A chemoenzymatic approach toward the identification of fucosylated glycoproteins and mapping of N-glycan sites

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Fucose (Fuc)-containing glycoconjugates play important roles in numerous physiological and pathological processes. Given the biological importance of post-translational glycosylation, a specific and robust strategy for the identification of fucosylated glycoproteins is highly desirable. In this study, we demonstrate an alternative way of labeling of fucosylated structures by metabolic engineering, using a chemoenzymatic approach. In this approach, the activities of *Bacteroides fragilis* 1-fucokinase/guanosine-5′-diphosphate-Fuc pyrophosphorylase and human α1,3-fucosyltransferase 9 are combined in a Namalwa cellular model. Interestingly, this system could be applied to labeling of alkyne-modified fucosylated glycoproteins. N-Glycan site mapping and identification were done using an in vitro selective chemical ligation reaction and isotope-coded glycosylation site-specific tagging, subsequent to liquid chromatography-tandem mass spectrometry analysis. This work illustrates the use of a click chemistry-based strategy combined with a glycoproteomic technique to get further insight into the pattern of Fuc-mediated biological processes and functions.

**Keywords:** alkynyl fucose / bio-orthogonal reaction / IGOT

**Introduction**

Fucosylated glycoproteins and glycolipids can be present in cell membranes or secreted into biological fluids. Various functions of fucose residues in biological processes have been established (Staudacher et al. 1999; Becker and Lowe 2003). The metabolic introduction of bio-orthogonal functional groups, such as azides and alkynes, into mammalian cell-surface oligosaccharides has become a powerful technique for studying glycosylation in native cellular environments; however, the fucose salvage pathway has also been demonstrated to accommodate azide and alkyne C-5 substituted analogs normally excluded by the de novo synthetic pathway (Prescher and Bertozzi 2006; Rabuka et al. 2006; Sawa et al. 2006; Hsu et al. 2007). This can be a hindrance, especially when trying to achieve specific labeling with high analog concentrations, which is necessary for avoiding cellular cytotoxicity and achieving enzymatic selectivity. Solving this practical problem leads to new challenges and opportunities. One of the issues faced by a practical-minded metabolic glycoengineer is finding the key enzymes that enhance the efficiency of the conversion of cellular unnatural sugars to nucleotide derivatives and subsequently the replacement of native glycans.

Previous studies have demonstrated the in vivo incorporation of fucose analogs into bacterial polysaccharides and an in vitro approach for the synthesis of guanosine-5′-diphosphate (GDP)-fucose, Lewis x (Le′x) structures [Galβ1-4(Fucα1-3)GlcNAc] (PubChem-CID: 4571095) and their derivative alkyne-bearing substituent at the fucose C-5 position, based on recombinant microbial 1-fucokinase/GDP-fucose pyrophosphorylase (FKP) and fucosyltransferase (Wang et al. 2009; Yi et al. 2009). Given this extensive toolkit of bio-orthogonal chemical probes, we devised an optimized chemoenzymatic labeling strategy for fucose-selective glycoprotein identification and N-glycan site mapping via isotope-coded glycosylation site-specific tagging, followed by liquid chromatography-mass spectrometry analysis (IGOT-LC-MS; Kaji et al. 2003). Toward this goal, the metabolic incorporation of alkynyl fucose was carried out in Namalwa cells, a human Burkitt lymphoma cell line that does not possess Leα, Leβ and sia1yl-Leα antigens (Isshiki et al. 1999), to potentially reduce alkynyl fucose consumption and to increase type 2 Lewis antigen labeling by facilitating better control over the activities of fucose metabolic enzymes (Figure 1). As a result, we investigated whether FKP would functionally accept a native fucose substrate, which would then allow GDP-fucose to be generated and subsequently used as a substrate for human α1,3-fucosyltransferase 9 (FUT9). FUT9 is the most likely enzyme that (i) generates distal Leα epitopes from many different acceptors, (ii) possesses inherent ability for the biosynthesis of internal Leα epitopes on growing poly lactosamine backbones and (iii) synthesizes the Leα epitopes in mammalian tissues (Kudo et al. 1998; Nishihara et al. 1999; Toivonen et al. 2002).

