A GDP-fucose:polypeptide fucosyltransferase was purified 5000-fold to homogeneity from Chinese hamster ovary cell extracts in the absence of detergent. The purification procedure included two affinity chromatographic steps using the acceptor substrate, a recombinant factor VII EGF-1 domain, and the donor substrate analog, GDP-hexanolamine, as ligands. The purified enzyme migrates as a single band of 44,000 daltons on SDS-polyacrylamide gel electrophoresis and is itself a glycoprotein with more than one high mannose type oligosaccharide chain with a total molecular weight of 4000. The $K_m$ values for factor VII EGF-1 domain and GDP-fucose are 15 and 6 $\mu M$, respectively. The $V_{max}$ is 2.5 $\mu mol/min\cdot mg^{-1}$. The presence of 50 mM Mn$^{2+}$ increased the enzyme activity 17-fold, but Mn$^{2+}$ was not absolutely required, since the enzyme exhibited some activity even in the presence of EDTA. The acceptor substrate specificity was studied using site-directed mutagenesis of human factor IX EGF domain. Only one of several differently folded species could serve as acceptor substrate, although they all had the same molecular weight as determined by liquid chromatography on-line with mass spectrometry. This indicates that the enzyme requires proper folding of the epidermal growth factor domain for its activity.

The identification of O-linked fucose attached to a specific protein was first made by Kentzer et al. (1), who found a residue of fucose covalently linked through an O-glycosidic bond to the EGF$^1$ domain of recombinant urokinase. The same modification was later identified to be on tissue plasminogen activator (2), human factor VII (3), human factor XII (4), and a plasminogen activator from the venom of the snake Bothrops jararacussu (5). O-linked fucose is present on an insect protease inhibitor (6), on a glycoprotein of the brain (7), and on a fragment of the epidermal growth factor (8).

Although all examples of mammalian O-fucosylation characterized to date have been modifications of EGF domains (8), it is possible that O-linked fucosylation occurs in non-EGF-containing proteins. Studies by two other laboratories using the lec1 mutant cell line indicated that O-fucosylation occurs on many proteins in Chinese hamster ovary cells (9, 10). These studies were not performed in a manner that would enable EGF domain-containing proteins to be distinguished from those without EGF domains. Structural analysis has also revealed that O-linked fucose is present on an insect protease inhibitor (11), although the flanking amino acids were not represented by the consensus sequence described above, and the protease inhibitor sequence is not homologous to an EGF domain.

We have focused our research mainly on the biosynthesis of this post-translational modification. Recently, we reported the development of an assay for the GDP-L-fucose:polypeptide fucosyltransferase, which catalyzes the attachment of fucose through an O-glycosidic linkage to the conserved serine or threonine residue in EGF domains (12). The assay uses a recombinant human factor VII EGF-1 domain as acceptor substrate and GDP-fucose as donor substrate. Using this assay, we were able to detect activity in extracts of Chinese hamster ovary cells and rat liver. In addition, when rat liver was homogenized 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. These results suggest 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 (13).

In this paper, we report the purification of this O-fucosyltransferase to apparent homogeneity. This study also includes the initial characterization of the purified enzyme and its acceptor substrate specificity.

**EXPERIMENTAL PROCEDURES**

**O-Fucosyltransferase Assay**

The assay procedure was essentially the same as described previously (12). The reaction mixture (50 $\mu l$) contained 0.1 M imidazole-HCl, pH 7.0, 50 mM MnCl$_2$, 0.1 mM of GDP-[14C]fucose (4000–8000 cpm/mmol), 20 mM recombinant human factor VII EGF-1 domain, and enzyme preparations typically containing 0.01–0.1 milliunit of activity. The mixture was incubated at 37 °C typically for 15 min. The reaction was stopped by placing the mixture on ice and then diluting with 950 $\mu l$ 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 H$_2$O, and the product was then eluted with 3 ml of 80% acetonitrile containing 0.052% trifluoroacetic acid. The eluant was mixed with 10 ml of Aquasol II (NEN Life Science Products) and counted using a liquid scintillation counter.

