O-Glucosylation and O-Fucosylation Occur Together in Close Proximity on the First Epidermal Growth Factor Repeat of AMACO (VWA2 Protein)*§

Jan M. Gebauer1, Stefan Müller3, Franz-Georg Hanisch1,5, Mats Paulsson1,5, and Raimund Wagener1,2

From the 1Center for Biochemistry and 2Center for Molecular Medicine, Medical Faculty, University of Cologne, Joseph-Stelzmann-Str. 52, D-50931 Cologne, Germany

AMACO (VWA2 protein) is an extracellular matrix protein of unknown function associated with certain basement membranes in skin, lung, and kidney. AMACO is a member of the von Willebrand factor A-like (VWA) domain containing protein superfamily and in addition to three VWA domains it also contains two epidermal growth factor-like domains. One of these contains the rare, overlapping consensus sequences for both O-glucosylation and O-fucosylation. In earlier studies of other proteins the attachment of either core glucose and fucose moieties or of the respective elongated glycans starting with these monosaccharides has been described. By a detailed mass spectrometric analysis we show that both elongated O-glucosylated (Xyl1–3Xyl1–3Glc) and elongated O-fucosylated glycan chains (NeuAc2–3Gal1–4GlcNAc1–3Fuc) can be attached to AMACO in close proximity on the same epidermal growth factor-like domain. It has been reported that the lack of O-fucosylation can markedly decrease secretion of proteins. However, the secretion of AMACO is not significantly affected when the glycosylation sites are mutated. The number of extracellular matrix proteins carrying the overlapping consensus sequence is very limited and it could be that these modifications have a new, yet unknown function.

This work was supported by Deutsche Forschungsgemeinschaft Grants WA1338/2-3, WA1338/2-4, and WA1338/2-6 and the PRO INNO II Program of the German Federal Ministry of Economics and Technology Grant KFO055203UL6. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†1 Member of the International Graduate School in Genetics and Functional Genomics at the University of Cologne.

‡1 To whom correspondence should be addressed. Tel.: 49-221-478-6990; Fax: 49-221-478-6977; E-mail: raimund.wagener@uni-koeln.de.

§1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

The abbreviations used are: VWA, von Willebrand factor A; EGF, epidermal growth factor; Q-TOF, quadrupole time of flight; MALDI, matrix-assisted laser desorption ionization; ESI, electrospray ionization; TSP-1, thrombospondin type 1; GC-MS, gas chromatography-mass spectrometry; LC, liquid chromatography; NeuAc, N-acetylleucosaminic acid; Hex, hexose; HexNAc, N-acetylgalactosamine; Xyl, xylose; Fuc, fucose; PNGase F, peptide N-glycosidase F.

© 2008 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

JUNE 27, 2008

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 26, pp. 17846 –17854, June 27, 2008

© 2008 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

17846

JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 283 • NUMBER 26 • JUNE 27, 2008

17846
and third conserved cysteines of the EGF-like repeat (13). Both modifications can be located in close proximity to each other on a single EGF domain (4). However, the presence of both glycan types on a single EGF domain has been shown only for factor VII and δ-like protein 1 and here the substitution was with monomeric glucose and fucose (14, 15). AMACO contains an overlapping consensus sequence for both modifications on its first EGF domain. We show for the first time that both fully elongated O-glucosylated and O-fucosylated glycan chains can occur on the same EGF domain and also that extracellular matrix proteins can be so modified.

MATERIALS AND METHODS

Expression and Purification of Recombinant AMACO Fragments—AMACO fragments (P1–P3) were generated by PCR on full-length cDNA clones with the following primers: P1 forward, 5’-GCT AGC CCC GAC CAT CTC TCT TCA G-3’; P1 reverse, 5’-GGA TCC GTC TGG ATC AGT GGT G-3’; P2 forward, 5’-GCT AGC CAC CAC TGC TGA TCC AGA C-3’; P2 reverse, 5’-GGA TCC TGG CTG GTC GAA TAG CCT C-3’; P3 forward, 5’-GCT AGC CCA GCC ACG GCC AGG CTG-3’; and P3 reverse, 5’-GGA TCC CTT GGC GGA GGA CAG GGC-3’. Full-length AMACO was generated using the primers P1 forward and P3 reverse. All forward primers introduced a 5’ terminal Nhel and the reverse primers introduced a 3’ terminal BamHI restriction site. The amplified PCR products for the AMACO fragments were cloned in a modified pCPE-Pu vector (16), containing an N-terminal BM-40 signal peptide and a C-terminal His6 tag. The amplified PCR products for the full-length AMACO were cloned in a modified pCPE-Pu vector (17), containing an N-terminal BM-40 signal peptide and a C-terminal 2 × Strep tag. The recombinant plasmids were transfected into human embryonic kidney 293/Epstein-Barr virus nuclear antigen cells (Invitrogen) using FuGENE 6 (Roche). Transfected cells were selected with 1 μg/ml puromycin and grown to confluence. Secretion of recombinant proteins into the cell culture medium was confirmed by SDS-PAGE followed by immunoblotting with specific antisera directed against full-length AMACO and by peptide mass fingerprinting. AMACO fragments were purified from serum-free cell culture supernatants using TALON metal affinity columns (Clontech) following the supplier’s protocol.