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**Results**

The effects of overexpression of FKP and FUT9 in the Namalwa cellular model

An increase in cellular fucosylation was observed, especially in the cytosolic subfraction (Figure 2A). Based on these results, we sought to determine the ability of FKP to convert alkynyl fucose to GDP-alkynyl fucose and that of FUT9 to transfer it to its corresponding acceptor substrates, thereby forming the alkyne-bearing fucosylated glycoconjugates in vivo. The bio-orthogonal probe incorporated can allow further elaboration by using click chemistry to introduce a biotin or fluorescence label for visualization by gel electrophoresis or cell image and, in principle, it should be possible to adapt this approach for the efficient labeling of fucosylated glycoconjugates (Figure 2B and C and Supplementary data, Figure S1). Our current cell biological data show that treating cells with alkynyl fucose does not afford optimal labeling of fucosylated glycoconjugates, probably because of a metabolic bottleneck at the native L-fucokinase/GDP-fucose pyrophosphorylase step of the fucose salvage pathway.

Having demonstrated the labeling of alkynyl fucose containing glycans, we next explored the N- or the O-glycan of the alkynyl fucose conjugating glycoproteins using the whole-cell lysate from cells stably overexpressing both FUT9 and FKP. When biotin-azide-tagged cell lysates were incubated with peptide N-glycosidase F (PNGase F) for up to 22 h, the streptavidin blot signals were found to gradually disappear, presumably reflecting the removal of N-glycans from their targets and confirming the ubiquitous occurrence of the fucosylated epitopes in a Namalwa cellular model (Figure 2D). Notably, it is important to consider that, during the introduction of chemically modified sugars, a change from a methyl group to a bulky biotin-tagged triazole ring might severely perturb the recognition of fucose lectin (Fujihashi et al. 2003; Wimmerova et al. 2003). Indeed, we found that the avidity of the Aleuria aurantia lectin (AAL) blot was significantly lower than that of the streptavidin blot whether in the total cellular lysates (Figure 3) or subcellular proteins (Figure 4). It is noted that the same phenomenon has also been reported in a previous study (Hsu et al. 2007).

N-Linked glycosylation site mapping and fucosylated glycoprotein identification by IGOT-LC-MS analysis

Next, to identify fucosylated glycoproteins and map N-glycan sites, Lys-C/tryptic peptides tagged with biotin were captured by streptavidin beads and then subjected to IGOT-LC-MS analysis similar to a previous strategy (Kaji et al. 2003). In all, from 2 mg of total cellular proteins, we identified 42 unique N-glycosylated peptides representing 36 fucosylated glycoproteins (36 gene symbols) and \( \approx 80\% \) (29/36) of those detected glycoproteins were also fucosylated before introducing FKP (Supplementary data, Table S1). The methods for
fucosylated glycoprotein preparation and validation are detailed in the Supplementary data. And not surprisingly, consistent with the known N-linked glycoprotein distribution (Zielinska et al. 2010), the majority of the N-glycan fucosylated proteins identified were membrane-bound transporters, receptors/ligands, adhesion molecules and components of subcellular locations rich in glycoproteins (endoplasmic reticulum/Golgi, lysosome and cytoplasmic vesicle; Figure 5A and Supplementary data, Table S1). As shown in Figure 5B and Supplementary data, Table S1, among the 45 potential N-linked glycosylation sites, 43 (96%, including one ambiguous assignment) fell into an 18O-assigned status. During the deamidation process, the asparagine (Asn) is converted into an aspartate with a stably incorporated 18O atom, which gives rise to a mass increase of 3 Da instead of 1 Da. This eliminates the possibility of false-positive protein identification arising from spontaneously occurring deamidation events. Although most of the sites have previously been considered as potential N-glycan sites, one of them had not been reported previously (Zielinska et al. 2010), which is why our 18O-labeling approach is necessary for the definitive identification of a protein as a fucosylated N-glycoprotein.

