The O-Linked Fucose Glycosylation Pathway

EVIDENCE FOR PROTEIN-SPECIFIC ELONGATION OF O-LINKED FUCOSE IN CHINESE HAMSTER OVARY CELLS

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O-Linked fucose is an unusual form of glycosylation recently shown to modify the hydroxyls of serine or threonine residues at a strict consensus site within epidermal growth factor-like domains of several serum proteins. Here we demonstrate that Chinese hamster ovary cells modify numerous proteins with O-linked fucose and that the fucose is elongated on specific proteins. We have identified at least two forms of O-linked fucose elongation in Chinese hamster ovary cells: a disaccharide (Glcβ1,3Fuc) and a larger oligosaccharide of indeterminate structure. Interestingly, it appears that the level of monosaccharide accumulates in the cells over time whereas the disaccharide does not. Analysis of the O-linked fucose-containing saccharides on individual proteins revealed that some proteins are modified with the monosaccharide only, whereas others are modified with monosaccharide and disaccharide, or monosaccharide and oligosaccharide. These results suggest that elongation of the O-linked fucose monosaccharide is a protein-specific phenomenon. The presence of elongated O-linked fucose moieties suggests that a novel glycosylation pathway exists in mammalian cells with O-linked fucose as the core.

In the past few years, a number of proteins have been shown to be modified with the monosaccharide L-fucose on the hydroxyls of serine or threonine residues (O-linked fucose) (1). Urokinase-type plasminogen activator was the first protein identified with this form of glycosylation (2) followed quickly by tissue-type plasminogen activator (3), factor VII (4), factor XII (5), and factor IX (6). Through comparison of the sites of glycosylation from these five proteins, a consensus sequence (CGXXG(S/T)C, where S/T is the modified residue) for the addition of O-linked fucose was proposed (7). Interestingly, in all cases this motif occurs within an epidermal growth factor-like (EGF)3 domain. Although these five proteins are all serum proteins involved in clot formation or dissolution, data base searches have revealed that many other types of proteins also contain this consensus sequence (1, 7).

Although L-fucose can be found internally linked in polysaccharides from plants and algae, in mammalian systems it is normally thought of as a terminal modification. Nonetheless, over 20 years ago the amino acid fucoside Glcβ1,3Fuc1-0-Ser/Thr was isolated from human urine, demonstrating that fucose residues can be found internally linked in mammalian systems (8, 9). More recently it was shown that the O-linked fucose moiety on factor IX is elongated into a tetrasaccharide with the structure NeuAcα2,6Galβ1,4GlcNAcb1,3Fucα1-0-Ser (7). To date, factor IX is the only protein reported to be modified with this tetrasaccharide structure and the only mammalian protein identified with an elongated fucose of any kind.

To study O-linked fucose in mammalian cells, we have used a Chinese hamster ovary (CHO) cell line, Lec1, which is deficient in the enzyme, N-acetylgalactosaminyltransferase I (10, 11). These cells are unable to synthesize complex or hybrid-type N-linked glycans. Since the addition of fucose to N-linked glycans was believed to occur only on complex or hybrid-type chains, we predicted fucose would be incorporated only into O-linked structures in these cells. Surprisingly, we found that small oligomannose N-glycans (Manα1,4Manα3) were labeled with fucose on the core GlcNAc in these cells suggesting that the core α1,6-fucosyltransferase does have some affinity for these structures (12). Nonetheless, the incorporation of fucose into N-glycans was greatly reduced and allowed for a better observation of O-linked fucose structures. Stults and Cummings (13) have utilized this same cell type for studying O-linked fucose, demonstrating that several proteins are modified with O-linked fucose monosaccharide in these cells.

We report here that CHO cells not only modify several endogenous proteins with O-linked fucose but that the O-linked fucose becomes elongated on a subset of the proteins. The major form of elongation is a simple disaccharide consisting of a β-linked glucose at the 3-position of the O-linked fucose (Glcβ1,3Fuc), identical with the structure found previously on amino acid fucosides (8, 9). A larger oligosaccharide also appears to exist, although in smaller and variable amounts. Over 15 proteins from CHO cells appear to be modified with O-linked fucose saccharides, and the elongation of O-linked fucose appears to be a protein-specific event. The presence of these elongated forms of O-linked fucose suggests the presence of a novel glycosylation pathway in mammalian cells with several potential end points all containing O-linked fucose as the core sugar.