Site-directed Mutagenesis—Mutagenesis was performed using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Single mutants were generated by exchanging the respective acceptor sites for alanine (S300A:Δglc, T308A:Δfuc) using the following primers: Δgltc forward, 5’-CCC CTG TGA CGC CCA GCC CTC CGC CC-3’; Δgltc reverse, 5’-GGG AGC GGT CCA CCC CTG TGT AAG GAG GG-3’; Δfuctc forward, 5’-CTG CCA AAA TGG AGG CGG CGC ATG CAT TCC AGA AGG TG-3’; Δfuctc reverse, 5’-CAC CTT CTA GAA TGC ATG CGC CTC CAT TTT GGC AG-3’. The double mutant S300A, T308A (Δgltc,Δfuc) was generated by sequentially using the same primers as for the single mutants. Mutant constructs were initially screened for the newly generated restriction site (marked in italic in primer sequence: Δgltc BsaHI, Δfuc NsiI) and confirmed by DNA sequencing. Full-length mutant constructs were generated by digesting AMACO-P2 constructs with SacI and Agel and ligating to full-length constructs.

Peptide N-Glycosidase F Release of N-Linked Oligosaccharides and Glycan Detection—N-Glycans were released by enzymatic cleavage with peptide N-glycosidase F (PNGase F, Roche Molecular Biochemicals). The protein (1.5 μg) was denatured in 15 μl of 1% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, for 15 min at 95 °C. After dilution digestion was performed in 0.1% SDS, 0.5% Nonident P-40 in Tris-buffered saline at 37 °C for 16–18 h using 1 unit of PNGase F.

The proteins were precipitated using 5% trichloroacetic acid and 0.1% Triton X-100 at 4 °C, washed with cold acetone, and subjected to SDS-polyacrylamide gel electrophoresis. Glycosylated proteins were detected on nitrocellulose membranes with the DIG Glycan Detection Kit (Roche) following the manufacturer’s protocol.

LC-MS/MS Analysis of Tryptic Peptides—Proteins were precipitated with 3 volumes of ice-cold acetone for 1 h at −20 °C, washed once with prechilled acetone, and left to dry at room temperature. Dried pellets were resuspended in 8 mM urea, 50 mM Tris-HCl, pH 8.0, 10 mM dithiothreitol and proteins were denatured and reduced by incubation at 60 °C for 45 min. To alkylate reduced cysteine residues, iodoacetamide was added to a final concentration of 25 mM and the samples kept for 30 min in the dark. The samples were diluted 1:4 with 50 mM Tris-HCl, pH 8.0, trypsin (sequencing grade, Promega) added to a final concentration of 12.5 ng/μl, followed by incubation at 37 °C overnight. The digestion was stopped by the addition of 0.1 volume of 1% trifluoroacetic acid.

Liquid chromatography (LC)-MS data were acquired on a Q-TOF II quadrupole-TOF mass spectrometer (Micromass) equipped with a Z spray source. Samples were introduced by an Ultimate Nano-LC system (LC Packings) equipped with the Famos autosampler and the Switchos column switching module. The column setup comprised a 0.3 × 5-mm trapping column and a 0.075 × 150-mm analytical column, both packed with 3 μm Atlantis dC18 (Waters). Samples were diluted 1:10 in 0.1% trifluoroacetic acid. A total of 10 μl was injected onto the trapping column and desalted for 1 min with 0.1% trifluoroacetic acid and a flow rate of 30 μl/min. The 10 port valve switched the trap column into the analytical flow path, and the peptides were eluted onto the analytical column by using a gradient of 5% acetonitrile in 0.1% trifluoroacetic acid to 40% acetonitrile in 0.1% trifluoroacetic acid over 35 min and column flow rate of approximately 200 nl/min, resulting from a 1:1000 split of the 200 μl/min flow delivered by the pump. The electrospray ionization (ESI) interface comprised a 20-μm inner diameter × 90-μm outer diameter tapered spray emitter (Carbotec) linked to the high performance liquid chromatograph flow path using a 7-μl dead volume stainless mounted onto the PicoTip holder assembly (New Objective). Stable nanospray was established by the application of 1.7 to 2.4 kV to the stainless steel union. The data-dependent acquisition of MS and tandem MS (MS/MS) spectra was controlled by the Masslynx software. Survey scans of 1 s covered the range from m/z 400 to 1,400. Doubly and triply charged ions rising above the threshold of 15 counts per second were selected for MS/MS experiments. In MS/MS mode the mass range from m/z 40 to 1,400 was scanned in 1 s, and 5
scans were added up for each experiment. Micromass-formated peaklists were generated from the raw data by using the Proteinlynx software module.