Discussion

Advances in our understanding of fucosylated glycans-dependent roles in the processes of tumorigenesis, neurite outgrowth and regeneration will provide new strategies for the treatment, diagnosis and understanding of malignant tumors and neural induction. Here, we have developed a chemoenzymatic labeling strategy for the characterization of fucosylated carriers in mammalian cells using alkynyl fucose that addresses these important needs by enabling better control over the activities of fucose metabolic enzymes.

The prokaryotic FKP gene expressed its function in eukaryotic cells. The key to the success of this chemoenzymatic approach lies in four points. (i) The FKP protein is located in
Fig. 3. AAL and streptavidin blots of alkynyl fucose-treated FUT9 and FKP overexpressing Namalwa cells. After 72 h of alkynyl fucose treatment, the total cellular lysates from FUT9 + FKP stable transfectants were labeled with biotinylated probe, enriched with streptavidin slurry beads and then incubated with AAL-HRP (upper-left panel) or streptavidin-HRP (upper-right panel). Input, aliquot of whole-cell lysate (1/50 of the input); FT, flow-through (1/50 of the fraction); Beads, streptavidin beads after incubation (1/50 of the beads); Eluent, elution fraction from streptavidin beads (1/50 of the elution fraction; elution buffer: 2 mM EDTA, pH 8.2 and 95% formamide). Each sample was stained with AAL-HRP, followed by chemiluminescent substrates; the same membrane was then stripped and rebotted with streptavidin-HRP. The signals of Input showed enhanced intensity in both blots when compared with those subjected to PNGase F treatment (Input”). The signals of Eluent showed significant intensity in the streptavidin blot when compared with the same fraction in the AAL blot. The lower-right panel shows silver staining of the corresponding samples.

Fig. 4. Comparison of cytosolic, membrane (A), nuclear and cytoskeletal (B) subcellular alkynyl fucosylated proteins from FUT9 and FKP overexpressing Namalwa cells by AAL and streptavidin blot analyses. Input, whole-cell lysate; FT, flow-through; Beads, streptavidin beads after incubation; Eluent, elution fraction from streptavidin beads (elution buffer: 2 mM EDTA, pH 8.2 and 95% formamide). Each fraction (~5 μg) was stained with AAL-HRP, followed by chemiluminescent substrates (A and B, upper-left panels); the same membrane was then stripped and rebotted with streptavidin-HRP (A and B, upper-right panels). In the Input of the cell membrane fraction, the protein bands showed enhanced intensity in both kinds of blots when compared with the other three fractions. Furthermore, the signals of the Eluent of membrane fraction also showed much stronger intensity in the streptavidin blot as compared with the PNGase F treatment (Eluent”) and the other three Eluent fractions, and no signals in the AAL blot, meaning that the alkynyl fucosylated proteins were mainly present on the membrane. Silver staining was also carried out on all of the samples (A and B, lower-right panels). The band corresponding to PNGase F is indicated by the arrowhead.
the cytoplasm and does not contain signal sequences for either location in the cell membrane or secretion. (ii) FKP is a highly functional, conserved enzyme of the fucose salvage pathway enzyme that metabolizes a common compound; thus, it would probably still be able to metabolize this compound in a eukaryotic cell. (iii) FKP is a protein that does not require other specific genes or molecules for its function. (iv) Because most glycosyltransferases can catalyze the hydrolysis of the corresponding nucleotide sugar donor substrate, continued addition of nucleotide sugars and in vivo generation of nucleotide sugars with special enzymes could be used as general strategies to improve the yields of glycosyltransferase-catalyzed reactions. GDP-fucose can be hydrolyzed by the overexpressed FUT9 enzyme, leading to low yields owing to consumption of the donor substrate by water. This can be overcome by the FKP enzyme producing an additional GDP-fucose or GDP-alkynyl fucose periodically during the reaction process. In situ generation of GDP-fucose from guanosine-5′-triphosphate and adenosine-5′-triphosphate using a bifunctional FKP enzyme helps to improve the yield. These concepts will be extremely useful when weak acceptors are used and water competes significantly for the same donor. Indeed, although previous results show that alkynyl fucose was the preferred fucose analog for metabolic labeling of mammalian-fucosylated glycoconjugates (Hsu et al. 2007), our approach may serve as an efficient labeling approach for fucosylated carriers in a mammalian system that had not been previously accessible.