**Recombinant Human Factor VII and IX EGF Domains and Mutants**

The recombinant forms of human factor VII EGF-1 domain used for assays and affinity chromatography were prepared as described previously (12). The construction of human factor IX EGF domain and its mutant genes were the same as for factor VII EGF-1 domain. The sequence of factor IX EGF domain was taken from residues 45–87 of the mature protein, with six histidine residues attached at carboxyl...
terminus of the sequence followed by three residues from the vector, YVDGDQCESNPCLNGSCCKDINSYECWCFPFEGKCNKELDVTY-HHHHHHGS. The potential glycosylation site is underlined. The gene encoding factor IX EGDF domain without the polyhistidine sequence was made first by annealing primers designed to form oligonucleotide cassettes as listed in Table I, using standard molecular biology techniques (14). The gene was then cloned into the pMIE and BamHI sites of a phagemid vector, pA4G32 (15), previously developed for bacteriophage display. The recombinant phagemid, p9EGF, contained the alkaline phosphatase promoter and the bacterial stII signal sequence upstream of the gene encoding the EGDF domain as well as the β-lactamase gene as a selectable marker. The construction of the gene for factor IX EGDF domain with a polyhistidine tag, p9EGF-His6, was made by replacing part of the COOH-terminal sequence with another oligonucleotide cassette containing the polyhistidine sequence, utilizing the restriction sites SstI and BamHI as shown in Table I. The mutants were constructed using the similar oligonucleotide cassettes with mutated sequences. The plasmids were then transformed into Escherichia coli strain 27C7 by electroporation. Expression of the proteins was carried out in LB medium, supplemented with 50 μg/ml of ampicillin, and 20% (w/v) glycerol, in a 40-liter bio-reactor. The cells were harvested when the density was 1.0×10^8 cells/ml, 98% viable, using a centrifugation method.

**Cell Culture Method**

Suspension culture of Chinese hamster ovary cells was maintained in serum-free Ham’s F12/DMEM (50:50) media, supplemented with insulin, putrescine, selenium, ferrous sulfate, and polyvinyl alcohol, in a 40-liter bio-reactor. The cells were harvested when the density reached 3.4×10^8 cells/ml, 98% viable, using a centrifugation method. The collected cell paste was frozen at -70 °C and used for purification of the O-fucosyltransferase without further treatment.

**Purification**

Buffer A was 25 mM imidazole-HCl, pH 7.0. Buffer B was 25 mM Tris-HCl, pH 8.0, 25 mM NaCl, and 25% (w/v) glycerol. Buffer C was 25 mM Tris-HCl, pH 8.0, 25 mM NaCl, 25% (w/v) glycerol, 1 mM MnCl2, and 0.1 mM GDP. Buffer D was 25 mM imidazole-HCl, pH 7.0, 25 mM NaCl, and 25% (w/v) glycerol. Buffer E was 25 mM imidazole-HCl, pH 7.0, 25 mM NaCl, 5 mM MnCl2, and 25% (w/v) glycerol.

**Step 1: Preparation of Chinese Hamster Ovary Cell Extract**—Frozen Chinese hamster ovary cell paste (100 g) was thawed at room temperature and kept on ice. The cells were homogenized by sonication (Vironic 550, at 20% output with a 1/8-inch probe) with three 30-s bursts in 300 ml of buffer A containing 25 mM NaCl. DNase I (2 mg/ml, 1 ml) and MgCl2 (1 mM, 0.4 ml) were added to the homogenate, which was then centrifuged at 10,000 × g (Sorvall RC-5, GSA rotor) for 45 min. The supernatant (355 ml) was used for further purification.

**Step 2: DE-52 and Affi-Gel Blue Chromatography**—Two columns, DE-52 (Whatman, 2.5 × 30 cm) and Affi-Gel Blue (Bio-Rad, 2.5 × 15 cm), were connected in series and equilibrated with buffer A containing 25 mM NaCl. The supernatant from the previous step was loaded onto the DE-52 column (1 ml/Min), and the column was washed with 500 ml of the same buffer. The DE-52 column was then detached from the Affi-Gel Blue column. The latter was washed with 200 ml buffer B of buffer A containing 125 mM NaCl and followed by 400 ml of high salt (buffer A with 1 M NaCl) elution. The eluted fractions containing enzyme activity were pooled and dialyzed against buffer B. The final volume was 40 ml.

**Step 3: Factor VII EGDF-His6-NTA-Agarose**—The affinity resin with the acceptor substrate as ligand was made by mixing 6 mg of factor VII EGDF-His6 with 10 ml of Ni2+-NTA-agarose resin in 0.1 M Tris-HCl, pH 8.0, for 4 h at 4 °C. The resin was then packed into a column (1.5 × 6 cm) and washed with 40 ml of 0.1 M Tris-HCl, pH 8.0, followed by another wash of 30 ml of 0.1 M Tris-HCl, 0.5 mM NaCl. It was then equilibrated with the same buffer used for dialysis in the previous step.