Reductive \( \beta \)-Elimination and Permethylation of Glycan Alditols—For structural studies the glycans were liberated by reductive \( \beta \)-elimination according to a protocol applicable to microscale samples (18). The O-glycoprotein (10–30 \( \mu \)g) was dried in a 0.5-ml Eppendorf vial and treated with freshly prepared 0.5 \( \text{M} \) NaBH\(_4\) in 50 \( \text{mM} \) NaOH (20 \( \mu \)l) overnight at 50 °C. After destruction of excess borohydride with glacial acetic acid the sample was desalted with a 50-\( \mu \)l aliquot of Dowex 50W-X8(H\(^+\)). To remove boric acid 100-\( \mu \)l aliquots of 1% acetic acid in methanol were added to the dry sample (\( \times 5 \)) and evaporated under nitrogen at 40 °C.

To the dry sample 50 \( \mu \)l of dispersed NaOH in dimethyl sulfoxide was added under argon and incubated for 30 min at room temperature with occasional shaking. Finally, a 25-\( \mu \)l aliquot of methyl iodide was pipetted to the frozen reaction mixture followed by incubation for a further 30 min at room temperature. After neutralization with dilute acetic acid the methylated glycans were extracted with chloroform/water. The chloroform phase was dried under nitrogen and glycans were solubilized in methanol.

Analysis of O-Glycans by Mass Spectrometry—Matrix-assisted laser desorption ionization (MALDI) mass spectrometry was performed on a Bruker Reflex IV instrument (Bruker Daltonics, Bremen, Germany). The methylated glycan samples (approximately 500 pmol/\( \mu \)l) contained in methanol were applied to the stainless steel target by mixing a 0.5-\( \mu \)l aliquot with 1.0 \( \mu \)l of matrix (saturated solution of 2,5-dihydroxybenzoic acid, 0.1% trifluoroacetic acid, 1:2). Analyses were performed by positive ion detection in the reflectron mode as described previously (19).

ESI mass spectrometry data were acquired on a Q-TOF II quadrupole-time of flight mass spectrometer (Waters, Eschborn, Germany) equipped with a Z spray source. ESI (Q-TOF) mass spectrometry was performed in the positive ion mode using previously described conditions (19). Collision energies varied in accordance with the type of molecular ion (\( M + Na \), 50–75 V; \( M + H \), 15–30 V).

Linkage Analysis of Permethylated Oligosaccharides by GC-MS—Partially methylated alditol acetates were prepared by hydrolysis of permethylated glycans with 2 \( \text{m} \) trifluoroacetic acid (Fluka) for 2 h at 121 °C followed by reduction with 10 mg/ml of sodium borodeuteride (Sigma) in 2 \( \text{m} \) aqueous ammonium hydroxide at room temperature for 2 h, and acetylation with acetic anhydride (Fluka) at 100 °C for 1 h (20). The partially methylated alditol acetates were extracted with chloroform/water, dried, and analyzed as a dichloromethane solution by GC-MS on a Fison MD800 (Thermo Electron, Dreieich, Germany) using a 15-m RTX5-SILMS column from Restek (Bad Homburg, Germany) and a temperature gradient from 60 to 100 °C (40 °C/min) followed by 100–280 °C (10 °C/min).

Analysis of AMACO Secretion—293 cells were grown in Dulbecco’s modified Eagle’s medium/F-12 (1:1) medium containing 10% (v/v) fetal calf serum, 2 \( \text{mM} \) glutamine, and 100 units/ml penicillin/streptomycin to about 60% confluence. The medium was changed shortly before transfection. 1 \( \mu \)g of full-length AMACO or AMACO-P2 constructs in the pCEP-Pu vector (17)(16) were transfected with FuGENE 6 using the manufacturer’s protocol. Shortly, 3 \( \mu \)l of FuGENE 6 was mixed with 97 \( \mu \)l of medium, incubated for 5 min, and added dropwise to 1 \( \mu \)g of DNA. After 15 min of incubation, the solution was slowly added to a single well of a 6-well plate (growth area 9.6 \( \text{cm}^2 \)). The supernatant was harvested after 3 to 4 days. The cells were washed in phosphate-buffered saline, solubilized by scraping in SDS-PAGE sample buffer, and homogenized by sonication. Supernatant and cell layer fractions were subjected to SDS-PAGE at a ratio of 3:1 (for AMACO fragments) or 3:2.5 (for full-length AMACO), to achieve comparable signal intensities. The proteins were transferred to nitrocellulose, and the blot was incubated with a specific antibody directed against AMACO-P2 followed by an antibody against rabbit.
immunoglobulins conjugated with Alexa Fluor 680 (Invitrogen). The signals were detected with an Odyssey scanner (Li-Cor).

