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## Human $\alpha$ 1,3/4 Fucosyltransferases

### CHARACTERIZATION OF HIGHLY CONSERVED CYSTEINE RESIDUES AND N-LINKED GLYCOSYLATION SITES\*

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Human  $\alpha$ 1,3 fucosyltransferases (FucTs) contain four highly conserved cysteine (Cys) residues, in addition to a free Cys residue that lies near the binding site for GDP-fucose (Holmes, E. H., Xu, Z., Sherwood, A. L., and Macher, B. A. (1995) *J. Biol. Chem.* 270, 8145–8151). The participation of the highly conserved Cys residues in disulfide bonds and their functional significance were characterized by mass spectrometry (MS) analyses and site-directed mutagenesis, respectively. Among the human FucTs is a subset of enzymes (FucT III, V, and VI) having highly homologous sequences, especially in the catalytic domain, and Cys residues in FucT III and V were characterized. The amino acid sequence of FucT III was characterized. Peptides containing the four conserved Cys residues were detected after reduction and alkylation, and found to be involved in disulfide bonds. The disulfide bond pattern was characterized by multiple stage MS analysis and the use of Glu-C protease and MS/MS analysis. Disulfide bonds in FucT III occur between Cys residues (Cys<sup>81</sup> to Cys<sup>338</sup> and Cys<sup>91</sup> to Cys<sup>341</sup>) at the N and C termini of the catalytic domain, bringing these ends close together in space. Mutagenesis of highly conserved Cys residues to Ser in FucT V resulted in proteins lacking enzymatic activity. Three of the four mutants have molecular weights similar to wild type enzyme and maintained an ability to bind GDP, whereas the other (Cys<sup>104</sup>) produced a series of lower molecular weight bands when characterized by Western blot analysis, and did not bind GDP. FucTs have highly conserved, potential N-linked sites, and our mass spectrometry analyses demonstrated that both N-linked sites are modified with oligosaccharides.

$\alpha$ 1,3/4 fucosyltransferases (FucTs)<sup>1</sup> have been cloned from a

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<sup>1</sup> The abbreviations used are: FucT, fucosyltransferase; ESI, electrospray ionization; H-type II, Fuc $\alpha$ 1,2Gal $\beta$ 1,4GlcNAc; LC, liquid chromatography; MS, mass spectrometry; NEM, N-ethylmaleimide; PBS,

variety of species and share significant sequence homology (see Refs. 1–5 and references therein). Among the conserved residues in FucTs (including human, mouse, chicken, and zebra fish) are four cysteine (Cys) residues (Fig. 1). Two of these Cys residues are located near the N terminus and two near the C terminus of the catalytic domain. The Cys residues found at the C terminus also are conserved in FucTs from *Caenorhabditis elegans* (6).

Among the human FucTs, FucTs III, V, and VI share substantial sequence homology. Within the catalytic domain only about 20 out of 300 amino acids vary among the three proteins. In addition, domain swapping experiments by our group (7) and Lowe and co-workers (9) have demonstrated that chimeric proteins composed of partial sequence from each of these FucTs are active, indicating that the minor differences in their amino acid sequences does not result in major alterations in their overall structure. Therefore, we have used two (FucT III and V) of these highly homologous proteins in the present study to evaluate the structure and functional significance of the highly conserved Cys residues.

Protein chemistry experiments, coupled with mass spectrometry analyses, have been used to locate all peptides containing Cys residues in human FucT III, allowing them to be assigned either as being involved in a disulfide bond or as a free Cys residue, and identifying which Cys residues are bound to each other in disulfide linkages. The results support our (7) previously stated hypothesis that amino acids affecting acceptor substrate specificity of human FucT III and V, located near the N and C termini of the catalytic domain, are brought close together in space by disulfide bonds between these highly conserved Cys residues.

To investigate the importance of these Cys residues, we have mutated each of these Cys residues in human FucT V and evaluated the activity and other properties of each resulting protein construct. The results demonstrate that these residues affect enzyme activity, but not the interaction of the protein with GDP-Fuc in three of the four cases. In the case of one of the mutant constructs (Cys<sup>104</sup>) protein folding/stability is altered compared with the wild type protein.

The amino acid sequences of FucTs have been predicted on the basis of cDNA sequences, but none of the amino acid se-

phosphate-buffered saline; PCR, polymerase chain reaction; type I, Gal $\beta$ 1,3GlcNAc; MS<sup>3</sup>, multiple stage mass spectrometry; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; PNGase F, peptide-N-glycanase F; MOPS, 4-morpholinopropanesulfonic acid; ASA, azidosalicylic acid; PEO, polyethylene oxide.

quences have been confirmed directly. Furthermore, little is known about sites of posttranslational modifications. Among the potential sites for posttranslational modification in FucTs, are Asn residues that may be substituted with *N*-linked glycans (Ref. 8 and references therein). Among FucTs III, V, and VI, there are two highly conserved *N*-linked sites, plus others that are less highly conserved (*i.e.* they occur in FucTs V and VI, but not FucT III). From previous analyses (7, 9), it is clear that some, but not all, of these sites are posttranslationally modified. In the current study, a combination of proteolytic digestions and MS/MS analyses have been used to analyze the amino acid sequence of FucT III. This methodology in combination with PNGase F treatment has been used to locate Asn residues in FucT III that are glycosylated.

#### EXPERIMENTAL PROCEDURES

**Materials**—Unlabeled GDP-Fuc was purchased from Calbiochem (San Diego, CA), and GDP-[<sup>3</sup>H]Fuc was purchased from NEN Life Science Products. The following items were obtained from HyClone Laboratories Inc. (Logan, UT): Dulbecco's modified Eagle's medium, phosphate-buffered saline (PBS; 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 2.7 mM KCl, pH 7.4), penicillin, streptomycin, and fetal calf serum. Rabbit IgG-agarose, anti-goat IgG-alkaline phosphatase conjugate, goat IgG, bovine serum albumin, 5-bromo-4-chloro-3-indolyl phosphate, nitro blue tetrazolium, and formamide were obtained from Sigma. PEO-maleimide-activated biotin was purchased from Pierce. PNGase F and chymotrypsin were purchased from Roche Molecular Biochemicals, and trypsin was obtained from Worthington Biochemical Corp. (Lakewood, NJ). SP-Sepharose was from Amersham Pharmacia Biotech, and the GDP affinity resin was a gift from Dr. Ole Hindsgaul (Department of Chemistry, University of Alberta, Alberta, Canada). All other chemicals were obtained from commercial sources and were of the highest purity available.

**FucT V Mutants**—The truncated, wild type FucT V was described previously (7) and used as a template to generate the Cys → Ser mutants at Cys<sup>94</sup>, Cys<sup>104</sup>, Cys<sup>351</sup>, and Cys<sup>354</sup> and the double mutant Cys<sup>351</sup> → Ser/Cys<sup>354</sup> → Ser by PCR. To facilitate cloning of the mutants, a modified form of the FucT V coding sequence containing a *NotI* site at nucleotide base 984 of the FucT V sequence was generated by PCR mutagenesis. This new restriction site was used in PCR mutagenesis (7, 10) experiments to generate mutations, which produced the Cys<sup>351</sup> → Ser, Cys<sup>354</sup> → Ser, and Cys<sup>351</sup> → Ser/Cys<sup>354</sup> → Ser FucTs. Mutations at the 5' end of the FucT V coding region (Cys<sup>94</sup> and Cys<sup>104</sup> of FucT V) were introduced by recombinant PCR as described previously (7), and the PCR products were subcloned between the 5' terminus (*EcoRI* site), and the *EcoRV* site (nucleotide base 373–378 in FucT V) that exists in the FucT V coding region.