EXPERIMENTAL PROCEDURES

Materials—Lec1 cells were developed by Dr. Pamela Stanley (11, 14). They were obtained from ATCC and grown as described (12). [6-3H]Fucose (86.5 Ci/mmol) was from DuPont NEN. β-Glucosidase (sweet almond), β-hexosaminidase (jack bean), and β-galactosidase (bovine testes) were from Sigma. Peptide N-glycosidase F (PNGase F) was purified from the culture filtrate of Flavobacterium meningosepticum as described (15). Alditol sugar standards were prepared by reduction of the corresponding sugar with sodium borohydride as described (16).

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The β-linked glucosylfucose standards (Glcβ1,2Fuc, Glcβ1,3Fuc, and Glcβ1,4Fuc) were synthesized, characterized, and generously provided by Dr. Khushi Matta (Roswell Park Memorial Institute, Buffalo, NY). All other reagents were of the highest quality available.

**Labeling of Lec1 Cells**—For short term labeling, cells were grown and radiolabeled essentially as described (12). For long term labeling, cells were incubated for 24 h in the presence of medium containing 50 μCi/ml [6-3H]fucose. For pulse-chase studies, cells were radiolabeled for 1 h in the presence of 100–300 μCi/ml [6-3H]fucose. The cells were collected by centrifugation, washed once with Tris-buffered saline (10 mM Tris-HCl, pH 7.5, 0.15 M NaCl), and resuspended in media containing 10 mM non-ionic detergent. The radiolabeled cells were then incubated for various chase times before washing and lysing.

**Lysis of Labeled Lec1 Cells**—Radiolabeled cells were collected by centrifugation and washed three times with cold Tris-buffered saline. Cells were lysed by addition of lysis buffer (Tris-buffered saline containing 1% (w/v) Nonidet P-40 and protease inhibitor mixtures I and II (17)) and incubated for 1 h with rocking at 4 °C. The lysate was clarified by centrifugation (12,000 × g, 10 min, 4 °C). The unincorporated radiolabel was separated from the protein fraction on a Sephadex G50–150 column (1 × 30 cm) developed in 50 mM ammonium formate containing 0.1% sodium dodecyl sulfate.

**Chromatographic Analysis of Sugars**—N-Linked oligosaccharides were removed from the [6-3H]fucose-labeled proteins by digestion with PNGase F as described (12). The O-linked sugars were released from proteins by alkali-induced β-elimination as described (12). The released O-linked sugar alcohols were size fractionated on a Superdex peptide column (Pharmacia Biotech Inc.) using partially hydrolyzed dextran as size standards (18). High pH anion-exchange chromatography (HPAEC) was performed on a Dionex DX300 HPLC system equipped with pulsed amperometric detection (PAD-2 cell). Samples were chromatographed on a CarboPac MA-1 column (Dionex Corp.) at 0.4 ml/min using the following gradient: 0–11 min, 0.1 M NaOH; 11–21 min, 0.1–0.7 M NaOH; 21–40 min, 0.7 M NaOH. Radioactive samples were mixed with standards (1 nmol each of fucitol, fucose, and glucose or the disaccharide standards Glcβ1,2fucitol, Glcβ1,3fucitol, and Glcβ1,4fucitol) prior to injection. Fractions (0.2–0.5 min) were collected and monitored for radioactivity by scintillation counting. Standards were followed by pulsed amperometric detection.

**Acid Hydrolysis**—The saccharides were hydrolyzed in 2 M trifluoroacetic acid at 100 °C for 2 h. The hydrolyzed samples were then dried in a Speed Vac evaporator (Savant) and resuspended in water prior to analysis by HPAEC.

**Exoglycosidase Digestions**—β-Glucosidase digestions were performed by incubating samples in β-glucosidase digestion buffer (50 mM sodium acetate, pH 5.0, 0.1 M NaCl, 0.1 mM zinc sulfate, 10 mg/ml bovine serum albumin) with β-glucosidase (25 units/ml) overnight at 37 °C. Mock digestions lacking enzyme were performed as a control. To stop the digestion, the sample was heated to 100 °C for 5 min. Digestions with β-galactosidase and β-hexosaminidase were performed as described (12). Digested samples were diluted into water and analyzed by HPAEC as described above.

**Analysis of O-Linked Fucose on Individual Proteins**—[6-3H]Fucose-labeled proteins (100 μg/lane), either treated with PNGase F or mock treated, were separated on 8% SDS-PAGE (19), stained with Coomassie Blue, and fluorographed. Additional samples (6 × 100 μg) of PNGase F-treated [6-3H]fucose-labeled proteins were separated on the same gel. After electrophoresis, the lanes containing these samples were cut into 2-mm horizontal slices, and the protein was extracted from the gel slices by incubation overnight in 1% SDS, 1% 2-mercaptoethanol at room temperature. The eluted proteins were concentrated by acetone precipitation (8 volumes, −20 °C, overnight) and analyzed for O-linked saccharides as described above.