RESULTS

AMACO is N- and O-Glycosylated—AMACO has a tendency to form very large protein aggregates (1) and as a consequence the pure monomer cannot be obtained in large amounts. Therefore, murine AMACO was recombinantly expressed in three parts. AMACO-P1 comprises the first VWA domain, AMACO-P2 the cysteine-rich, the first EGF and the second VWA domain, and AMACO-P3 the third VWA, the second EGF, and the unique C-terminal domain (Fig. 1A). cDNAs encoding the respective sequences of mouse AMACO were cloned into the pCEP-Pu vector utilizing the BM40 secretion signal sequence and a C-terminal His8 tag (16). The recombinant plasmids were introduced into human embryonic kidney 293 EBNA cells and maintained in an episomal form. The recombinant AMACO proteins were secreted into the cell culture medium and subsequently purified by affinity chromatography on a Talon column. The purified proteins appeared in nonreducing SDS-PAGE as single bands that have moderately higher apparent molecular masses than the predicted 24.6, 33.6, and 30.9 kDa (Fig. 1B), indicating glycosylation of all AMACO fragments. Glycan staining of the fragments immobilized on nitrocellulose after SDS-PAGE confirmed that all fragments are indeed glycosylated. PNGase F digestion caused a shift in the apparent molecular mass of AMACO-P1 of ~3–5 kDa. A similar shift was not observed for AMACO-P2 or AMACO-P3 (Fig. 1B), indicating that only the N-terminal fragment P1 is N-glycosylated. After PNGase F digestion, AMACO-P1 was still positive in the glycan detection reaction, indicating additional O-glycosylation on AMACO-P1. As murine AMACO contains two predicted N-glycosylation sites, in AMACO-P1 at Asn146 and in AMACO-P3 at Asn730, only the one at Asn146 but not the one at Asn730 seems to be used in 293 EBNA cells.

To further support this finding, we analyzed proteolytic fragments of AMACO-P3 by LC-MS/MS analysis. We could identify the unglycosylated proteolytic fragment containing the predicted N-glycosylation site Asn 730 (results not shown), indicating that this site is either not or only rarely used in 293 EBNA cells. Thus, AMACO-P3 and AMACO-P2 are exclusively O-glycosylated.

AMACO Contains Rare Consensus Sequences for O-Glycosylation—A bioinformatics search for potential O-glycosylation sites in AMACO led to the detection of two overlapping, highly conserved consensus sequences (13). These were C1XXC2 for O-glucosylation and C2XXGG(S/T)C3 for O-fucosylation, where C1, C2, and C3 are the first, second, and third conserved cysteines of the EGF1 domain of fragment AMACO-P2 (Fig. 2). In AMACO, the consensus sequences are strongly conserved between species. Only one amino acid in the O-fucosylation site of the green blowfish (Tetraodon nigroviridis) is altered, resulting in the sequence C2XXSG(S/T)C3 (Fig. 2).

The Consensus Sequences on EGF1 Are Glycosylated—As O-fucosylation has been shown to be of functional importance, at least in Notch (21), we studied the glycosylation of the AMACO-P2 fragment in detail using LC ESI-MS and MS/MS. After digestion with trypsin the resulting peptides were separated by liquid chromatography on a C18 column and the fractions directly analyzed by ESI-MS.
**Glycosylation of AMACO**

deconvoluted spectrum of one fraction showed three major peaks at 2775.5, 2921.0, and 3083.1 m/z (Fig. 3). MS/MS analysis (results not shown) revealed that all three peaks result from a single tryptic peptide /H9004/292/H9004/1CPGPCSQPCQNGGTCIPEGVDR/H11004/, bearing different post-translational modifications. This peptide originates from the region of the EGF domain that contains the potential O-glucosylation and O-fucosylation acceptor sites (in bold). The calculated mass of the carbamidomethylated, non-glycosylated tryptic peptide is 2775.5 m/z. The glycan analysis revealed that for O-glucosylated and O-fucosylated EGF domains could account for the higher mass peaks of the spectrum. The 2921.0 m/z peak (+146 m/z) could represent the peptide carrying a deoxyhexose, probably fucose, and the 3083.1 m/z peak (+162 m/z) the peptide carrying a hexose, probably glucose. Other, higher mass peaks have additional mass increments of 2 × 132, 162, 203, and 291 m/z indicating the presence of additional pentoses (xylose), hexoses (galactose), HexNAc (N-acetylgalactosamine), and NeuAc, respectively. All mass increments are consistent with the monosaccharides known to occur on EGF domains (13) and are indicated in the spectra (Fig. 3). Interestingly, forms that contain only glucosylation could not be detected (Fig. 3).