Recombinant PCR was used to create Cys<sup>94</sup> → Ser and Cys<sup>104</sup> → Ser mutants. The flanking primers (A and D) were used in the second round of PCR to create the full-length PCR product that was inserted into pPROTA. The underlined segments are restriction sites that were used in subcloning or to screen for recombinants (GAATTC (*EcoRI*), ATTAAT (*AseI*), GGTACC (*KpnI*), GATATC (*EcoRV*)). Standard PCR was used to create Cys<sup>351</sup> → Ser, Cys<sup>354</sup> → Ser, and Cys<sup>351</sup> → Ser/Cys<sup>354</sup> → Ser mutants. The underlined segments are restriction sites that were used in subcloning or to screen for recombinants. TGCGGCCG (*NotI*), GTCGAC (*SalI*), AAGCTT (*HindIII*). Each mutant coding region was verified by DNA sequencing.

The primers used are: primer A forward, 5'-GGAATTCGCTATTAATCCTGCTGTGGACGTGGCCTTTT-3'; primer B reverse, 5'-CGCGGC-GCCGGGTACCATCTCTGAGGAGCGGGCAGAGCCAC-3'; primer C forward, 5'-GTGGCTCTGCCCGCTCCTCAGAGATGGTACC CGCG-3'; primer D reverse, 5'-CATGATATCCCAGTGGTGCACGAT-3'; primer E reverse, 5'-GTCGGCAGTGATGTTGGAGTCCCGCGC-CGGGTACCATCTC-3'; primer F forward, 5'-GAGATGGTACCCGGC-GCCGCGGACTCCAACATCACTGCCGAC-3'; primer G forward, 5'-C-GCTGCGGCGCGCTCCTCAGCTGGGCACTGGCTTTCTCCAAG-GTTGCTGG-3'; primer H reverse, 5'-GCCGACGCTCGACTCAGGTGACCAAGCCGCTATGCTGCGCACCGT-3'; primer I forward, 5'-CGCT-GCGGCCGCGCTCCTCAGCTGGGCACTGGCTTTCTGCAAGCTT-CCTGGAAG-3'; primer J forward, 5'-CGCTGCGGCCGCGCTCCTCAGCTGGGCACTGGCTTTCTCCAAGCTTCTGGAAG-3'.

The resulting plasmids were propagated in the JM109 strain of *E. coli* and transfected into COS-7 cells. Wild type and mutant proteins were expressed as soluble proteins with an N-terminal protein A, IgG

binding domain (11). The fusion proteins were purified from the cell culture media by IgG-agarose column chromatography. Recombinant FucTs were detected and quantified via Western blot analysis (16).

**Fucosyltransferase Assays**—The standard reaction mixture contained: 50 mM MOPS/NaOH buffer, pH 6.5, 6.25 mM MnCl<sub>2</sub>, 0.05% bovine serum albumin, 3.0 nmol of GDP-Fuc, 0.01 μCi of GDP-[<sup>3</sup>H]Fuc, 20 nmol of acceptor, and 5 μl of the enzyme, and reactions were terminated by adding 400 μl of water. The reaction product was separated from substrate by reverse phase chromatography (Sep-Pak C<sub>18</sub>) and quantified as described previously (12).

**Preparation of [<sup>125</sup>I]-GDP-hexanolamine-ASA**—GDP-hexanolamine-ASA was iodinated (see Ref. 13) in reaction mixtures that contained two IODOBEADS (Pierce), 5 μmol sodium phosphate buffer, pH 7.0, 1 mCi of Na<sup>125</sup>I, and H<sub>2</sub>O in a total volume of 60 μl. At the end of 15 min, 0.6 μmol of GDP-hexanolamine-ASA in 35 μl of H<sub>2</sub>O was added and incubated for an additional 15 min at room temperature. The reaction was stopped by removal of the solution from the IODOBEADS and labeled GDP-hexanolamine-ASA used in photolabeling experiments.

**Photoaffinity Labeling of Beaded Enzyme**—Wild type FucT V and FucT V Cys mutant constructs expressed in COS-7 cells and adsorbed onto IgG-agarose beads were suspended in a 25% bead slurry in PBS. Aliquots (10 μl), each containing 350–540 ng of expressed protein, were dispensed in reaction mixtures, which also contained 0.5 μmol of sodium phosphate buffer, pH 7.0, 0.625 μmol of [<sup>125</sup>I]-GDP-hexanolamine-ASA (approximately 1 × 10<sup>7</sup> cpm/nmol), with or without 20 μmol of GDP-Fuc, and H<sub>2</sub>O in a total volume of 50 μl in wells of a round-bottomed 96-well plate. The reaction mixtures were allowed to stand for 30 min at room temperature in the dark prior to photolysis at 254 nm for 1 min with a hand-held UV lamp placed directly above the wells. The beads were quantitatively transferred to 0.5-ml Eppendorf tubes, washed in PBS, and SDS gel sample buffer (Bio-Rad; 30 μl) was added. The tubes were heated at 100 °C for 10 min and centrifuged, and 25-μl aliquots were electrophoresed on 8.5% polyacrylamide gels. After electrophoresis, the gels were stained with Coomassie Blue, destained, and dried. The dried gels were exposed to X-Omat AR film to locate labeled protein. Labeled protein bands were cut out and quantified by counting in a γ counter.

**FucT III Expression and Purification**—FucT III was expressed in *Pichia pastoris* as described previously (14). To purify FucT III, pellets were resuspended to an OD<sub>600</sub> = 100 with 50 mM cacodylate, pH 7.0, 0.1 mM dithiothreitol. Lysis was achieved by glass bead beating using a bead beater. The beating chamber was filled with a 50/50 mixture of glass beads (Sigma acid-washed; 425–600 μm) and the resuspended pellet. Four lysis cycles were used to free the enzyme from the pelleted material (1 min beating and 2 min icing). Cell debris was removed by centrifugation at 27,500 × *g* for 1 h, and the supernatant was used as the enzyme source. The enzyme was purified in a two-step procedure that included cation exchange (SP-Sepharose) and a GDP-resin affinity chromatographic procedures. The crude pellet lysate was loaded onto the SP-Sepharose column, which was equilibrated in 50 mM cacodylate, pH 7.0 buffer containing 1.0 mM dithiothreitol. The column (approximately 50 ml) was washed with 200 ml of the loading buffer, and the enzyme was eluted with loading buffer containing 0.3 M NaCl. Fractions containing FucT III activity were pooled and loaded onto a 4-ml GDP-hexanolamine affinity resin (~3 μmol of ligand/mol of resin), which was equilibrated with a loading buffer containing 10 mM cacodylate, pH 7.0, 10 mM MgCl. The sample was applied at a flow rate of 0.2 ml/min and the column washed with 12 ml of loading buffer at the same flow rate. FucT III active fractions were eluted from the column with loading buffer containing 2 mM GDP at 0.2 ml/min. Fractions were analyzed for enzyme activity, and proteins by SDS-PAGE.

**Modification of Cys Residues**—One-ml fractions of FucT III (~50 ng/μl) from the GDP-affinity column were concentrated by Centricon filtration to 40 μl (1 μg/μl), 10 mM cacodylate, 10 mM MgCl<sub>2</sub>, 2 mM GDP at pH 7.0). A 5-μl sample of the concentrated FucT III (~5 μg) was incubated with a 20-fold molar excess of PEO-maleimide-activated biotin for 30 min in the dark at room temperature (total volume 6 μl). Biotin-labeled FucT III was denatured with urea (8.4 M) in a final volume of 9.1 μl for 30 min before tryptic digestion.