Compared with previous studies using unnatural sugars (Hanson et al. 2007; Zaro et al. 2011), the fucose analog allowed us to identify fewer glycoproteins by MS analysis. The possible reasons for this phenomenon lie in three points. (i) The alkynyl fucose or GDP-alkynyl fucose can be tolerated by FKP or FUT9, but not as well as its native substrate, resulting in low metabolic conversion of the unnatural sugar and low efficiency of natural substrate replacement. (ii) The dominant de novo pathway, which provides up to more than 90% of the cellular GDP-fucose pool (Yurchenco and Atkinson 1977), had not been effectively diminished in the no-glucose culture medium at 72 h. (iii) These modifications are generally substoichiometric, because of the competition between natural and unnatural substrates in the biosynthetic pathway.

In summary, we anticipate that metabolic labeling of corresponding carriers of Lewis antigens using our chemoenzymatic method, coupled with subsequent analysis by IGOT-LC-MS, will provide a powerful strategy for analyzing Lewis antigen-dependent roles in various experimental contexts. Identification of these specific fucose linkage-type biomarkers and targets for therapeutic intervention is one of the key objectives of our strategy.

Materials and methods

Cell culture and electroporation

Namalwa cells were maintained in a 5% CO2, humidified atmosphere at 37°C in RPMI medium 1640 (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and antibiotics. The transfection of Namalwa cells stably expressing FUT9 has been described previously (Kaneko et al. 1999). FKP-transfected Namalwa cells or FUT9 stable transfectants were prepared by co-introducing Bacteroides fragilis 9343 full-length FKP cDNA (Liu et al. 2011; in pCMV-Tag2B-FLAG vector, Agilent Technologies, Palo Alto, CA) and a puromycin resistance gene carrier plasmid (pBABE-puro; the mole ratio of plasmids was pCMV-Tag2B-FLAG-FKP/pBABE-puro 40:1) using the Bio-Rad Gene Pluser II Electroporation System (the optimal setting is 0.3 kV, 250 μF), followed by puromycin selection (1–2 μg/mL), limited dilution and expansion. Stable expression of the FLAG-tagged target gene was confirmed by western blot analysis with antibody (Sigma, St. Louis, MO) against the FLAG peptide. Namalwa cells transfected with empty vector were also created by using the same methods.