The dialyzed sample was supplemented with 1 mM MnCl2 and 0.1 mM GDP (Sigma) and loaded onto the affinity column at a flow rate of 0.5 ml/min followed by 40 ml of buffer C. The column was then washed with 45 ml of buffer C containing 0.5 mM NaCl and 45 ml of buffer D, respectively. The enzyme was then eluted off the column with 90 ml of 0.25 M imidazole-HCl, pH 7.0, 25% (w/v) glycerol. The fractions containing activity were pooled and dialyzed against buffer D and ready for the next step.

**Step 4: GDP-hexanolamine-Agarose**—The affinity resin with donor substrate analog as ligand was made by coupling GDP-hexanolamine (30 mg/ml, provided by Dr. R. L. Hill, Duke University) to CNBr-activated Sepharose 4B resin (10 ml, Amersham Pharmacia Biotech) according to the manufacturer’s instructions. The resin was then packed in a column (inner diameter, 1.5 cm) and equilibrated with buffer F.

The dialyzed sample (13 ml) was supplemented with 5 mM MnCl2 and loaded onto the column at 5 ml/h. The column was then washed with 30 ml of buffer E, followed by 45 ml of the same buffer with 125 mM NaCl and then another 10 ml of buffer E. The elution was carried out by using a linear gradient from 0–2 mM GDP, it started with 100% of buffer E and finished with 100% of buffer E containing 2 mM GDP in a total volume of 50 ml. The column was washed with another 40 ml of the latter buffer. Fractions containing activity were first examined by silver-stained SDS-PAGE, and those with only a single band (44,000 Da) were pooled. Glycerol was added to a final concentration of 50% (w/v) for storage at -20 °C.

**Step 5: Gel Filtration Removal of Recombinant Factor VII EGDF-His6**—The flow-through fractions containing enzyme activity from the first GDP-hexanolamine-agarose chromatography were pooled (14 ml total). A column (2.5 × 20 cm) of Sephacryl G-50 fine was equilibrated and run with buffer B at a flow rate of 15 ml/h. Half of the pooled sample was loaded at a time, and the column effluent was monitored for absorbance at 280 nm. Two well separated peaks were identified. Reverse phase HPLC analysis of the fractions within the second peak determined that it mainly contained factor VII EGDF-His6. The fractions within the first peak were then pooled, supplemented with 5 mM MnCl2, and loaded onto the GDP-hexanolamine-agarose column as described above.

**Glycosidase Digestion of the Purified O-Fucosyltransferase**

PNGase F Digestion—Pure protein in storage buffer (0.4 μg, 50 μl) was first precipitated with 250 μl of acetone at -20 °C for 30 min and then spun in a microcentrifuge for 15 min. The pellet was washed with 200 μl of acetone and air-dried. The protein was then redissolved in 10 μl of 0.5% SDS, 10 mM β-mercaptoethanol and 0.15 mM Tris-HCl, pH 8.0, and heated at 100 °C for 3 min. The digestion was carried out by adding 0.5 unit of PNGase F (Boehringer Mannheim) in 20 μl of 2% Nonidet P-40, 30 mM EDTA, pH 8.0, to the denatured protein mix, and the solution was incubated at 37 °C overnight. The digested sample (10 μl) was directly analyzed by SDS-PAGE.

Endoglycosidase H Digestion—The protein was denatured as described above. Digestion was carried out with 1 milliunit of endoglycosidase H (Boehringer Mannheim) in 30 μl of 50 mM sodium citrate, pH 5.5, 2 mM phenylmethylsulfonyl fluoride, 0.25% Nonidet P-40 at 37 °C for 4.5 h. An aliquot (10 μl) of the sample was analyzed by SDS-PAGE.
Kinetic Characterization of the Purified O-Fucosyltransferase

Assays for measuring $K_m$ and $V_{\text{max}}$ were carried out essentially as described above except with variations in substrate concentrations. The amount of enzyme applied was limited to give no more than 10% conversion of substrate to product to quantitatively retain on Affi-Gel Blue resin. As described in our previous paper (12), the enzyme was enriched approximately 8-fold. The objective of the present study was the purification of an assay for the $O$-fucosyltransferase that modifies certain serine or threonine residues of proteins (12). During the course of that work, several properties of the enzyme were observed. Most of the enzyme activity was recovered in the soluble fraction of Chinese hamster ovary cell lysates. The activity did not bind to DE-52 anion exchange column but was quantitatively retained on Affi-Gel Blue resin. As described in our previous paper (12), the enzyme was enriched approximately 8-fold. The objective of the present study was the purification of this enzyme to homogeneity. The specifics of the purification were as follows.