**Digestion with Trypsin**—The concentration of urea in the denatured FucT III sample was reduced to 2 M by adding water. Trypsin (1/5 ratio w/w of trypsin/protein) was added and the mixture (36.6 μl) was incubated overnight at 37 °C.

**Endoproteinase Glu-C Digestion**—Fractions of the tryptic digest of biotin modified FucT III eluted at 34–38 min were collected and dried by vacuum, and dissolved in 20 μl of ammonium bicarbonate buffer (100 mM, pH 7.9), and the endoproteinase Glu-C (1/10, w/w, enzyme/protein) was added. Digestion was carried out overnight at 37 °C.

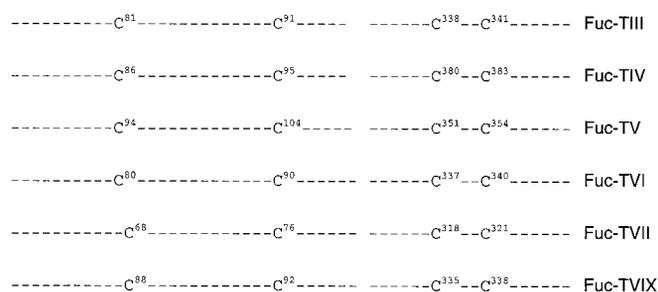


FIG. 1. Alignment of conserved Cys residues (labeled C) in human  $\alpha$ 1,3/4 fucosyltransferases.

**PNGase F Digestion**—PNGase F was dissolved in 100 mM sodium phosphate, 25 mM EDTA at pH 7.2 at a concentration of 200 units/ml. PNGase F digestion was done on tryptic digests of FucT III by adding PNGase F to a final concentration of 20 units/ml, and the mixture was incubated overnight at 37 °C.

**Sequence Analysis of Peptides by HPLC/MS/MS**—LC/MS/MS analysis was performed using an LCQ ion trap mass spectrometer (Finnigan, San Jose, CA) with a modified electrospray ionization (ESI) source. A positive voltage of 3 kV was applied to the electrospray needle, and the temperature of the stainless steel heating capillary was maintained at 220 °C. A  $N_2$  sheath flow (65 scale) was applied to stabilize the ESI signal. The voltage at the exit of the heating capillary and the tube lens was held at 13 and 5 V, respectively, to minimize the source-induced dissociation and optimize the ESI signal of the analyte. The ion injection was controlled by automatic gain control to avoid the space charge effects. The full scan mass spectrum was acquired from  $m/z = 300$  to  $m/z = 2000$ . The MS/MS experiments were executed with a relative collision energy of 38%. The LC/MS analysis was conducted using a 1050 HPLC system (Hewlett-Packard, Palo Alto, CA) coupled to the LCQ. The HPLC system was operated at a flow rate of 0.25 ml/min. The mobile phase was split before the injector by a tee-connector. One end of the tee was connected to a capillary C18 column (150  $\times$  0.18 mm; Nucleosil, 5- $\mu$ m particle size) and a flow rate of 2  $\mu$ l/min was established. The enzymatically digested peptides were eluted from the column using 0.5% formic acid in water (mobile phase A) and 0.5% formic acid in acetonitrile (mobile phase B) with a three-step linear gradient of 5–10% B in the first 10 min, 10–35% B in the next 40 min and 35–40% B in the last 5 min. The LC/MS/MS analysis was accomplished using an automated data acquisition procedure, in which a cyclic series of three different scan modes were performed. Data acquisition was conducted using the full scan mode to obtain the most intense peak (signal  $> 1.5 \times 10^5$  counts) as the precursor ion, followed by a high resolution zoom scan mode to determine the charge state of the precursor ion and an MS/MS scan mode to determine the structural fragment ions of the precursor ion. The resulting MS/MS spectra were then searched against a protein data base (Owl) by Sequest to confirm the sequence of tryptic peptides. Multiple stage ( $MS^3$ ) analysis was performed by selecting a fragment ion from the MS/MS analysis as the precursor ion to generate the  $MS^3$  spectrum. The relative collisional energy for the MS/MS and  $MS^3$  analyses was set from 30% to 43%.

## RESULTS

Six human FucTs have been cloned (FucT III to FucT VII and FucT IX). A comparison of the distribution of Cys residues among these enzymes demonstrates that there are four highly conserved Cys residues (see Fig. 1), which are also highly conserved in FucTs from other species. The importance of these conserved Cys residues for protein structure (e.g. disulfide bond formation) and enzyme activity is unknown. Therefore, a series of protein chemistry/mass spectrometry and site-directed mutagenesis studies have been carried out to provide the first information on the structure/function relationships of these highly conserved Cys residues.

**Amino Acid Sequence Analysis of FucT III**—The availability of a yeast expression system for FucT III provided an opportunity to obtain a sufficient quantity of enzyme for a complete characterization of the protein's amino acid sequence, its disulfide bond pattern and the location of *N*-linked glycosylation. FucT III was expressed as a soluble protein in *P. pastoris* (14)

and purified in a two-step process that yielded a single Coomassie-stained band by SDS-PAGE. Fractions containing this protein band were highly active (specific activity: 1200 nmol/min mg protein) for fucosyltransferase activity with a type I acceptor. In-gel tryptic digestion products of the band detected on the SDS-PAGE contained peptides derived from the FucT III sequence, and there was no conclusive evidence for the presence of any other protein contaminants (data not shown). A complete peptide sequence analysis of the purified protein solution (reduced with dithiothreitol and alkylated with iodoacetamide) was done using a combination of tryptic digestion and tandem mass spectrometry analysis. More than 95% of the amino acid sequence predicted from the cDNA for FucT III was confirmed by using the protein data base searching program, Sequest (data not shown). Two peptides (amino acids 152–160 and 161–189) that contain an *N*-linked consensus sequence were not detected. However, after treatment with PNGase F, modified (i.e. peptides with Asn converted to Asp) peptides corresponding to these sequences were detected. MS/MS analysis confirmed that the detected amino acid sequence contained Asp in place of Asn. Thus, the peptide containing amino acids 152–160 (YF(N  $\rightarrow$  D)LTMSYR) gave a  $y_7$  ion with  $m/z = 885.4$  instead of 884.4 predicted for the peptide with Asn present, and the peptide containing amino acids 161–189 (SDS-DIFTPYGWLEPWSGQPAHPPL(N  $\rightarrow$  D)LSAK) gave a  $y_8$  ion with  $m/z = 840.2$  instead of 839.2 predicted for the peptide with Asn present. Thus, both of the highly conserved (found in FucT III, V, and VI) *N*-linked glycosylation sites are glycosylated when FucT III is expressed in the yeast system.

**Identification of Free and Disulfide-bonded Cys Residues**—Purified FucT III was reacted with PEO-maleimide-activated biotin to label any free Cys residues (one was predicted to occur at Cys<sup>143</sup>; see Ref. 15), denatured and digested with trypsin under non-reducing conditions. The tryptic digest was separated by liquid chromatography and analyzed for peptides containing modified Cys residues by ESI-MS/MS. A triply charged ion (Fig. 2A) for the Cys-containing peptide (132–151) at  $m/z = 1002.3$  and a doubly charged ion at  $m/z 1503.0$  were detected. The MS/MS analysis (Fig. 2B) of the ion specie at  $m/z 1002.3$  conclusively demonstrated that Cys<sup>143</sup> was conjugated to the biotin reagent. This is illustrated by the observed mass of the fragment ions ( $y_{12}$  and  $y_{13}$ , doubly charged ion at  $m/z 959.6$  and 1008.1, respectively) containing the biotin modified Cys residue. These ions have a mass that is 525.2 Da greater than expected if the Cys residue were unmodified; this mass corresponds to that expected for the peptide containing a modified Cys residue. Thus, the results presented in the MS/MS spectrum clearly show that Cys<sup>143</sup> is a free Cys residue in the native protein as we previously predicted. This Cys residue is known to lie in or near the GDP-Fuc binding site (15). No other biotinylated Cys-containing peptides were detected, indicating that the four other highly conserved Cys residues are involved in disulfide bridges.