Lectin blot analysis

Whole-cell extracts were prepared by resuspending ~1 × 10⁷ cells in 1 mL of lysis buffer [1% sodium dodecyl sulfate (SDS)/protease inhibitor cocktails (1/50, Sigma, St. Louis, MO)/250 U benzonase (Merck, Darmstadt, Germany)/10 mM NaF/0.1 mM Na₃VO₄/0.02% (w/v) NaN₃/50 mM Tris–HCl, pH 8.0]. The solution was mixed well via pipetting up and down several times and incubated on ice for 15 min. The suspensions were centrifuged at 17,800 × g at 4°C for 5 min in an angle centrifuge, and the clear supernatant fluid was harvested. Cell fractionation was performed below 4°C using a ProteoExtract Subcellular Proteome Extraction kit (Merck) according to the manufacturer’s instructions. Samples were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) under reducing conditions and then transferred onto a
polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA) for AAL blot analysis. After blocking with phosphate-buffered saline Tween-20 (PBST; 0.1% Tween-20) containing 3% bovine serum albumin (BSA; w/w) overnight at 4°C, the membrane was incubated in diluted horseradish peroxidase (HRP)-conjugated AAL (1:2000, made in our lab) for 30 min at room temperature (RT). It was then washed three times with PBST and developed with an enhanced chemiluminescence (ECL) system. For the internal control assay, the PVDF membrane was stripped by Restore PLUS western Blot Stripping Buffer (Thermo Scientific, Rockford, IL, USA). The blocking procedure (using 5% skim milk in PBST) was carried out again, followed by blotting with mouse monoclonal anti-β-actin antibody and HRP-conjugated rabbit anti-mouse IgG (GE Healthcare, Piscataway, NJ).

Culturing of Namalwa transfectant cells and metabolic labeling conditions

Stable Namalwa transfectants expressing FUT9 only were cultured in RPMI 1640 medium supplemented with 10% FBS and 0.6 mg/mL of neomycin (G418); stable Namalwa transfectants expressing FUT9 + FKP were cultured in the RPMI 1640 medium supplemented with 10% FBS, 0.6 mg/mL of G418 and 2 μg/mL of puromycin at 37°C in a humidified atmosphere of 5% CO₂. For labeling experiments, cells were seeded in T-75 flasks (BD Falcon, San Jose, CA) in the above medium and when ~60% confluency was obtained, the culture medium was replaced with the RPMI 1640 medium lacking glucose (Invitrogen) but supplemented with 10% FBS and either dimethyl sulfoxide (DMSO; 1:1000) or peracetylated alkyne-fucose (Click-iT fucose alkyne, Invitrogen) prepared at 35 mM in DMSO for stock and diluted to the indicated concentrations (1:1000, 35 μM). Cells were labeled for 72 h, at which time their density was ~1.0 × 10⁶ cells/mL.

Click reaction on labeled proteins and detection by streptavidin affinity blotting

After 72 h, whole-cell extracts were prepared by resuspending ~1.0 × 10⁶ cells in 1 mL of lysis buffer [1% SDS/protease inhibitor cocktails (1/50, Sigma)/250 U Benzonase (Merck); 10 mM NaF/0.1 mM Na₃VO₄/0.02% (w/v) NaN₃/50 mM Tris–HCl, pH 8.0]. The solution was mixed well via pipetting up and down several times and incubated on ice for 15 min. The suspensions were centrifuged at 17,800 × g at 4°C for 5 min in an angle centrifuge, and the clear supernatant fluids were harvested. Cell lysate was obtained by transferring the supernatant to another tube and pre-cleaning the supernatant with streptavidin beads (GE Healthcare) to remove non-specific proteins. These pre-cleaned glycoproteome samples (~1.5–2.0 mg/mL) were divided into 0.2 mL aliquots and treated sequentially with 100 μM biotin-azide (stock prepared in DMSO, Invitrogen), 250 μM Tris-triazoleamine ligand (TBTA, stock prepared in 1BuOH/DSMO 4:1, Sigma) and 100 μM CuSO₄ (stock prepared in ddH₂O), all diluted from ×50 stocks. The reactions were thoroughly mixed, equilibrated for 2–3 min and treated with 250 μM tris(2-carboxyethyl)phosphine reducing agent (freshly prepared in ddH₂O, Sigma) and then incubated for 2 h at RT with gentle vortex mixing. The proteins were then precipitated by using an MeOH/CHCl₃/H₂O method. The pelleted proteins were washed twice by adding 0.4 mL of MeOH and allowed to air dry. Negative controls were performed under identical conditions, except that peracetylated alkyne-fucose was left out of the cell culture. These dry samples were solubilized with an appropriate volume of 0.5% SDS in 50 mM Tris–HCl, pH 8.0, to obtain a ~1 mg/mL of protein solution. The samples were resolved on SDS–PAGE and transferred to PVDF (Millipore). After blocking with PBST (0.1% Tween-20) containing 5% BSA (w/w) overnight at 4°C, the membrane was incubated in diluted streptavidin-HRP (1:100,000, GE Healthcare) for 30 min at RT. It was then washed three times with PBST and developed with an ECL system.