**RESULTS**

**Lec1 Cells Synthesize at Least Three O-Linked Fucose-containing Saccharides**—Previously, we have shown that the majority (68%) of the [3H]fucose incorporated into proteins of Lec1 cells could be released by digestion with PNGase F (12). Subsequent analysis showed these glycans to be core-fucosylated oligomannose-type N-linked glycans of the Manα1,3-Manα2 size (12). The majority of the PNGase F-resistant [3H]fucose could be released by alkaline-induced β-elimination, implying an O-linkage to the protein (12). Analysis of the material released by

**FIG. 1. Size chromatography of O-linked saccharides from Lec1 cells labeled with [3H]fucose.** Lec1 cells were labeled with [3H]fucose under short term (5 h, 100–300 μCi/ml, panel A) or long term (24 h, 50 μCi/ml, panel B) conditions. After removal of N-glycans with PNGase F, O-linked saccharides were released by alkali-induced β-elimination as described under “Experimental Procedures.” The β-eliminated material was then separated on a calibrated Pharmacia Superdex sizing column. The migration positions of partially hydrolyzed dextran standards (in glucose units) are indicated at the top of the graph. The migration positions of the [3H]fucose-labeled peaks are also labeled MS, DS, and OS.

β-elimination on a calibrated Superdex sizing column revealed three radiolabeled species (Fig. 1A, peaks MS, DS, and OS). MS represents a monosaccharide as it migrates at a position slightly larger than 1 glucose unit. DS migrates at approximately 2 glucose units, representing a disaccharide. OS is a disaccharide migrating at a position larger than 10 glucose units. The amount and migration position of the oligosaccharide species varied with the labeling conditions (Fig. 1B) and the preparation.

To determine which of the three species was linked to protein through an O-linked fucose, each peak was analyzed for the presence of fucitol after acid hydrolysis. The sodium borohydride in the β-elimination solution converts the reducing end sugar to the alditol form (e.g., fucitol to fucitol) after being released from the protein. Therefore, fucitol, which had been directly linked to protein (as in O-linked fucose), would be converted to fucitol. All other forms of fucose (terminal modifications) should remain unreduced (fucose). Acid hydrolysis was performed to break the di- and oligosaccharide species into monomers, and HPAEC was used to separate fucitol from fucitol. The monosaccharide comigrated with the fucitol standard before and after acid hydrolysis, suggesting that this species is derived from O-linked fucose (Fig. 2A). When the disaccharide was analyzed prior to acid hydrolysis, it migrated at a unique position relative to fucitol, fucitol, and glucose (Fig. 2B). Acid hydrolysis of the disaccharide converted all of the radioactivity to fucitol, indicating that the disaccharide contains O-linked fucose (Fig. 2C). Unhydrolyzed oligosaccharide was not recovered from the HPAEC analysis, suggesting that it might be negatively charged. This observation was supported by the analysis of oligosaccharide material on QAE-Sephadex. The majority of the oligosaccharide material bound to QAE-Seph-

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1 R.K. Vig and K. Matta, unpublished data.
FIG. 2. MS, DS, and OS all contain O-linked fucose. The components of the three peaks from the sizing column (MS, DS, and OS) were analyzed by HPAEC before and after acid hydrolysis as described under “Experimental Procedures.” The migration positions of standard sugars are shown (1, fucitol; 2, fucose; 3, glucose). A, MS peak from Fig. 1A; B, DS peak from Fig. 1A prior to acid hydrolysis; C, DS peak from Fig. 1A after acid hydrolysis; D, OS peak from Fig. 1A after acid hydrolysis.

adex and eluted with 70–140 m\(\text{M}\) NaCl (data not shown). Acid hydrolysis of the oligosaccharide converted the majority of the radiolabel to fucitol, again indicating that the oligosaccharide material contains O-linked fucose (Fig. 2D). A small amount of unreduced fucose is also recovered from the oligosaccharide fraction. This may be due to a modification of the O-linked fucose oligosaccharide with another fucose, or there may be some contaminants with terminal fucose residues present in this fraction. Further work is required to determine the structure(s) of the O-linked fucose-containing saccharides in the oligosaccharide peak. Thus, all three peaks from the Superdex column represent O-linked fucose-containing saccharides: the monosaccharide, a disaccharide, and an oligosaccharide.