To directly demonstrate that these Cys residues are indeed involved in disulfide bonds and determine how these Cys residues are linked, the  $m/z$  values of possible disulfide-linked peptide combinations were calculated and the data generated from the LC/ESI-MS/MS analysis of a nonreduced preparation of FucT III was searched for possible matches. The search identified ions (detected as both triply  $m/z 1171.9$  and quadruply  $m/z 879.1$  charged species) that would result if the three Cys-containing peptides (amino acids 81–97, 330–339, and 340–343; see Fig. 3A) were eluted as a disulfide-linked, tripeptide complex. MS/MS analysis of the quadruply charged ion at  $m/z 879.1$  (data not shown) further supported the presence of a complex of the three Cys-containing peptides with the most

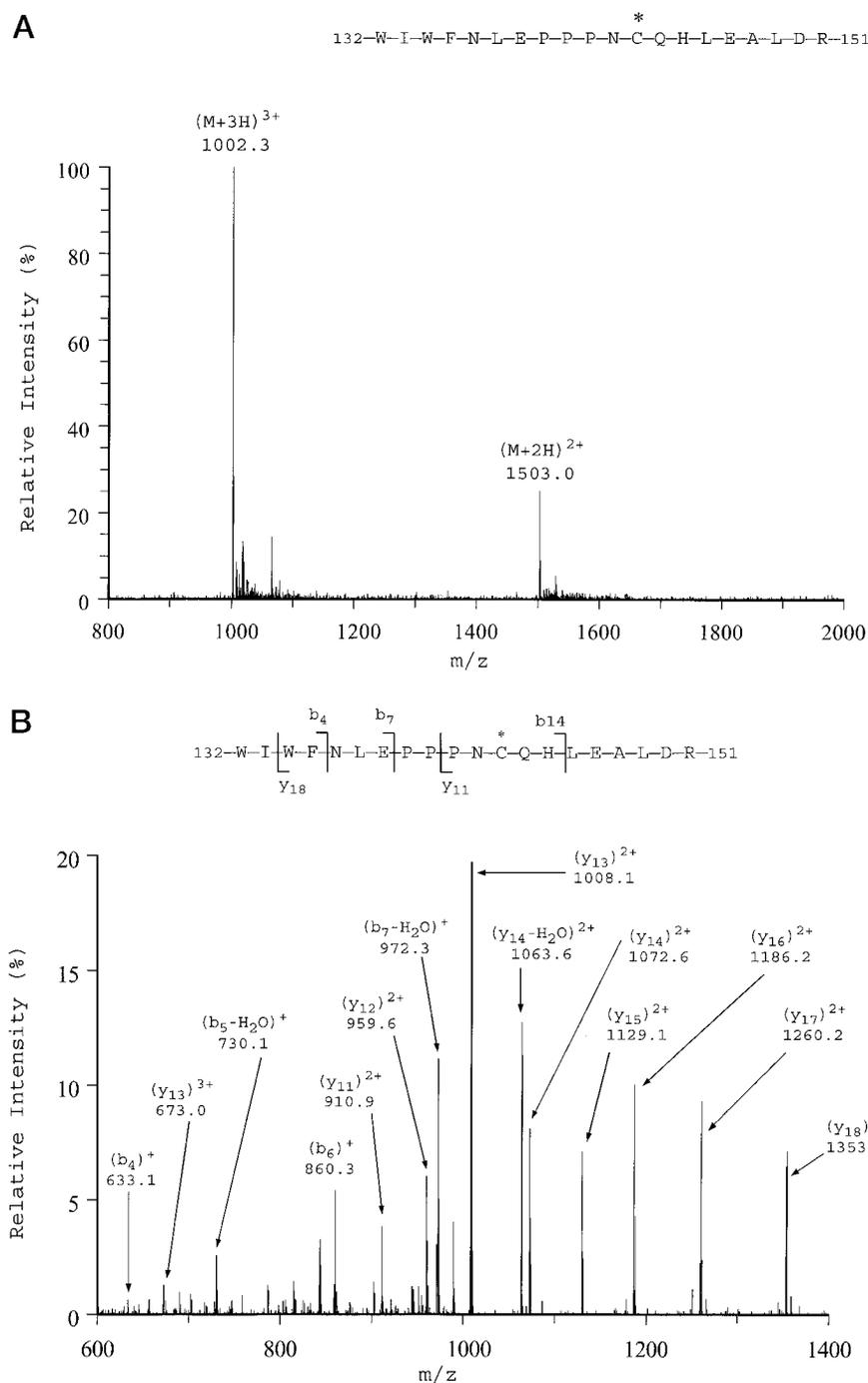


FIG. 2. *A*, the full scan mass spectrum of the modified Cys<sup>143</sup>-containing tryptic peptide (amino acids 132–151) of FucT III at  $m/z$  1503.0 for the doubly charged ion and  $m/z$  1002.3 for the triply charged ion. *B*, the corresponding MS/MS spectrum for  $m/z$  1002.3 contains both  $b_n$ - and  $y_n$ -dominant ( $b_n$ ,  $n = 4-7$  and 14;  $y_n$ ,  $n = 11-18$ ) fragment ions, confirming the sequence and Cys modification. The position of the modified Cys residue is designated with an asterisk (\*).

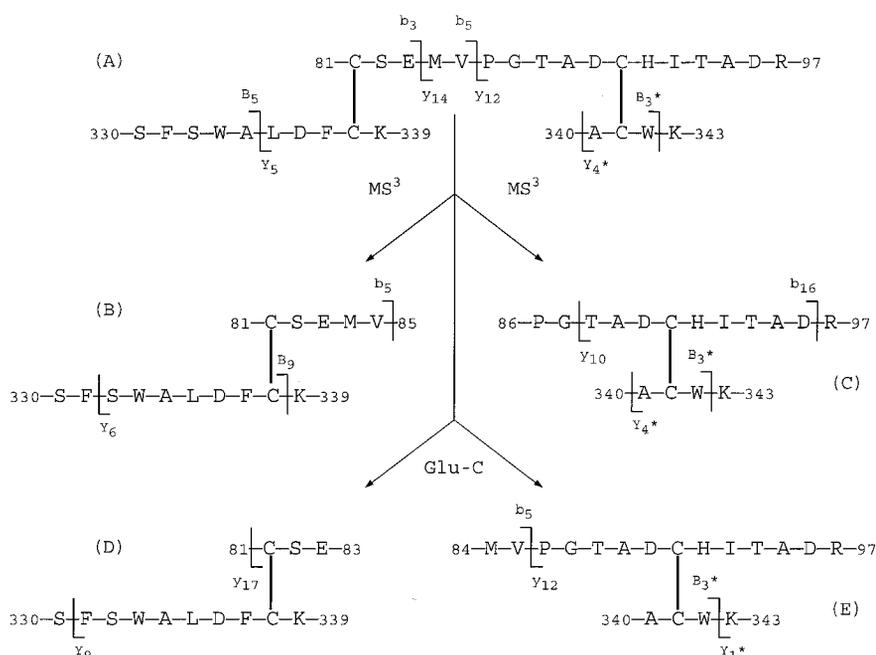
abundant fragments generated by a loss of amino acids from N terminus of peptide 330–339. Low energy collision-induced dissociation resulted in a small amount of S-S bond dissociation, which was only observed at  $m/z$  1288.3 ( $y_{12}+S$ ).