To further confirm the assumed glycosylation pattern AMACO-P2 mutants were generated, in which the glucose acceptor site Ser300 (Δglc), the fucose acceptor site Thr308 (Δfuc) or both acceptor sites (Δglc, Δfuc) were mutated to alanine residues (Fig. 4). All mutations led to the expected loss of the characteristic peaks for the respective glycosylated forms. LC-ESI-MS spectra of the double mutant (Δglc, Δfuc) showed only one peak at 2728.8 m/z (Fig. 4), which corresponds to the carbamidomethylated, non-glycosylated peptide. The mutant lacking the O-fucose acceptor site (Δfuc) displayed a major peak at 2745.7 m/z, matching the mass of the carbamidomethylated, non-glycosylated peptide. Two additional prominent peaks at 2907.9 and 3171.0 m/z (Fig. 4) are likely to represent the glucosylated and the fully xylosylated forms, whereas mass increments indicating fucosylation were not detected. The spectrum of the mutant lacking the O-glucose acceptor site (Δglc) showed the peak of the carbamidomethylated, non-glycosylated peptide at 2759.0 m/z and a minor peak at 2905.0 m/z, which is likely to be the fucosylated form. The peak at 3270.0 m/z corresponds to the mass of the peptide bearing fucose, N-acetylgalactosamine, and galactose. Peaks, which would indicate glucosylation were not observed (Fig. 4).

**AMACO-P2 Is O-Fucosylated and O-Glucosylated—Attempts were made to determine the structure of the sugar chains on AMACO-P2. As the mutant AMACO-P2 fragments each lack a specific glycan chain, we analyzed the differences in the pattern of the cleaved-off glycans. Reductive β-elimination was performed on all AMACO-P2 proteins and the released glycans were methylated using methyliodide. The permethylated glycans were subsequently analyzed by MALDI-MS (Fig. 5, supplemental Fig. S1, and Table 1). All constructs revealed a similar set of molecular ions in addition to those derived from the specific O-fucosylation and O-glucosylation indicating further mucin-type O-glycosylation. This fits with the observation that the double mutant Δglc,Δfuc was still positive in the glycan detection reaction (Fig. 1B). However, a unique 1069 m/z molecular ion, corresponding to NeuAc, dHex, Hex, and HexNAc, was detected only in wild type and Δglc. This would be consistent with the expected fucose containing glycan NeuAc-O-Gal-O-GlcNAc-O-Fuc and the structure was therefore further analyzed. ESI-MS/MS fragmentation
(Fig. 6A) revealed the sequence NeuAc-O-Hex-O-HexNAc-O-deoxyhexitol by a series of characteristic ions. In particular, the B₃ ion indicates the absence of dHex in the non-reducing trisaccharide and the Y₁ and Y₃ ions unequivocally localize the deoxyhexitol to the reducing terminus of the oligosaccharide (Fig. 6A). A characteristic molecular ion representing the glucose-containing oligosaccharides from Δfuc was found at 609 m/z. Further analysis by ESI-MS/MS fragmentation (Fig. 6B) revealed the trisaccharide structure Pen-O-Pen-O-hexitol, which would be consistent with the published glucose-based structure Xyl-O-Xyl-O-Glc. The Y₁,Y₂ and C₁,C₂ ions confirm the suggested structure.

To further analyze the linkage patterns of the sugars, the methylated glycans of both Δfuc and Δglc were hydrolyzed, reduced, and acetylated and analyzed by GC-MS (Table 2). Due to the bulk of sugars derived from the mucin-type O-glycans the differences detected between the mutants were small. Terminal and 3-linked xylose were detected only in the Δfuc mutant, indicating the presence of a glycan containing xylose only on Δfuc, consistent with the proposed glycan structure. Sugars specific for the Δglc mutant could not be identified.

Neither O-Glucosylation nor O-Fucosylation Affect Secretion—Recently, a role of the O-fucosylation in protein quality control was proposed (9). To analyze the possible function of O-fucosylation and O-glucosylation in AMACO secretion, equal amounts of full-length AMACO or AMACO-P2 constructs were transfected to 293 EBNA cells, and analyzed for differences in the ratio between protein levels in the supernatant and cell layer. Neither the lack of O-glucosylation, nor O-fucosylation, or the lack of both gave a significant difference in the rate of secretion (Fig. 7), indicating that both glycans do not influence the secretion of AMACO.

DISCUSSION

In this study we analyzed the glycosylation pattern of AMACO and show for the first time that a single EGF-like module can carry both an O-glucose-based glycan and an O-fucose-based glycan in their fully elongated forms. Furthermore, AMACO is the first extracellular matrix protein shown to be modified by this rare kind of glycosylation.

A survey of AMACO glycosylation revealed the presence of both N- and O-glycan chains. Murine AMACO contains a single potential N-glycosylation site located in the first VWA domain. Interestingly, this site is conserved between human, mouse, and chicken, indicating a potential functional relevance. Mucin-type O-glycosylation is present on each of the three AMACO fragments, but was not further characterized.

