of the same sample with orifice potential set at 90 V. The four labeled ions in (A) were from a single polypeptide of molecular weight, 5838.8 with three to six charges, respectively. The two new ions in (B), 1422.4 (four charges) and 1139.1 (five charges), were from a polypeptide with molecular weight of 5692.6. The mass difference between these two polypeptides was 146.2.

the orifice potential to 90 V (Figure 4 B), two new major ion species were observed (m/z 1422.4 and m/z 1139.7). These ions corresponded to five and six charged states of a peptide with molecular weight of 5692.6. Thus, increasing the orifice potential decreased the mass of the peptide by 146.2 mass units, which corresponds to the ions of one fucose residue (146.1). Because the glycosidic linkage is relatively labile, it is common to observe the cleavage of a sugar moiety from a peptide with increased orifice potential (Carr *et al.*, 1993). The results clearly indicated that enzymatic fucosylation was complete, and that only one fucose was added to the recombinant factor VII EGF-1 polypeptide.

Further information was obtained on the attachment position of the fucose residue by digesting the reaction product with the protease Asp-N. After Asp-N digestion, a radioactive peptide was obtained, and amino acid analysis confirmed that it was

the peptide DQCASSPCQNGGSCK, which included the glycosylation site serine residue (underlined). Mass spectrometry of this peptide revealed that it had a molecular weight of 1805.5, which corresponds well with the predicted molecular weight (1804.8) of the peptide plus one residue of fucose.

In summary, mass spectrometry confirmed the transfer of a single fucose residue to the factor VII EGF-1 polypeptide, and this modification was localized to a peptide containing the 'consensus' *O*-fucosylation site. Thus, the characterization of the *O*-fucosyltransferase reaction product gave results that were entirely consistent with previously characterized *O*-fucosylated proteins (Harris and Spellman, 1993).

Is O-fucosyltransferase a membrane protein?

As described above, *O*-fucosyltransferase activity was found in homogenates of rat and hamster liver as well as in CHO cell lysates. The *O*-fucosyltransferase used for the characterization work described above was originally prepared from the aqueous fraction of CHO cell extracts without using any detergent; this preparation was used because it was relatively pure and had good activity. However, most mammalian glycosyltransferases characterized to date are type II membrane proteins, consisting of an amino-terminal cytoplasmic domain, a membrane-spanning sequence, a 'stem' region, and the catalytic domain (for a review, see Paulson and Colley, 1989). Because the *O*-fucosyltransferase activity from CHO cells was in the soluble fraction, we were interested in examining whether the *O*-fucosyltransferase is different from most of the other glycosyltransferases in this respect.

To address this question, frozen rat liver tissue was homogenized in the presence of a protease inhibitor cocktail, subjected to repeated PBS extractions, and then subjected to extraction with Triton X-100 (Table III). The total activity recovered in the fractions (the PBS homogenate supernatant and two subsequent washes) was around 50% of the activity in the original homogenate. The second and third washes contained much less activity than the initial supernatant. The Triton X-100 extraction after three PBS washes still contained 37% of the total activity.

These results suggest that the enzyme is probably a membrane protein, with a stem region very susceptible to proteolysis. If so, it is similar to other mammalian glycosyltransferases described to date (Paulson and Colley, 1989). In the CHO cell preparations, the protein was probably proteolyzed at the 'stem' region, and most of the activity released from the membrane, during the initial homogenization or the thawing proce-

Table III. Fractionation of the *O*-fucosyltransferase from rat liver

Fraction	Total activity (munit*)	Percent of total activity (%)
Homogenate	25.4	100
Supernatant	8.7	34
First wash	2.8	11
Second wash	1.2	5
Triton X-100 extract	9.3	37

Preparation procedure was described in *Materials and methods*. First wash is the supernatant of resuspension of the pellet from initial homogenate. Second wash is the supernatant of the pellet suspension from the first wash. Assays were carried out as described in *Materials and methods*, all the reaction mixtures contained 1% Triton X-100.