Interestingly, the MS/MS spectrum of the triply charged ion for the tripeptide complex at  $m/z$  1172.0 (see Fig. 4) shows that the dominant fragment ions are generated from a preferential cleavage at the amide bond of Pro<sup>86</sup> within the peptide containing Cys<sup>81</sup> and Cys<sup>91</sup>. This MS/MS fragmentation pattern is consistent with previous studies of Pro-containing peptides by Loo *et al.* (16), who observed a similar pattern with peptides derived from several proteins. The fragment ions,  $m/z$  1750.3 ( $b_5Y_{10}$ ) and 881.1 ( $y_{12}Y_4^*$ ), correspond to singly and doubly charged ions of disulfide-containing peptides, respectively. The ion at  $m/z$  881.1 is predicted to result from the combination of the two peptides containing Cys<sup>91</sup> and Cys<sup>343</sup> (peptides con-

taining amino acids 86–97 and 340–343, respectively), and the ion at  $m/z$  1759.1 is predicted to result from the combination of the two peptides containing Cys<sup>81</sup> and Cys<sup>338</sup> (peptides containing amino acids 81–85 and 330–339, respectively). These results suggest that the disulfide linkage patterns are Cys<sup>81</sup>-Cys<sup>338</sup> and Cys<sup>91</sup>-Cys<sup>343</sup>. Two additional sets of experiments were used to verify the identity of these ion species as the tripeptide complex and to validate the assigned disulfide bond pattern.

*Analysis of the Tripeptide Complex by MS<sup>3</sup>*—An ion trap mass spectrometer is capable of performing multiple stage MS/MS analysis to obtain structural information. MS<sup>3</sup> analysis for the MS/MS fragment ions,  $m/z$  881.1 and 1750.3 was employed to confirm the assignment of the Cys<sup>91</sup>-Cys<sup>343</sup> and Cys<sup>81</sup>-Cys<sup>338</sup> disulfide bonding pattern. The dominant product ion in the MS<sup>3</sup> spectrum of  $m/z$  881.1 ( $y_{12}Y_4^*$ ) is the doubly

FIG. 3. Scheme of (A) the disulfide-linked tripeptide from FucT III and the products observed in the MS<sup>3</sup> (B and C) and Glu-C experiments (D and E).



Fragment	Charge	Mass	Fragment	Charge	Mass
1 B <sub>5</sub> -H <sub>2</sub> O	+	561.0	6 M+3H-H <sub>2</sub> O	3+	1165.4
2 Y <sub>7</sub> Y <sub>4</sub> *	2+	660.4	7 Y <sub>3</sub> Y <sub>17</sub> B <sub>3</sub> *	2+	1280.0
3 b <sub>12</sub> Y <sub>4</sub> *	2+	872.0	8 Y <sub>3</sub> Y <sub>17</sub> Y <sub>4</sub> *	2+	1353.4
4 Y <sub>12</sub> Y <sub>4</sub> *	2+	881.1	9 Y <sub>4</sub> Y <sub>17</sub> Y <sub>4</sub> *	2+	1410.3
5 b <sub>5</sub> Y <sub>3</sub>	+	944.1	10 Y <sub>5</sub> Y <sub>17</sub> Y <sub>4</sub> *	2+	1467.0
			11 Y <sub>10</sub> Y <sub>17</sub> +S	2+	1520.5
			12 b <sub>16</sub> Y <sub>10</sub> Y <sub>4</sub> *	2+	1669.8
			13 b <sub>5</sub> Y <sub>10</sub>	+	1750.3
			14 Y <sub>12</sub> Y <sub>4</sub> *	+	1761.5

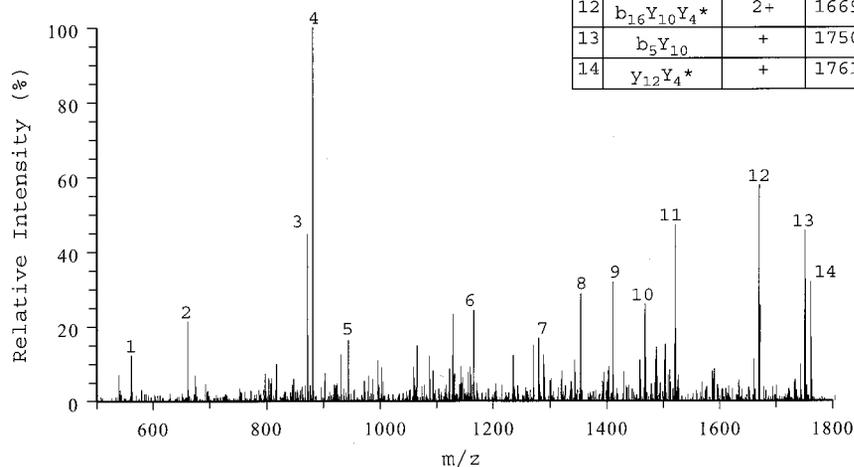


FIG. 4. MS/MS of the disulfide-linked tripeptide for the ion at  $m/z$  1172.0. Details of the fragments observed are shown in Fig. 3A and in the *inset* table.

charged ion ( $y_{12}Y_4-H_2O$ ) due to the loss of 18 Da (water) from the precursor ion (Fig. 5). All prominent ions observed, as shown in Fig. 3, can be derived from the sequence of disulfide paired peptides (86–97 and 340–343), confirming the disulfide pattern of Cys<sup>91</sup>-Cys<sup>343</sup>. The MS<sup>3</sup> spectrum of the singly charged ion of  $m/z$  1750.3 ( $b_5Y_{10}$ ) is shown in Fig. 6. As predicted, the dominant fragments are derived from the sequence of the disulfide paired peptides 81–85 and 330–339, with sequential loss of N-terminal amino acids from peptide 330–339; confirming the disulfide pattern of Cys<sup>81</sup>-Cys<sup>338</sup>.

**Analysis of the Tripeptide Complex by Endoprotease Glu-C Digestion**—To further confirm the identity of the disulfide-containing tripeptide complex, fractions of the tryptic digest obtained from the biotin-modified FucT III that eluted between 34 and 38 min from the LC run were collected and digested with Glu-C. The resulting peptides were analyzed by ESI-

MS/MS (Fig. 7). As would be predicted from the specificity of Glu-C, ions corresponding to two disulfide-containing peptides (Cys<sup>91</sup>-Cys<sup>343</sup> and Cys<sup>81</sup>-Cys<sup>338</sup>) were detected. These peptides produced doubly charged ions at  $m/z$  769.7 and 995.8, corresponding to calculated values ( $m/z$  769.9 and 995.8, due to the cleavage at the C terminus of Glu<sup>83</sup> found in the peptide containing amino acids 81–97 and Cys<sup>81</sup> and Cys<sup>91</sup> (see Fig. 3). The MS/MS analysis (data not shown) of the doubly charged ion at  $m/z$  995.8 for the disulfide-containing peptides Cys<sup>91</sup>-Cys<sup>342</sup> showed that the dominant product ions are generated from a fragmentation event at the amide bond of Pro<sup>86</sup> in the peptide containing amino acids 84–97 and Cys<sup>91</sup>, confirming the disulfide pattern of Cys<sup>91</sup>-Cys<sup>341</sup>. The MS/MS spectrum of the doubly charged ion at  $m/z$  769.7 (Fig. 7) for the disulfide-containing peptide (Cys<sup>81</sup>-Cys<sup>338</sup>) shows that the dominant product ions are the singly charged ions ( $Y_{17}Y_n$ ,  $n = 2-9$ ), that result

FIG. 5. MS<sup>3</sup> spectrum of doubly charged ion at  $m/z$  881.1. Fragments generated from the  $(y_{12}Y_4)^{2+}$  ion, peptide C in Fig. 3.  $y_{12}Y_4-H_2O$  is the dominant ion.