Fluorescence microscopy

Stable transfectants expressing FKP only, FUT9 only and FUT9 + FKP were treated with 35 μM alkynyl fucose for 72 h. The cells were washed three times with ice-cold 1% BSA/PBS solution and resuspended in PBS solution to a final concentration of ~1.0 × 10⁶ cells/mL; cyt centrifuge slides were then prepared. The cells were centrifuged on the glass slide for 4 min at 400 × g and allowed to air dry for 5 min. The slides prepared from the treated cells were fixed with absolute acetone for 15 min at −20°C. After natural air-drying of the fixative, the slides were washed with PBS at RT for 5 min with two renewals of the washing fluid and then labeled at RT for 45 min in a moist dark box by using a Click-iT Cell Reaction Buffer Kit (Invitrogen) for the Alexa Fluor 488 azide probe (Invitrogen) according to the manufacturer’s instructions. Subsequently, the fixed and labeled cells were rinsed with PBS and stained with monoclonal rabbit anti-GM130 (Golgi marker, Epitomics, Inc, Burlingame, CA; 4 μg/mL in 1% BSA/PBST), followed by Alexa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen; 0.4 μg/mL in 1% BSA/PBST) and Hoechst 33342 (Invitrogen) (2 μg/mL in PBS). The slides were then rinsed once for 5 min in ddH₂O and covered with a few drops of Prolong Gold antifade reagent (Invitrogen) to reduce fading of the fluorescence and with a coverslip that was allowed to air dry. Fluorescent images were captured by a Keyence (BZ-8000) laser scanning confocal microscopy system.

Preparation and enrichment of alkyne-labeled glycopeptides for proteomics

The dry biotin-labeled glycopeptides (~2 mg total) were dissolved in a 7 M guanidine-HCl (Gu-HCl), 10 mM ethylene-diaminetetraacetic acid (EDTA), 0.5 M Tris–HCl, pH 8.6, solvent (50 μL). Ten microliters of dithiothreitol (100 mM, prepared fresh) was added to the solution and reduction proceeded by vortexing at 37°C for 1 h in the dark. Ten microliters of iodoacetamide (200 mM, prepared fresh) was then added to the solution and alkylation proceeded by vortexing at 37°C for another 1 h in the dark. The concentration of Gu-HCl in solution was diluted to ~1.6 M with 150 μL of freshly prepared 50 mM NH₄HCO₃ (pH 8.5) containing 20 μg MS grade Lys-C endoproteinase (Wako Corp., Tokyo, Japan; 1:100 w/w protein) and vortexed at 37°C for 5 h. A fresh pre-mixed trypsin solution (220 μL), consisting of 20-μg sequence grade modified trypsin (Wako Corp.; 1:100 w/w protein), and
20 mM CaCl$_2$ in 50 mM NH$_4$HCO$_3$ (pH 8.5), was added to the mixture, and the digestion was allowed to proceed overnight at 37°C. An aliquot of 0.5 M phenylmethylsulfonyl fluoride in EtOH was added to the mixture at a final concentration of 2 mM to stop the digestion. Biotin-labeled glycopeptides were enriched using streptavidin-Sepharose beads (GE Healthcare). Beads (50 µL) pre-equilibrated in PBS (1 mL, ×2) were treated with Lys-C/trypsin digested samples diluted to 0.5 M Gu-HCl (~700 µL) by rotating for 2 h at 4°C. The beads were washed with 1 M Gu-HCl in PBS (1 mL, ×3), 0.4 M NaCl (1 mL, ×2) and ddH$_2$O (1 mL, ×2). Centrifugation of the beads between steps was carried out by using a fixed angle rotor (5,000 × g, 2 min), and the beads were finally dried in a SpeedVac centrifuge.