*One unit represents 1 μ mol of fucose transferred per minute.

ture before that. It has been reported previously that the catalytic domains of other such glycosyltransferases retain catalytic activity after proteolysis of their stem region (Paulson and Colley, 1989). It is likely that the *O*-fucosyltransferase studied here is also similar to most previously characterized mammalian glycosyltransferases with respect to subcellular localization (i.e., membrane proteins located in the endoplasmic reticulum or the Golgi apparatus, Roth *et al.*, 1994). This conclusion is supported by the fact that all five proteins so far identified to have this novel modification are secreted from cells and contain *N*-linked glycosylation and the fact that the *O*-linked fucose residue of factor IX is further modified by GlcNAc, Gal, and NeuAc (Harris *et al.*, 1993).

In summary, we have reported here for the first time the development of an assay for, and the initial characterization of, the *O*-fucosyltransferase activity that attaches *O*-linked fucose to EGF domains. The establishment of the assay should make it possible to purify the *O*-fucosyltransferase to homogeneity and study the structure and function of this novel posttranslational modification.

Materials and methods

Materials

GDP- β -L-fucose was purchased from Sigma, GDP- ^{14}C - β -L-fucose was purchased from NEN. Chinese hamster ovary (CHO) cell paste was kindly provided by Dr. Hardayal Prasad at Genentech, Inc. Rat liver was purchased from Pel-Freez. Peptides used in this study were synthesized at Genentech, Inc. by Dr. Cliff Quan. C18 cartridges (Extract Clean, C18, 200 mg) used in the assay were purchased from Alltech. Reversed phase C18 HPLC columns (2.1 \times 250 mm, 5 μm) were purchased from Vydac Separation Group. DE-52 resin was purchased from Whatman, and Affi-Gel Blue (100–200 mesh) was from Bio-Rad.

Recombinant human factor VII first EGF domain

A recombinant form of the first EGF domain from human factor VII was produced in *E. coli*. This construct included residues 45–87 of the intact protein, an amino-terminal hexahistidine tag and three additional residues not of factor VII origin at the carboxy terminus, for the following primary sequence: HHHHHHSDGDQCASSPCQNGGSCCKDQLQSYICFLPAFEGRNCETHKDDGSA. The theoretical mass of the oxidized form of the peptide is 5691. The potential *O*-fucosylation site is underlined. The details of oligonucleotide cassette-based gene construction and protein expression are described in a separate paper (Lee *et al.*, unpublished observations). The EGF domain was purified from periplasmic shockates, produced by osmotic shock of 100 g cell pellet in 0.5 liter 20 mM Tris-HCl, pH 8, containing 1 mM each PMSF and benzamide, after a single freeze-thaw cycle. The shockate was loaded to a 30 ml column of chelating Sepharose 6B fast flow resin (Pharmacia), previously charged with 50 mM NiSO₄ and equilibrated in 20 mM Tris-HCl, pH 8, 0.5 M NaCl. The column was then washed with 10–15 column volumes 20 mM Tris-HCl, pH 8, 0.5 M NaCl, followed by 10 volumes of 20 mM Tris-HCl, pH 8, 1 M NaCl, and 10 volumes of 20 mM Tris-HCl, 0.5 M NaCl, 25 mM imidazole, pH 8. The EGF domain was eluted in 3 column volumes of 20 mM Tris-HCl, 0.5 M NaCl, 0.3 M imidazole, pH 8. EGF domain-containing fractions were pooled, concentrated, and then loaded to a 2.5 \times 50 cm Sephadex G-50 (Pharmacia) column equilibrated in PBS, to remove higher molecular weight contaminants. Chromatographic separation was achieved at a flow rate of 0.5 ml/min in PBS. The peak eluting at \approx 325 min was collected and used in subsequent experiments.

O-Fucosyltransferase assay

The reaction mixture (50 μl) contained 0.1 M imidazole-HCl, pH 7.0, 50 mM MnCl₂, 0.1 mM of GDP- ^{14}C -Fucose (4000–8000 c.p.m./nmol), 20 μM recombinant human Factor VII EGF-1 domain, and enzyme preparations typically containing 0.01–0.1 mU. The mixture was incubated at 37°C for 10–20 min. The reaction was stopped by placing the mixture on ice, and then diluted with 950 μl of 0.25 M EDTA, pH 8.0. Separation of incorporated fucose from GDP-fucose, fucose-phosphate, and free fucose was carried out by passing the solution through a C18 cartridge (Alltech, Extract Clean, C18, 200 mg). The

cartridge was washed with 5 ml of H₂O, and the product was then eluted with 3 ml of 80% acetonitrile containing 0.052% TFA. The eluant was mixed with 10 ml Aquasol II (NEN/Du Pont) and counted using a liquid scintillation counter.