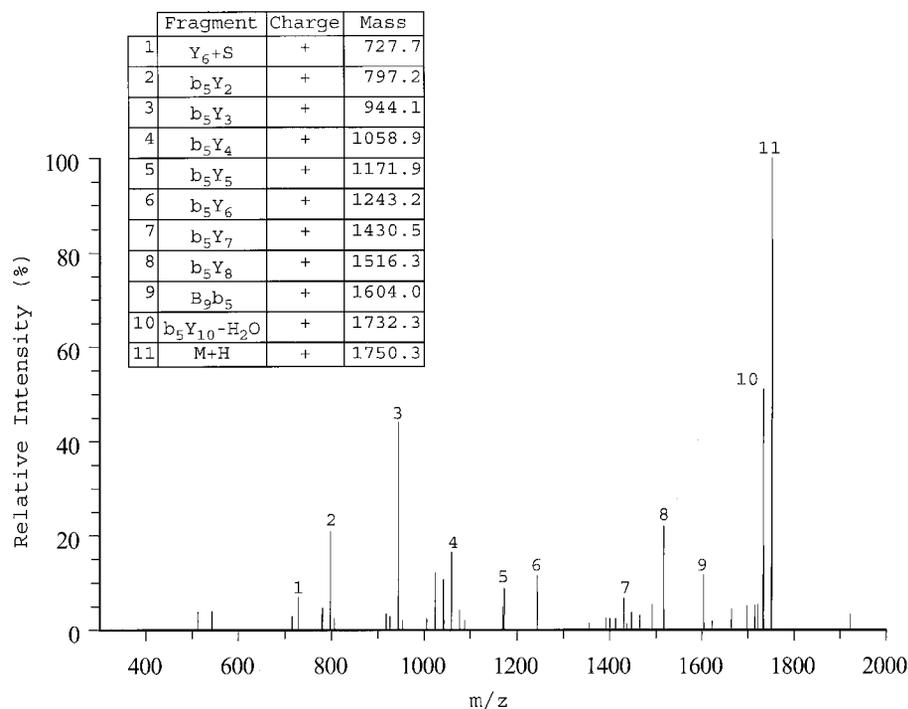
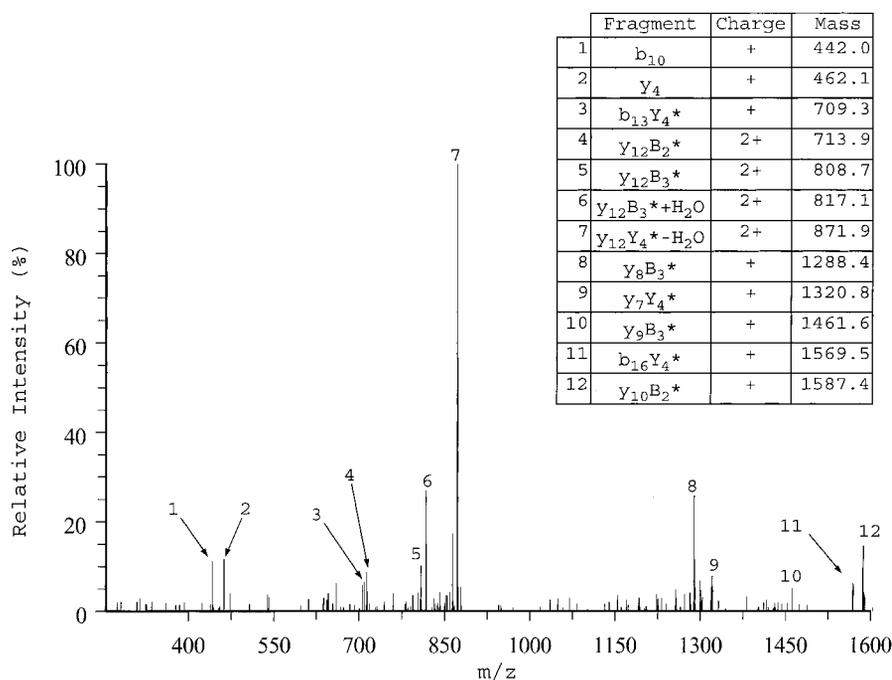


FIG. 6. MS<sup>3</sup> spectrum of the singly charged ion at  $m/z$  1750.3. Fragments generated from the  $(b_5Y_{10})^+$  ion, peptide B in Fig. 3. The ions  $b_5Y_n$  ( $n = 2-8$ ) are the dominant fragment ions.

from the sequential loss of N-terminal amino acids from peptide 330–339, confirming the disulfide pattern of Cys<sup>81</sup>-Cys<sup>338</sup> between peptides 81–83 and 330–339.

**Site-directed Mutagenesis of FucT V**—To evaluate the functional significance of the highly conserved Cys residues, site-directed mutagenesis studies were carried out using a FucT (FucT V) that is highly homologous to FucT III, differing at only 21 amino acid residues within the catalytic region. A truncated form of the enzyme, containing only the catalytic domain of the enzyme, coupled to a peptide tag corresponding to the protein A, IgG binding domain was used. We have previously established that this tag does not alter the activity or substrate specificity of any of the human FucTs. Furthermore, the tag provides a means of purifying and detecting the enzyme. Fi-

nally, the tagged FucT is isolated after secretion into the cell growth medium. Therefore, any expressed form (*i.e.* wild type or mutant) must traverse the entire protein secretory pathway before it is isolated for analysis.

Site-directed mutagenesis was used to change each of the conserved Cys residues in FucT V independently to Ser residues, and a double mutant (Cys<sup>351</sup> → Ser/Cys<sup>354</sup> → Ser) was also produced (Fig. 8, lane 6). As shown in Fig. 8, each mutant construct produced a protein that could be isolated from the cell growth medium by affinity chromatography. Three of the four single mutants, and the double mutant, produced proteins with a molecular weight similar to that of the wild type FucT V (Fig. 8, lane 1). Treatment of the wild type enzyme and these mutants with PNGase F demonstrated that all of these pro-

	Fragment	Charge	Mass
1	Y <sub>4</sub> +S	+	542.8
2	B <sub>5</sub> -H <sub>2</sub> O	+	561.0
3	Y <sub>17</sub> Y <sub>2</sub>	+	585.0
4	Y <sub>17</sub> Y <sub>8</sub>	2+	652.7
5	Y <sub>17</sub> Y <sub>3</sub>	+	732.1
6	Y <sub>17</sub> Y <sub>4</sub>	+	847.1
7	Y <sub>17</sub> Y <sub>5</sub>	+	960.2
8	Y <sub>17</sub> Y <sub>6</sub>	+	1031.2
9	Y <sub>17</sub> Y <sub>7</sub>	+	1217.3
10	Y <sub>17</sub> Y <sub>8</sub>	+	1304.3
11	Y <sub>17</sub> Y <sub>9</sub>	+	1392.1

FIG. 7. MS/MS of the Glu-C product (see Fig. 3D), a doubly charged ion at  $m/z$  769.9. The dominant ions observed are Y<sub>17</sub>Y<sub>n</sub> ( $n = 2-9$ ) using the notation of fragments in Fig. 3A.