The first EGF-like domain carries overlapping consensus sequences for O-glucosylation and O-fucosylation (Fig. 2). The sequence of the O-glucosylation site C₁DSQPC₂ of AMACO, where C₁ and C₂ are the first and second cysteines of an EGF-like domain, is completely conserved in evolution, indicating a functional role. The O-fucosylation site is identical in human, mouse, chicken, and zebrafish (C₂QNGGTC₃), but differs

**Glycosylation of AMACO**

**TABLE 1**

MALDI-MS of methylated glycan aldithols derived from AMACO-P2 constructs

Parentheses indicate weak signals; species specific for certain protein forms are in bold face.

| M+Na | Proposed composition of glycan alditol | Detected in AMACO constructs | WT | Δglc | Δfuc | Δglc/fuc |
|------|--------------------------------------|-----------------------------|----|------|------|----------|
| 609  | Pen₃ Hex                             |                             | + | -    | +    | -        |
| 779  | Hex HexNAc₂                          | (+)                         | (+)|      |      |          |
| 895  | NeuAc Hex HexNAc                     | +                           | + |      |      |          |
| 983  | Hex₂ HexNAc₂                         | +                           | - |      |      | (+)      |
| 1024 | Hex HexNAc                          | +                           | + |      |      | (+)      |
| 1069 | NeuAc dHex Hex HexNAc                | +                           | - |      |      |          |
| 1140 | NeuAc Hex HexNAc₂                    | +                           | (+)|      |      |          |
| 1198 | dHex Hex HexNAc₂                     | +                           | + |      |      |          |
| 1256 | NeuAc₃ Hex HexNAc                    | +                           | + |      |      | (+)      |
| 1344 | NeuAc Hex HexNAc                     | +                           | + |      |      |          |
| 1385 | NeuAc Hex HexNAc₂                    | +                           | + |      |      |          |
| 1559 | NeuAc dHex Hex HexNAc                | +                           | + |      |      |          |
| 1705 | NeuAc₂ Hex HexHexNAc                | +                           | + |      |      |          |
slightly in *Xenopus laevis* and *T. nigroviridis*. The glutamine residue at position 2 is exchanged to a lysine or leucine residue, respectively. Only in *T. nigroviridis* is the well conserved glycine residue at position 4 exchanged to a serine residue. However, studies of murine Notch 1 have shown that the consensus could be more vague, i.e. C$_2$XXG(S/T)C$_3$ (22) instead of the originally proposed C$_2$XXG(S/T)C$_3$ (13).

The list of proteins with experimentally confirmed O-fucosylation is short and this kind of modification was so far only found on EGF-like domains or thrombospondin type 1 (TSP-1) repeats (6). Recently, it was shown that two distinct O-fucosylation pathways exist, specific either for EGF-like domains or TSP-1 repeats (23, 24). In contrast to the modification on TSP-1 repeats, which can only be elongated by a glucose moiety, the fucose moiety on EGF-like domains can be elongated to a tetrasaccharide with N-acetylglucosamine, galactose, and N-acetylneuraminic acid.

N-acetylglucosamine, the second sugar moiety, is added by Fringes$^{1}$ and the elongated sugar chain modulates the affinity of Notch to its ligand (25–27). It has been proposed that the activities of the Fringe enzymes are dependent on two amino acid residues in the EGF repeats, which are located in the vicinity of the consensus sequence (8). In AMACO, only the proline residue adjacent to the fifth cysteine residue is conserved (Fig. 2).

The list of proteins with experimentally demonstrated O-glucosylation sites is even shorter. To date only six proteins are shown to carry this modification: Notch (28), factor VII and factor IX (13, 29), protein Z (30), thrombospondin-1 (31), and δ-like protein 1 (15). All O-glucosylations are reported to occur on EGF repeats. Interestingly, only thrombospondin-1 does not contain the neighboring consensus sequence for O-fucosylation, indicating the presence of an isolated O-glucosylation on this protein.

Although most EGF-like domains that carry an O-glucosylation consensus sequence also contain an O-fucosylation

---

**TABLE 2**

Methylation analysis by GC-MS

| PMAA$^{a}$ | RT$^{b}$ | Δglc | Δfuc |
|------------|---------|-------|-------|
| Xyl-term   | 8.8     | -     | +     |
| 3-Xyl      | 9.8     | -     | +     |
| Fuc-term   | 9.2     | +     | -     |
| Gal-term   | 10.7    | +     | -     |
| 3-Gal      | 11.7    | +     | -     |
| 6-Gal      | 11.9    | -     | -     |
| 3,6-GalNAc-ol | 14.0 | +     | -     |
| 4-GlcNAc   | 14.4    | +     | -     |
| 3,4-GlcNAc | 15.1    | +     | -     |

$^{a}$-term, terminal sugar residue; the numbers indicate the site of linkage. 
$^{b}$Retention time.
Recently it was suggested that O-fucosylation may play a general role in quality control of O-fucosylated proteins (9). It is known that O-fucosylation is dependent on the structural integrity of the EGF-like domain (36) and that it takes place in the ER compartment (37). Furthermore, lack of O-fucosylation leads to decreased cell surface expression of Notch (8). Analogous results were obtained recently for a similar modification, the O-fucosylation of certain TSP-1 repeats. The lack of O-fucosylation of certain TSP-1 repeats on ADAMTS-13 and punctin-1 severely affects secretion (9, 10). The authors suggested a general role of O-fucosylation in protein quality control. For AMACO we could not observe a significant influence of O-fucosylation on secretion. However, in contrast to Notch, ADAMTS-13, and punctin-1, AMACO does not contain repetitive EGF-like domains and, therefore, quality control of properly folded EGF-like domains might not be so critical.