Partial purification of *O*-fucosyltransferase from CHO cell paste

All procedures were performed at 4°C unless otherwise noted. Frozen CHO cell paste (20 g) was thawed at room temperature, suspended in three volumes of 20 mM imidazole-HCl, pH 7.0, 30 mM NaCl, and homogenized by sonication (Virsonic 550, 1/2 inch probe) at 20% output power with three 30 s bursts. The suspension was centrifuged at 20,000 \times *g* for 45 min using an SS-34 rotor in a model RC-5B centrifuge (Sorvall/Du Pont). The supernatant, containing most of the activity, was then loaded onto a DE-52 column (2.5 \times 10 cm) equilibrated with 20 mM imidazole-HCl, pH 7.0, 30 mM NaCl. The column was then washed with 250 ml of the same buffer. The flow-through fractions containing activity were pooled and loaded directly onto an Affi-Gel Blue column (1.5 \times 15 cm) equilibrated in the same buffer. The column was washed with 100 ml of the same buffer, and the activity was eluted using 20 mM imidazole-HCl, pH 7.0, 1.0 M NaCl. The high salt eluant containing enzyme activity was pooled and dialyzed against 25 mM imidazole-HCl, pH 7.0, 25% (v/v) glycerol. The dialyzed solution was then made to contain 50% glycerol (v/v) by adding one half volume of 100% glycerol for storage at –20°C and future use.

Preparation of *O*-fucosyltransferase from rat liver

The procedure was very similar to the method used to prepare Triton X-100 extracts for purification of a sialyltransferase (Sadler *et al.*, 1979). In brief, frozen rat liver (about 5 g) was thawed, minced and homogenized in 15 ml of PBS with 1 ml of 25 \times protease inhibitor cocktail (Complete Protease inhibitor cocktail tablet, Boehringer Mannheim) in a Waring blender, using a 15 s burst at high and two 15 s bursts at low setting. The homogenate was then centrifuged at 35,000 \times *g* (SS-34) for 45 min. The pellet was suspended in 15 ml PBS with 10 mM MnCl₂ by vortexing. The suspension was then centrifuged as described above, and the same process was repeated once more. Aliquots were taken from each supernatant for later assays. The washed pellet was resuspended in 10 ml PBS with 10 mM MnCl₂. Triton X-100 (2 ml of 10% w/v) was then added, and the mixture stirred at 4°C overnight. The Triton X-100 extract was obtained by centrifugation of the mixture as described above.

HPLC purification of the *O*-fucosylated EGF-1 domain

The reaction was set up essentially as described above for the fucosyltransferase assay. After incubation at 37°C for 1 h, the mixture (50 μl) was injected directly onto a 2.1 \times 250 mm C18 reverse phase column (Vydac), running a water/acetonitrile/TFA gradient at 0.2 ml/min. Fractions of 0.4 ml were collected, and half of the amount was counted using a liquid scintillation counter. The other half of the radioactive fractions was dried and redissolved in water/acetonitrile 9:1 containing 0.06% TFA, for further analysis.

Mass spectrometry

The purified polypeptide (50 pmol in 50 μl) was infused at 5 $\mu\text{l}/\text{min}$ into a Sciex AP300 triple-quadrupole mass spectrometer with a regular ion sprayer. The orifice potential was set at 50 V and the ion-spray potential was set at 4500 V. The scan was performed from 400 to 2000 *m/z* with step size of 0.1 *amu* and dwell time of 0.1 ms. The data were analyzed using BioMultiView (Perkin-Elmer).

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Abbreviations

EGF, epidermal growth factor; CHO, Chinese hamster ovary; Glc, glucose; Fuc, fucose; NeuAc, N-acetylneuraminic acid; Gal, galactose; GlcNAc, N-acetylglucosamine; HPLC, high pressure liquid chromatography.

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