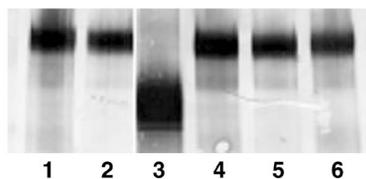
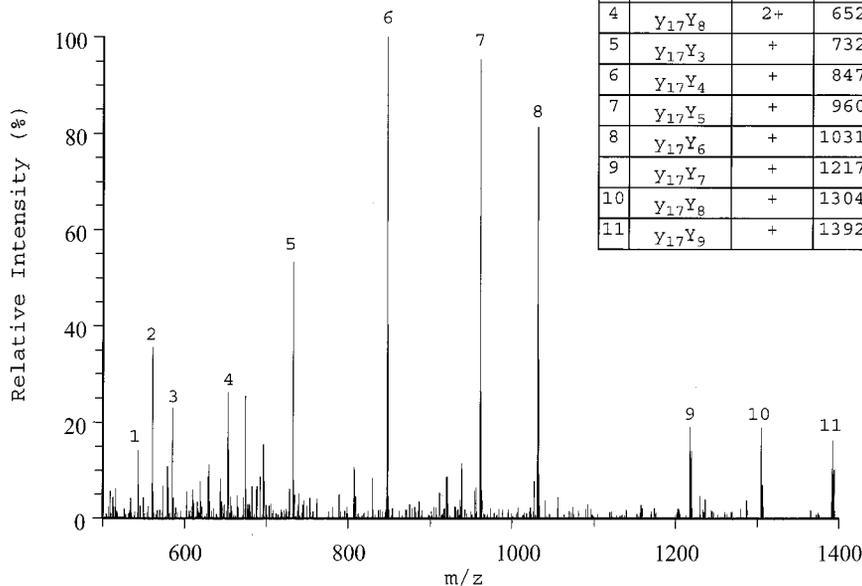


FIG. 8. Western blot of Cys mutants of human FucT V. FucT V mutants were prepared by a double PCR mutagenesis technique and expressed as chimeric proteins (catalytic domain of FucT V fused to the IgG binding domain of protein A) in COS-7 cells. The proteins were purified from the growth medium with an IgG resin and chromatographed by SDS-PAGE. Lane 1, wild type FucT V; lane 2, mutant Cys<sup>94</sup>; lane 3, mutant Cys<sup>104</sup>; lane 4, mutant Cys<sup>351</sup>; lane 5, mutant Cys<sup>354</sup>; lane 6, double mutant Cys<sup>351</sup>/Cys<sup>354</sup>.

teins are modified by N-linked oligosaccharides (data not shown). The other mutant (Cys<sup>104</sup> → Ser) produced a broad band on the Western blot that had a faster mobility than the wild type FucT V (Fig. 8, lane 3), and some of these bands had a higher gel mobility after treatment with PNGase F. Each protein was assayed for enzyme activity using a range of known acceptor substrates, including the preferred acceptor substrate for FucT V, H-type II. None of the mutant proteins had detectable enzyme activity, even upon prolonged incubation conditions (data not shown). The lack of enzyme activity could result from a variety of reasons (e.g. disruption of the protein structure or elimination of an essential functional group that assists in substrate binding or the catalytic mechanism). Therefore, further studies were carried out in an effort to better understand the basis for the lack of enzyme activity.

**Photolabeling Experiments**—Although the FucT V Cys mutants were enzymatically inactive, it is possible that they may still bind substrate. Since the  $K_m$  for binding the nucleotide sugar donor, GDP-Fuc, is significantly lower than that of the acceptor substrate, photolabeling experiments were conducted using the photoaffinity probe <sup>125</sup>I-GDP-hexanolamine-ASA (13). Wild type FucT V and FucT V Cys mutants were photolabeled with 12.5 μM <sup>125</sup>I-GDP-hexanolamine-ASA (equivalent to 0.5 times the  $K_i$  of GDP-hexanolamine with respect to GDP-Fuc; Ref. 13) in the absence and presence of 400 μM GDP-Fuc.

TABLE I  
Photoaffinity labeling of wild type FucT V and FucT V Cys mutants with <sup>125</sup>I-GDP-hexanolamine-ASA

Enzyme	Specific labeling <sup>a</sup>	Protection <sup>b</sup>
	cpm / 100 ng protein	%
FucT V	609	58
C94S	220	36
C104S	26	11
C351S	283	29
C354S	251	42

<sup>a</sup> Values are derived from the difference in extent of labeling of enzyme protein in photolabeling reaction mixtures conducted in the absence and presence of 400 μM GDP-Fuc (specific labeling = cpm incorporated in the absence of GDP-Fuc minus cpm incorporated in the presence of 400 μM GDP-Fuc).

<sup>b</sup> Percentage of protection of photolabeling by GDP-Fuc [(cpm incorporated in the absence of GDP-Fuc – cpm incorporated in the presence of 400 μM GDP-Fuc)/cpm incorporated in the absence of GDP-Fuc] × 100).

After photolysis, the enzyme fractions were separated by SDS-PAGE, and the amount of labeled photoprobe incorporated into protein quantified. The results shown in Table I indicate that a significant level of specific labeling of wild type FucT V occurred (defined as the cpm incorporated into protein in the absence of GDP-Fuc minus the cpm incorporated into protein in parallel reactions in the presence of GDP-Fuc). The extent of protection by GDP-Fuc for wild type FucT V was 58%. The remainder of the cpm incorporated into protein most likely represents nonspecific labeling as the highly reactive nitrene generated upon photolysis reacts with protein sites at random.

The level of specific labeling of the FucT V mutants Cys<sup>94</sup> → Ser, Cys<sup>351</sup> → Ser, and Cys<sup>354</sup> → Ser was lower (35–50% of wild type) when compared with wild type FucT V, but still significant. In addition, substantial protection from photolabeling was observed with excess GDP-Fuc, demonstrating that the photoprobe was competing with GDP-Fuc for binding to each protein. In contrast, there was very little specific labeling observed with the FucT V Cys<sup>104</sup> → Ser mutant, suggesting this enzyme has lost the ability to bind GDP-Fuc. Since much (>90% estimated from a Coomassie-stained gel) of this protein

was apparently degraded, it was not surprising that there was little incorporation of photoprobe.

#### DISCUSSION

The existence of a family of FucTs was initially proposed on the basis of differences observed in substrate specificity and sensitivity to inhibition by an amino acid modifying agent (*i.e.* NEM) (see Ref. 17 and references therein). However, it was not possible to establish how the enzymes differed until they were cloned. The predicted amino acid sequence information from their cloning provided several clues that have allowed us and others to begin to identify which amino acids give each enzyme its distinct properties (7, 9, 12, 15, 18–21). For example, among the human FucTs, only some (FucTs III, V, and VI) are inhibited by NEM, and we (15) have established that a free Cys residue accounts for this property in the sensitive enzymes, whereas those that are insensitive (22–24) to NEM have a corresponding Ser (FucT IV) or Thr (FucT VII) residue. More importantly, the identification of the NEM-sensitive Cys residue allowed us to pinpoint an amino acid that lies in or near the binding site for GDP-Fuc. In the current study, we have now chemically established that FucT III does contain a single, free Cys residue at this site. We have also identified a Lys residue that also lies in or near the GDP-Fuc binding site. These two residues occur at a significant distance (equivalent to amino acids Cys<sup>143</sup> and Lys<sup>259</sup> in FucT III) from one another in the FucT sequence and therefore, the native fold of the FucTs must bring these residues near one another in space. Furthermore, we have verified that the amino acid sequence predicted from the corresponding DNA is correct and that the only amino acids modified posttranslationally in the yeast expression system are Asn residues at the two predicted *N*-linked sites (Asn<sup>154</sup> and Asn<sup>185</sup>). These are the first results available to verify the complete amino acid sequence of any fucosyltransferase, and provide the first information on the location of *N*-linked site, oligosaccharide occupancy for a fucosyltransferase.