The frozen Chinese hamster ovary cell paste was thawed at room temperature and kept at 4 °C afterward for the entire procedure. Low ionic strength buffer was used during homogenization to help break the cells, and DNase I was added to the homogenate to reduce its viscosity and facilitate the following chromatography steps. As shown in Table II, 86% of the activity was recovered after the first step, which achieved 2.2-fold purification.

Since preliminary experiments had demonstrated that the enzyme flowed through DE-52 at pH 7 and bound to Affi-Gel Blue, the two columns were connected in series for loading and initial washing steps. The DE-52 column was detached from the Affi-Gel Blue column after the initial wash (Fig. 1, point A). Some nonspecifically bound protein was eluted when the salt concentration was increased from 25 mm to 125 mm NaCl. The enzyme was then eluted with 1 mm NaCl (Fig. 1, point B). The combined purification for the two columns was 7.3-fold with 70% yield. The total volume of the preparation was reduced from 350 to 40 ml.

In efforts to further purify the O-fucosyltransferase, we determined that the enzyme had high affinity toward both its acceptor substrate, the recombinant EGF domain, and a donor substrate analog, GDP-hexanolamine. Hence, affinity resins were made with these two molecules as ligands.

The affinity resin with factor VII EGF-1 domain as ligand was made by taking advantage of the polyhistidine sequence originally designed for purification of the recombinant protein. The $O$-fucosyltransferase was found to bind to the resin better when the polyhistidine tag was at the carboxyl terminus of the EGF domain rather than at its amino terminus; hence, the former was used for the purification. The coupling of the EGF to Ni$^{2+}$-NTA-agarose was almost quantitative, and the resin was very stable; no leakage of recombinant EGF domain was detected after extensive washes. As shown in Fig. 2, the binding of the O-fucosyltransferase activity to this affinity resin was quantitative. At point A, the column was washed with the buffer containing 0.5 mm NaCl, and a large amount of nonspecifically bound protein was eluted. The binding of enzyme to the EGF domain was found to be strong enough to withstand 2 mm NaCl. Because the association between the enzyme and the EGF domain was so strong, an alternative method of elution was used to avoid denaturing the enzyme. Since the linkage of EGF domain to Ni$^{2+}$-NTA-agarose was noncovalent, the recovery of the enzyme activity was achieved by dissociating the EGF domain from the resin by using imidazole to displace the polyhistidine binding to the Ni$^{2+}$-NTA-agarose. The column was washed at point B with buffer containing 25 mm imidazole to elute more nonspecifically bound protein. At point C, a solution of 0.3 mm imidazole was used to elute the polyhistidine-tagged EGF domain together with the enzyme off the column. The step purification was actually significantly higher than 16-fold as stated in Table II, since there was almost 6 mg of recombinant factor VII EGF-1 domain present in the eluant.

### Table II

**Summary of the purification of O-fucosyltransferase**

| Preparation                        | Total protein | Total volume | Total activity | Specific activity | Step purification | Total purification | Step yield | Yield |
|------------------------------------|---------------|--------------|----------------|-------------------|-------------------|--------------------|-------------|-------|
| Homogenate                         | 5736          | 400          | 0.911          | 0.00016           | 2                 | 2                  | 2           | 2     |
| 1. Supernatant                     | 2239          | 350          | 0.785          | 0.00035           | 2.2               | 2.2                | 86          | 86    |
| 2. DE-52/Affi-Gel Blue             | 215           | 40           | 0.550          | 0.0026            | 7.3               | 16.1               | 71          | 80    |
| 3. Factor VII-EGF-Ni$^{2+}$-NTA-agarose | 9.81        | 13           | 0.401          | 0.041             | 16                | 256                | 73          | 44    |
| 4. GDP-hexanolamine-agarose        | 0.237         | 21           | 0.186          | 0.784             | 19                | 4937               | 46          | 20    |
The pooled activity from the factor VII EGF-1 affinity purification step was applied to a column of GDP-hexanolamine-agarose (Fig. 3). Unexpectedly, at least half of the enzyme activity flowed through this column. Experiments examining this flow-through activity will be described below. At point A, the column was washed with buffer containing 125 mM NaCl to elute nonspecifically bound protein. Then a GDP gradient (0–2 mM) was used for specific elution of the enzyme (point B). The fractions collected contained very limited amounts of protein. SDS-PAGE with silver staining was used to assess the purity of the preparation as shown in Fig. 4. Only one band, with a molecular weight of 44,000, was visible on the gel. The variation of the band intensity also corresponded to that of the enzyme activity among different fractions.