Unfortunately, the function of AMACO is still unknown and accordingly the functional role of its glycans cannot yet be analyzed. AMACO is the first extracellular matrix protein shown to be modified with both O-glucose-based glycans and O-fucose-based glycans and it could be that these modifications have a new, yet unknown function on such proteins.

**REFERENCES**

1. Sengle, G., Kobbe, B., Morgelin, M., Paulsson, M., and Wagener, R. (2003) *J. Biol. Chem.* 278, 50240–50249
2. Xin, B., Platzer, P., Fink, S. P., Reese, L., Nosrati, A., Willson, J. K., Wilson, K., and Markowitz, S. (2005) *Oncogene* 24, 724–731
3. Eller, E., Vardi, P., Daly, M. J., Babu, S., Roberts, C., Yang, F., Eisenbarth, G. S., and Fain, P. R. (2004) *Ann. N. Y. Acad. Sci.* 1037, 145–149
4. Peter-Katalinic, J. (2005) *Methods Enzymol.* 405, 139–171
5. Shao, L., Luo, Y., Moloney, D. J., and Haltiwanger, R. (2002) *Glycobiology* 12, 763–770
6. Panin, V. M., Shao, L., Lei, L., Moloney, D. J., Irvine, K. D., and Haltiwanger, R. S. (2002) *J. Biol. Chem.* 277, 29945–29952
7. Haines, N., and Irvine, K. D. (2003) *Nat. Rev. Mol. Cell. Biol.* 4, 786–797
8. Rampal, R., Li, A. S., Moloney, D. J., Georgiou, S. A., Luther, K. B., Nita-Lazar, A., and Haltiwanger, R. S. (2005) *J. Biol. Chem.* 280, 42454–42463
9. Ricketts, L. M., Dlugosz, M., Luther, K. B., Haltiwanger, R. S., and Majerus, E. M. (2007) *J. Biol. Chem.* 282, 17014–17023
10. Wang, L. W., Dlugosz, M., Somerville, R. P., Raed, M., Haltiwanger, R. S., and Apte, S. S. (2007) *J. Biol. Chem.* 282, 17024–17031
11. Hofsteenge, J., Huwiler, K. G., Macek, B., Hess, D., Lawler, J., Mosher, D. F., and Peter-Katalinic, J. (2001) *J. Biol. Chem.* 276, 6485–6498
12. Gonzalez de Peredo, A., Klein, D., Macek, B., Hess, D., Peter-Katalinic, J., and Hofsteenge, J. (2002) *Mol. Cell. Proteomics* 1, 11–18
13. Harris, R. J., and Spellman, M. W. (1993) *Glycobiology* 3, 219–224
14. Bjorner, S., Foster, D. C., Thim, L., Wiberg, F. C., Christensen, M., Komiyama, Y., Pedersen, A. H., and Kvisel, W. (1991) *J. Biol. Chem.* 266, 11051–11057
15. Krogh, T. N., Bachmann, E., Teisner, B., Skjodt, K., and Hojrup, P. (1997) *Eur. J. Biochem.* 244, 334–342
16. Maertens, B., Hopkins, D., Franzke, C. W., Keene, D. R., Bruckner-Tuderman, L., Greenspan, D. S., and Koch, M. (2007) *J. Biol. Chem.* 282, 10647–10659
17. Gara, S. K., Grumati, P., Urciuolo, A., Bonaldo, P., Kobbe, B., Koch, M., Paulsson, M., and Wagener, R. (2008) *J. Biol. Chem.* 283, 10658–10670
18. Schulz, B. L., Packer, N. H., and Karlsson, N. G. (2002) *Anat. Chem.* 74, 6088–6097
19. Engelmann, K., Kinlough, C. L., Muller, S., Razawi, H., Baldus, S. E., Hughey, R. P., and Hanisch, F. G. (2005) *Glycobiology* 15, 1111–1124
20. Albersheim, P., Nevins, D. J., English, P. D., and Karr, A. (1967) *Carbohydrate Res.* 5, 340–345
21. Shi, S., and Stanley, P. (2003) *Proc. Natl. Acad. Sci. U. S. A.* 100, 5234–5239