We and others have also identified amino acids that affect acceptor substrate specificity and thus are most likely close to one another in the native protein (7, 9, 12, 18, 20). Our previous studies of FucTs III and V have shown that amino acids affecting acceptor substrate specificity lie at the two ends of the catalytic domain of these two enzymes (7, 20). Based on this observation, and the fact that all mammalian FucTs have two sets of conserved Cys residues at the N and C termini of their catalytic domain, we (see discussion of Ref. 7) proposed that disulfide bonds between one or both pairs of the highly conserved Cys residues are responsible for bringing the two ends of the catalytic domain close together in the native protein, and thus bringing the residues identified as being important for acceptor substrate specificity together. The results reported in this study verify that this hypothesis is true. Furthermore, the results demonstrate that both pairs of conserved Cys residues are involved in disulfide bonds and that Cys<sup>91</sup>-Cys<sup>341</sup> and Cys<sup>81</sup>-Cys<sup>338</sup> of FucT III are bonded together. The latter point was unequivocally established by a combination of MS experiments including MS<sup>3</sup> analyses of the disulfide-linked tripeptide tryptic peptide isolated from nonreduced FucT III, and MS/MS analyses of the GluC products obtained from the disulfide-linked tryptic peptide. These results establish the maximum distance (not more than 35 Å) that amino acids we had previously demonstrated to affect acceptor substrate specificity (His<sup>73</sup>-Ile<sup>74</sup> and Asp<sup>336</sup> in FucT III, separated by more than 250 amino acid residues in the linear sequence) can be from one

another in space when the disulfide bonds are formed.

We<sup>3</sup> have recently demonstrated that FucT VII has a different disulfide bond pattern than that reported here for FucT III. This protein contains the four highly conserved Cys residues found in other FucTs (Fig. 1) plus two additional, closely spaced Cys residues (Cys<sup>211</sup> and Cys<sup>214</sup>) in the middle of its catalytic domain. Our results demonstrate that all six Cys residues form disulfide bonds and that each closely spaced pair (*i.e.* Cys<sup>68</sup> to Cys<sup>76</sup>, Cys<sup>211</sup> and Cys<sup>214</sup>, and Cys<sup>318</sup> to Cys<sup>321</sup>) of Cys residues are linked together. This results in a protein containing three short loops, in contrast to the large, single loop pattern that occurs in FucT III. Although we currently do not know whether the Cys residues in other FucTs are involved in disulfide bonds, it seems reasonable to expect that FucT V and VI would have the same disulfide bond pattern as FucT III since these enzymes have very highly conserved amino acid sequences, especially within their catalytic domain. Furthermore, we (12) and Lowe and co-workers (9) have demonstrated that it is possible to swap segments of the corresponding amino acid sequences of these proteins and create active domain swap mutants (*i.e.* swapping segments that do not contain all of the conserved Cys residues). Thus, it seems likely, although still unproven, that FucTs III, V, and VI would share a common disulfide bond pattern. Since human FucT IV and FucTs expressed in other species share ≤50% sequence homology with FucT III and VII, it is difficult to predict whether they will share the disulfide bond pattern of FucT III or VII, or have a distinct pattern.

The fact that both FucT III and VII bind GDP-Fuc but do not share a common disulfide bonding pattern suggests that the disulfide bonding pattern in these enzymes is not required for or involved in the formation of the GDP-Fuc binding pocket. The results obtained from our photolabeling experiments are consistent with this since the Cys mutants could still be labeled with the GDP-photo probe and labeling was blocked by GDP-Fuc.

Even though the mutant enzymes can bind GDP-Fuc, they are inactive. This may indicate that the acceptor substrate binding pocket for FucT V (and by analogy FucT III and VI) is dependent on the proper formation of disulfide bonds. Since these enzymes have a broader acceptor substrate specificity than FucT VII, one would anticipate that their acceptor substrate binding pockets would differ from that of FucT VII (see Ref. 11 and references therein; see also Refs. 22 and 23). The difference in the disulfide bond pattern of these FucTs could be critical for the formation of these distinct acceptor substrate binding sites. Further studies will be necessary to establish whether this is the case.

Another interesting observation from the mutagenesis studies is that only one of the mutants, FucT V Cys<sup>104</sup> → Ser, gave a Western blot pattern that differed significantly from the wild type enzyme. This protein was always found to be substantially degraded, whereas all of the other mutants, including the double mutant, gave a pattern essentially identical to the wild type enzyme. It is not clear why the conversion of one of the highly conserved Cys residues in the FucTs sequence should lead to a misfolded protein that is degraded more rapidly than the other mutants or the wild type protein. However, it is interesting to point out that this Cys residue lies near a potential *N*-linked glycosylation site (not one of those conserved in FucT III); a site that we have previously shown is not glycosylated in wild type FucT V (7). Thus, it is possible that substitution of a Ser residue for the Cys residue closest to this *N*-linked site causes some alteration that allows *N*-linked glycosylation to occur and

<sup>2</sup> Sherwood, A. L., Davis, W. C., Ho, S., Macher, B. A., Stroud, M. R., Upchurch, D. A., and Holmes, E. H. (2000) *Biochem. Biophys. Res. Commun.*, in press.

<sup>3</sup> T. de Vries, T.-Y., Yen, R. K. Joshi, J. Storm, D. H. van den Eijnden, R. M. A. Knegt, H. Bunschoten, D. H. Joziassse, and B. A. Macher, submitted for publication.

prohibits proper folding of the protein. Further studies are required to establish the actual cause of the misfolding and degradation of the FucT V Cys<sup>104</sup> → Ser mutant.

Comparisons of FucT amino acid sequences from several species have suggested that the  $\alpha$ 1,3 fucosyltransferase family of enzymes were derived from a common ancestral gene, and that the enzymes found today have evolved by gene duplication and divergence (1–3). Gene duplication of a common ancestral gene originated the leukocyte (FucT VII), myeloid (FucT IV), and Lewis (FucT III, V, and VI) subfamilies. Based on the results reported in this study, and those to be reported elsewhere on FucT VII's disulfide bond pattern, not only have the amino acid sequences of the FucTs diverged during evolution but, so have the pattern of their disulfide bonds. It will be interesting to determine the disulfide bond pattern of the other members of the FucT families and incorporate the resulting information into an assessment of the evolution of this family of enzymes. It is interesting to note that the FucT reported by DeBose-Boyd *et al.* (6) from *C. elegans* does not contain the N-terminal Cys residues conserved in other species and, therefore, would not be capable of forming the disulfide bond pattern found for FucT III, but could form a disulfide bond equivalent to that found in FucT VII, between the highly conserved Cys residues near the C terminus of the catalytic domain. It will be interesting to determine if this is the case or if FucTs from lower order organisms have a completely different pattern.

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**Human  $\alpha$ 1,3/4 Fucosyltransferases: CHARACTERIZATION OF HIGHLY CONSERVED CYSTEINE RESIDUES AND N-LINKED GLYCOSYLATION SITES**

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