The Disaccharide Is Glucose \(\beta_{1,3}\)-Fucitol—We initially suspected the disaccharide to be a precursor in the synthesis of the known O-linked fucose tetrasaccharide on factor IX (7). If this were the case, then the disaccharide would consist of an O-linked fucose elongated with a \(\beta\)-linked N-acetylglucosamine. Upon digestion with jack bean \(\beta\)-hexosaminidase, we observed no shift in migration of the disaccharide (data not shown); we also did not observe a shift after digestion with \(\beta\)-galactosidase.

Surprisingly, when we subjected the disaccharide to digestion with \(\beta\)-glucosidase, we observed a shift to fucitol (data not shown) indicating that the disaccharide is fucitol modified with a \(\beta\)-linked glucose.

In an effort to determine the exact structural identity of the O-linked fucose-containing disaccharide, standards for the three possible configurations where glucose is \(\beta\)-linked to fucose were synthesized. The three disaccharide standards were each reduced with alkaline borohydride, yielding Glc\(\beta_1\)2fucitol, Glc\(\beta_1\)3fucitol, and Glc\(\beta_1\)4fucitol. These alditol standards were analyzed by HPAEC as described under “Experimental Procedures” and shown to migrate at unique positions (Fig. 3). Analysis of the disaccharide from Lec1 cells showed that it migrates exclusively with the Glc\(\beta_1\)3fucitol standard, demonstrating that the glucose is linked to the third position of the fucose.

The Disaccharide to Monosaccharide Ratio Decreases with Time—Although Stults and Cummings (13) also utilized Lec1 cells to examine O-linked fucose, they did not observe the disaccharide or oligosaccharide species. We believe that the difference in our findings results mainly from a difference in labeling conditions. Our initial studies were done on cells labeled for 3–5 h with fairly high concentrations of \(\text{[6-3H} \text{]fucose}\) (100–300 \(\mu\text{Ci/ml}\)). Stults and Cummings (13) labeled Lec1 cells under equilibrium conditions (24 h, 10 \(\mu\text{Ci/ml}\)). We examined incorporation of \(\text{[3H} \text{]fucose}\) into the disaccharide under both conditions. Interestingly, we observed a marked decrease in the amount of labeled disaccharide relative to monosaccharide in the long term labeling (compare Fig. 1B with Fig. 1A). This suggests that the monosaccharide accumulates during the longer labeling whereas the disaccharide does not. To more closely examine this phenomenon, we labeled cells for 1 h with \(\text{[3H} \text{]fucose}\) and then chased them with cold fucose for 0–5 h. Analysis of the O-linked fucose saccharides clearly shows that the ratio of disaccharide to monosaccharide decreases during the chase, consistent with the idea that the monosaccharide accumulates whereas the disaccharide does not (Fig. 4). Although the oligosaccharide does not change significantly in amount between long and short term labelings, it does appear to change in size (compare Fig. 1, A and B). We are currently investigating this phenomenon.

Lec1 Cells Synthesize Numerous O-Linked Fucose-modified Proteins Bearing Different Combinations of O-Linked Fucose Saccharides—We next sought to begin identifying proteins in Lec1 cells that contain these O-linked fucose modifications. Radiolabeled proteins from Lec1 cells were treated with or without PNGase F and separated by SDS-PAGE (Fig. 5A). The
labeled bands that remained after PNGase F treatment represent O-linked fucose-containing proteins. Over 15 protein species can be identified that appear to be modified with O-linked fucose saccharides. Identical PNGase F-treated samples were separated on the same gel, the gel was cut into 2-mm strips, and each fraction was analyzed for the presence of MS and DS as described in Fig. 1. The ratio of the peak areas of DS to MS is plotted versus the chase time. The line represents a best fit linear regression of the data.

**DISCUSSION**

In this report we have demonstrated that CHO cells not only modify numerous proteins with O-linked fucose but that the O-linked fucose is elongated on a subset of these proteins. At least two forms of elongation occur in these cells: a disaccharide and an oligosaccharide. The elongation appears to be protein-specific since some proteins bear only monosaccharide, some monosaccharide and disaccharide, and some monosaccharide and oligosaccharide in addition. The ratio of disaccharide to monosaccharide over time could have several potential explanations. For instance, it is possible that the change in ratio of disaccharide to monosaccharide could be the result of the removal of the glucose residue by a novel processing glucosidase activity. Alternatively, the disaccharide-modified proteins could be preferentially secreted from the cells. Another potential explanation is that the proteins modified with disaccharide turn over more rapidly than the proteins modified with monosaccharide. Finally, the disaccharide could be a precursor to the oligosaccharide. Further work is necessary to test these possibilities and determine an explanation for the disappearance of the disaccharide.