As described above, approximately half of the enzyme activity was in the flow-through fractions of the GDP-hexanolamine-agarose column. Possible explanations for this observation included the following: 1) the binding capacity of the GDP-hexanolamine column had been exceeded; 2) the column had resolved different fucosyltransferase activities that had been co-purified throughout the EGF affinity column; and 3) the presence of a large molar excess of the recombinant factor VII EGF-1 domain had interfered with binding of the O-fucosyltransferase to the GDP-hexanolamine column. Of these possibilities, the first was very unlikely, since preliminary experiments performed in the absence of the factor VII EGF-1 domain showed that the resin had higher capacity than would be required to bind all of the fucosyltransferase activity in the sample. To examine the latter two possibilities, the flow-through fractions from the GDP-hexanolamine agarose column were pooled and loaded on a Sephadex G-50 column as described under “Experimental Procedures.” Two protein peaks were identified. One was in the void volume and contained enzyme activity, presumably now in a 1:1 stoichiometry of enzyme and the recombinant factor VII EGF-1 domain; the other eluted later and consisted of the recombinant factor VII EGF-1 domain, as assessed by HPLC. The fractions of the first peak were pooled and rerun on the GDP-hexanolamine-agarose column using the conditions described above. All of the activity was now retained on the column. SDS-PAGE analysis of eluted fractions gave a single band with the same molecular weight (44,000) as had been observed with the bound fraction of the first run of the GDP-hexanolamine column. These results indicated that the presence of a large molar excess of the acceptor substrate had interfered with the efficiency of binding of the
enzyme to the GDP-hexanolamine column and that the bound versus unbound fractions did not represent different enzymes or molecular forms.

The final purification of the enzyme was nearly 5000-fold (Table II). The combined enzyme activity from two GDP-hexanolamine-agarose runs gave a total purification yield of about 20%. The O-fucosyltransferase accounted for approximately 0.02% of total Chinese hamster ovary cell protein at the start of the purification.

Characterization of the Purified Enzyme

Glycosidase Digestion—The nature of glycosylation on the purified O-fucosyltransferase was examined using two endoglycosidases, PNGase F and endoglycosidase H, as described under “Experimental Procedures.” Fig. 5 shows that after PNGase F digestion, the molecular weight of the protein was reduced from 44,000 to 40,000 (lane 2), suggesting the presence of N-linked oligosaccharides. Digestion with endoglycosidase H gave rise to two bands (lane 3) of lower molecular weight than the undigested protein (lane 1). These results were consistent with the presence of more than one high mannose type oligosaccharide on the enzyme.

Kinetic Parameters and Enzymatic Properties—The \( K_m \) and \( V_{max} \) for factor VII His6-EGF-1, factor VII EGF-1 and GDP-fucose were measured and are listed in Table III. The enzyme activity was inhibited at high concentrations (>15 \( \mu M \)) of recombinant factor VII EGF-1 domains (data not shown). The parameters for the EGF domains obtained here were calculated from data generated with acceptor substrate concentrations up to 8 \( \mu M \), in which the initial rate increased linearly with substrate concentration. The presence of the six histidine residues at the amino terminus of the EGF domain did not have a significant effect on enzyme activity. Because of the acceptor substrate inhibition, two different concentrations of factor VII His6-EGF-1 were used to measure the parameters for GDP-fucose (Table III). As a consequence, the \( V_{max} \) values obtained for GDP-fucose are much lower than the ones for the recombinant factor VII EGF-1 domain. Although the \( K_m \) values for GDP-fucose did not change with different factor VII EGF-1 domain concentrations, the \( V_{max} \) values decreased with the increased concentrations of the acceptor substrate. To understand the exact nature of the substrate inhibition, more detailed kinetic studies would be required.