**TABLE 3**

Proteins containing the consensus site CX(S/P)CX(4,5)(TS)C

| Receptor/ligands | CRB1 | CRB2 | Cubilin | Dlk1 | DI1 | DI4 | Dner | FAT1 | FAT2 | FAT4 | Heg1 | Jagged1 | Jagged2 | Notch1 | Notch2 | Notch3 | Notch4 | SLIT-1 | SLIT-2 | SLIT-3 |
|------------------|------|------|---------|------|-----|-----|------|------|------|------|------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Proteins         | Factor VII | Hepatocyte growth factor activator | Protein Z | Extracellular matrix proteins | Agrin | AMACO | Multimerin1 | Polydom (Svep1) | Sncd | Versican | Unknown function | RAMP |

sequence, only factor VII and δ-like protein 1 have been experimentally shown to carry both modifications on the same EGF-like domain (14, 15). Interestingly, in all cases only monosaccharide modifications could be detected, leaving the question open, whether the full-length modifications can occur on a single EGF-like domain.

We could observe a diverse pattern of differently glycosylated tryptic peptides in LC-ESI-MS/MS analysis; however, it remains unclear if this pattern is physiological. The heterogeneity could also be the consequence of underglycosylation of the overexpressed recombinant proteins. Nevertheless, our results show that both glycan types are independently elongated. Interestingly, peptides bearing only O-glucosylation could not be detected in the spectrum of wild type AMACO-P2 protein. This could indicate that O-fucosylation facilitates the addition of O-glucose-based glycans. However, the isolation of O-glycosylated peptides from the Δfuc mutant shows that both glycosylations can occur independently.

A database search using a combined consensus sequence for both glycosylations detects only a few proteins. These can be separated into three groups: plasma membrane receptors and their ligands, plasma proteins, and extracellular matrix proteins (Table 3). For some members of the first two groups a function is known for at least one of the modifications. O-Fucosylation is essential for Notch signaling and Notch-ligand interactions (21, 32–34) and is required for uPA-induced signaling (35) and the lack of O-glucosylation in factor VIIa was shown to reduce the coagulant activity to ~60% of the wild type activity (14).
Glycosylation of AMACO

22. Shao, L., Moloney, D. J., and Haltiwanger, R. (2003) *J. Biol. Chem.* **278**, 7775–7782
23. Luo, Y., Nita-Lazar, A., and Haltiwanger, R. S. (2006) *J. Biol. Chem.* **281**, 9385–9392
24. Luo, Y., Koles, K., Vorndam, W., Haltiwanger, R. S., and Panin, V. M. (2006) *J. Biol. Chem.* **281**, 9393–9399
25. Bruckner, K., Perez, L., Clausen, H., and Cohen, S. (2000) *Nature* **406**, 411–415
26. Moloney, D. J., Panin, V. M., Johnston, S. H., Chen, J., Shao, L., Wilson, R., Wang, Y., Stanley, P., Irvine, K. D., Haltiwanger, R. S., and Vogt, T. F. (2000) *Nature* **406**, 369–375
27. Xu, A., Haines, N., Dlugosz, M., Rana, N. A., Takeuchi, H., Haltiwanger, R. S., and Irvine, K. D. (2007) *J. Biol. Chem.* **282**, 35153–35162
28. Moloney, D. J., Shair, L. H., Lu, F. M., Xia, J., Locke, R., Matta, K. L., and Haltiwanger, R. S. (2000) *J. Biol. Chem.* **275**, 9604–9611
29. Hase, S., Kawabata, S., Nishimura, H., Takeya, H., Sueyoshi, T., Miyata, T., Iwanaga, S., Takao, T., Shimonishi, Y., and Ikenaka, T. (1988) *J. Biochem. (Tokyo)* **104**, 867–868
30. Nishimura, H., Kawabata, S., Kisiel, W., Hase, S., Ikenaka, T., Takao, T., Shimonishi, Y., and Iwanaga, S. (1989) *J. Biol. Chem.* **264**, 20320–20325
31. Nishimura, H., Yamashita, S., Zeng, Z., Walz, D. A., and Iwanaga, S. (1992) *J. Biochem. (Tokyo)* **111**, 460–464
32. Sasamura, T., Sasaki, N., Miyashita, F., Nakao, S., Ishikawa, H. O., Ito, M., Kitagawa, M., Harigaya, K., Spana, E., Bilder, D., Perrimon, N., and Matsuno, K. (2003) *Development* **130**, 4785–4795
33. Okajima, T., and Irvine, K. D. (2002) *Cell* **111**, 893–904
34. Okajima, T., Xu, A., and Irvine, K. D. (2003) *J. Biol. Chem.* **278**, 42340–42345
35. Rabbani, S. A., Mazar, A. P., Bernier, S. M., Haq, M., Bolivar, I., Henkin, J., and Goltzman, D. (1992) *J. Biol. Chem.* **267**, 14151–14156
36. Wang, Y., Lee, G. F., Kelley, R. F., and Spellman, M. W. (1996) *Glycobiology* **6**, 837–842
37. Luo, Y., and Haltiwanger, R. S. (2005) *J. Biol. Chem.* **280**, 11289–11294