The oligosaccharide reported here may be related to the tetrasaccharide found on factor IX, since preliminary results indicate that the oligosaccharide is negatively charged and therefore may contain sialic acid. Alternatively, the oligosaccharide may be a novel structure unrelated to the tetrasaccharide. As mentioned above, the oligosaccharide could result from elongation of the disaccharide, although further work needs to be done to analyze the relationship between these two structures. Work on the structure and composition of this oligosaccharide is underway.

The fact that the disaccharide and the oligosaccharide are found on discrete populations of proteins suggests there may be specific signals for the elongation of O-linked fucose on certain proteins. In such a model, an enzyme that adds a sugar to O-linked fucose would need to recognize the fucose as well as some protein determinant that directs the elongation. This paradigm is not without precedent, as both the lysosomal enzyme GlcNAc-1-phosphotransferase (21) and the glycoprotein
O-Linked Fucose Glycosylation Pathway

![Diagram of the O-linked fucose glycosylation pathway](image)

**Fig. 6. The O-linked fucose glycosylation pathway.** This pathway shows the synthesis of the O-linked fucose-containing saccharide structures reported to date. The letters represent the enzymes responsible for catalyzing that step. A, GDP-Fuc; polypeptide fucosyltransferase (24); B, proposed UDP-Glc, O-fucose β1,3-glucosyltransferase; C, proposed UDP-GlcNAc, O-fucose β1,3-N-acetylgalactosaminyltransferase; D, β1,4-galactosyltransferase; and E, α2,6-sialyltransferase. Details are discussed in the text.

The identification of the various O-linked fucose-containing saccharide structures has revealed an entirely new glycosylation pathway (see Fig. 6). The first step in the pathway is the addition of O-linked fucose to a consensus sequence by an O-fucosyltransferase (Fig. 6, see A). This enzyme has recently been identified and was initially characterized (24). Interestingly, it appears to require an intact EGF domain containing the consensus site for efficient glycosylation to occur. For some proteins, this is the end of the pathway. For others, the fucose can be elongated on the 3-position by either a Glc or a GlcNAc. These reactions are catalyzed by as yet undescribed enzymes: an O-linked fucose β1,3-glucosyltransferase or an O-linked fucose β1,3-N-acetylgalactosaminyltransferase, respectively (Fig. 6, see B and C). Other yet to be identified forms of elongation are also possible (indicated by the question marks). For instance, an unidentified, neutral, O-linked fucose-containing structure (“TS” for triasaccharide) was reported by Morton and Steiner (20) and may represent an elongated form of the disaccharide or a novel structure. The oligosaccharide we observed here may also result from elongation of Glcβ1,3Fuc, or it may be related to the tetrasaccharide. For the GlcNAcβ1,3Fuc-modified proteins the pathway continues with the addition of galactosyl and sialic acid (presumably by standard Golgi galactosyl- and sialyltransferases) to form the tetrasaccharide structure represented on factor IX (7). The entire pathway most likely occurs in the Golgi apparatus since there are apparently no GDP-fucose transporters in the endoplasmic reticulum (25). The O-linked fucose pathway appears to be widespread in biology. Proteins bearing O-linked fucose have been reported from insects to humans (1, 7, 26).

Although a specific function has not yet been assigned to any of the O-linked fucose saccharides, several intriguing observations have been made in the past few years. First, two interesting studies have shown that O-linked fucose on the EGF domain of urokinase is necessary for a mitogenic/growth factor-like activity of the domain (27, 28). Growth factor domains lacking the fucose could bind to urokinase receptors but could not induce mitogenesis. These results suggest that the fucose is necessary for transmission of the mitogenic signal into the cell. Hajjar and Reynolds (29) have suggested that O-linked fucose within the EGF domain of tissue plasminogen activator plays a role in the serum half-life of the protein, although other workers have suggested that this is not the case (30). Also, the list of proteins that are predicted to be modified contains several cell surface receptors, which are involved in cell signaling and developmental regulation (7). Interestingly, some of these proteins have multiple O-linked fucose consensus sites (e.g. Notch-12 sites (31), Delta-6 sites (32), Jagged-1 sites (33)) and are known to interact with each other through their EGF domains (34). Modification of these domains with O-linked fucose saccharides may provide a means of modulating these interactions. Ultimately, the functional importance of O-linked fucose and its variations will become clearer as we learn more about this unusual form of glycosylation.

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