The purified O-fucosyltransferase was active from pH 5.5 to pH 8.0. It exhibited the highest activity in cacodylate and imidazole buffers (data not shown). As listed in Fig. 6, the presence of Mn\(^{2+}\) and several other divalent ions could increase its activity. However, the purified enzyme was active without the addition of any divalent ions and even in the presence of 25 mM EDTA, and therefore, does not appear to require divalent metal ions for its activity. Several ions (Cu\(^{2+}\), Fe\(^{3+}\), and Zn\(^{2+}\)) appeared to inhibit the enzyme activity.

The donor substrate specificity of the purified enzyme was examined by performing the assays using the following nucleotide sugars in place of GDP-fucose. They were GDP-mannose, UDP-glucose, UDP-N-acetylgalactosamine, UDP-galactose, UDP-N-acetylgalactosamine, and UDP-xylose. No activity was observed in assays with these donor nucleotide sugars.

Acceptor Substrate Specificity—As described previously (12), all examples of O-fucosylation on EGF domains characterized to date occur within the putative consensus sequence CXXG-G(S/T)C. To determine if the two glycine residues are required for O-fucosylation, human factor IX EGF domain mutants were constructed using alanine to replace either or both glycine residues (Table IV), although the total amount of fucose incorporated varied from one construct to another. It appeared, therefore, that the two glycine residues are not absolutely required for activity.

Analysis of the recombinant factor IX EGF domains using reverse phase HPLC revealed that upon the change of glycine to alanine, the mutant EGF domains exhibited multiple peaks on the chromatograms, whereas the wild type had only one peak (Fig. 7). Further characterization of the different peaks using LC/MS showed that all peaks from each mutant had same molecular weight. The most likely explanation for this observation is that the multiple peaks represented the same protein with different pairing of disulfide linkages and, there-
Factor IX EGF domain and its mutants were constructed and expressed as described under "Experimental Procedures." The sequences shown here are part of the consensus region for O-fucosylation of EGF domains. The two residues where changes were made are shown in boldface type. The glycosylation site is underlined. EGF(wt) is the wild type sequence. Their abilities to serve as substrates for O-fucosyltransferase are shown as fucose transferred under standard assay conditions.

| Name     | Sequence | \( M_r \) | Fucose |
|----------|----------|-----------|-------|
| EGF.AA   | -CLNAGSC- | 5816.3    | 1818  |
| EGF.AG   | -CLNAGSC- | 5802.3    | 4585  |
| EGF.GA   | -CLNAGSC- | 5802.3    | 6480  |
| EGF(wt)  | -CLNAGSC- | 5788.2    | 12,062|

FIG. 7. Reverse phase HPLC of factor IX EGF domain and its mutants. The recombinant proteins are listed in Table III. Chromatography was carried out as described under "Experimental Procedures." Peaks labeled with retention times are recombinant proteins as verified by electrospray mass spectrometry. In each chromatogram all labeled peaks have the same molecular weight.

| Mutants | Retention time | Molecular weight |
|---------|----------------|-----------------|
| EGF.AA  | 28.7           | 5818            |
|         | 29.3           | 5817            |
|         | 30.4           | 5965            |
|         | 33.0           | 5818            |
| EGF.AG  | 28.4           | 5804            |
|         | 28.8           | 5803            |
|         | 30.2           | 5950            |
|         | 32.8           | 5804            |
| EGF.GA  | 28.4           | 5804            |
|         | 29.6           | 5950            |
|         | 32.4           | 5804            |

DISCUSSION

A GDP-fucose-polypeptide fucosyltransferase was purified to apparent homogeneity from Chinese hamster ovary cell extracts in the absence of detergent. Our previous study showed that the same enzyme in rat liver was probably a membrane-bound protein (12). Most of the glycosyltransferases studied to date are type II membrane proteins with a short cytoplasmic amino-terminal domain followed by a transmembrane region very susceptible to proteolysis, and a large carboxyl-terminal catalytic domain that retains enzymatic activity in the absence of other domains (13). These glycosyltransferases are usually localized in endoplasmic reticulum or Golgi apparatus. It is likely that the protein obtained using the purification procedure described here is a truncated form of an originally membrane-bound enzyme. The fact that the purified O-fucosyltransferase was itself a glycoprotein with high mannose type oligosaccharide chains also supported this suggestion, since glycoproteins are usually membrane-bound or secretory proteins. The presence of mostly high mannose type oligosaccharides on O-fucosyltransferase suggests that it is probably localized in either the endoplasmic reticulum or cis-Golgi region (19).

The affinity resin made with recombinant factor VII EGF-1-His, and Ni\(^{2+}\)-NTA-agarose had several advantages over conventional covalent cross-linking. First, the EGF ligand was attached to the resin in a defined orientation, according to the position of the polyhistidine sequence. We observed that bind-
ing of the enzyme to the resin was more efficient when the polyhistidine tag was at the carboxyl terminus of factor VII EGF-1 domain; hence, this construct was used in the purification. Second, the binding of polyhistidine tag to Ni²⁺-NTA resin, and high concentrations of the EGF domain inhibited the enzyme activity. Although a more detailed kinetic study would be needed to determine the exact nature of this substrate inhibition, the fact that 2 x NaCl failed to elute the enzyme off the EGF column suggests that the binding of EGF domain to O-fucosyltransferase has a very low dissociation constant. Therefore, high concentrations of the EGF domain may inhibit the interaction between GDP-fucose and the enzyme.

As described under “Results,” the presence of an excess amount of EGF domain reduced the binding of O-fucosyltransferase to GDP-hexanolamine agarose, and high concentrations of the EGF domain inhibited the enzyme activity. Although a more detailed kinetic study would be needed to determine the exact nature of this substrate inhibition, the fact that 2 x NaCl failed to elute the enzyme off the EGF column suggests that the binding of EGF domain to O-fucosyltransferase has a very low dissociation constant. Therefore, high concentrations of the EGF domain may inhibit the interaction between GDP-fucose and the enzyme.

Preliminary studies of the acceptor substrate specificity of the purified O-fucosyltransferase revealed some unexpected properties. Although all of the EGF domain O-fucosylation characterized to date occurred within the putative consensus sequence CXGXXG(S/T)C, the results presented here clearly showed that the two glycine residues are not absolutely required. Even a mutant in which both glycines were replaced by alanines was O-fucosylated. It is certainly possible that substitution of the glycine residues with amino acids other than alanine may abolish the O-fucosylation. For example, protein Z and protein C in which one of the glycines is replaced by asparagine and histidine, respectively, are not O-fucosylated (8). It is also reasonable to suggest that the enzyme may fucosylate sequences with amino acids other than glycine or alanine at the corresponding positions as long as they are properly folded. If this is true, the number of potential candidates for EGF domain O-fucosylation would increase substantially, although no such cases have been identified so far.

O-Fucosylation has also been reported on the insect protease inhibitor PMP-C peptide (11). This is the first report of O-fucosylation of a non-EGF structure. We have observed that a recombinantly expressed mature PMP-C peptide in E. coli failed to serve as an acceptor substrate for the purified enzyme.² It is not known if a precursor of the PMP-C peptide would serve as substrate. It is also possible that the modification reported for the PMP-C peptide is the result of an insect O-fucosyltransferase with different acceptor substrate specificity from the mammalian enzyme.

This study showed that proper disulfide pairing of EGF domains is critical for them to serve as acceptor substrates for the O-fucosyltransferase. This is in contrast to the oligosaccharyl transferase (20) and the β-1,4-GalNAc transferase (21), in which the specific peptide sequence, rather than a particular disulfide pairing or folding pattern, determines if the sites are glycosylated. The mechanism of the O-fucosyltransferase recognition of its substrates is not yet known. Further experiments involving changing the positions of the cysteine residues and their flanking sequence may give important insights to this question.

The experiments reported here also support the studies published previously by Stults and Cummings (9) and Maloney et al. (10), in which it was reported that many proteins from Chinese hamster ovary cells were modified with O-linked fucose. The amount of O-fucosyltransferase present in Chinese hamster ovary cells is about 0.02% of total protein, which is more than most of the glycosyltransferases studied to date. A GenBank™ search resulted in many proteins with the putative consensus sequence for O-fucosylation. It is possible that O-fucosylation could be a very common post-translational modification.

Purification of the O-fucosyltransferase permits further studies on structure and function of the enzyme and on the biological implications of O-fucosylation. We are currently isolating the gene encoding the enzyme and further characterizing the sequence or structure requirements of the acceptor substrates for the enzyme activity.

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² Y. Wang and M. W. Spellman, unpublished observation.
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