Preparation and glycosylation site-specific stable isotope-labeling of streptavidin-captured glycopeptides carrying alkynyl-fucosylated N-glycans
The dried beads were treated with PNGase F (TakaraBio, Kyoto, Japan) [25 mU in 0.1 M Tris–HCl, pH 7.5, made of stable isotope-labeled water (H$_2$O$_{18}$O, 99 atom % 18O) (Taiyo Nippon Sanso Corp., Tokyo, Japan), 50 µL] at 37°C overnight. The beads were put into a spin column and centrifuged at 600 × g for 5 min at 4°C and the filtrate was recovered. The beads were resuspended with 50 µL of 0.1% formic acid, vortexed for 2 min and left to stand for 10 min. The filtrate was recovered in the same way and combined with the first filtrate.

LC/MS analysis of $^{18}$O-labeled peptides
The stable isotope-labeled peptides were analyzed on an automated nanoflow LC/MS system. The peptide mixture was acidified with 1% formic acid and injected onto a reverse-phase trap column (0.2 mm i.d. × 10 mL, MonoCap C18, GL Science, Tokyo, Japan) on the LC system (LC Assist, Shimadzu, Kyoto, Japan) at a flow rate of 15 µL/min. After washing with 0.1% formic acid for 15 min (15 µL/min), the trap column was connected to the nanoflow LC system via a switching valve, and the peptides were separated on a reverse-phase tip column (150 µm i.d. × 50 mm, Mightysil-C18, 3 µm particle; LC Assist) by a linear gradient of MeCN (0–35% in 0.1% formic acid) for 35 or 70 min at 4°C. The beads were switched with 0.1% formic acid for 15 min (15 µL/min), the trap column was connected to the nanoflow LC system via a switching valve, and the peptides were separated on a reverse-phase tip column (150 µm i.d. × 50 mm, Mightysil-C18, 3 µm particle; LC Assist) by a linear gradient of MeCN (0–35% in 0.1% formic acid) for 35 or 70 min at a flow rate of 100 nL/min. The eluted peptides were sprayed directly into a hybrid mass spectrometer (LTQ Orbitrap Velos, Thermo Scientific) set to positive-ion mode. The spectrometer was operated in a data-dependent MS/MS mode, where a full scan on the orbitrap analyzer (profile mode; resolution, 15,000; m/z range, 500–1500) was followed by 10 MS/MS scans on the ion-trap analyzer (centroid mode). The 10 most intensive precursor ions with a charge state (z) of 2+ or 3+ were dynamically selected and subjected to collision-induced dissociation (CID) with a collision energy recommended by the manufacturer (35 V) and a dynamic exclusion duration of 60 s.

Identification of glycopeptides by Mascot search
MS raw data were converted to a Mascot generic format file using the software Proteome Discoverer 1.1 (Thermo Scientific, San Jose, CA) and searched using the software Mascot (ver.2.3, Matrix Science, London, UK) against the NCBI refseq protein sequence database (27176 entries, downloaded on 25 February 2011). The search conditions were as follows: precursor ion mass tolerance, 15 ppm; tolerance of CID fragment ions detected in the linear ion trap, 0.8 Da; fixed modification, carbamidomethylation of cysteine (Cys); variable modifications including oxidation of methionine, deamination of peptide N-terminal glutamine and carbamidomethylated Cys; IGOT (deamidation and incorporation of stable isotope $^{18}$O) of Asn, maximum miss cleavage. Peptide identification with a lower expectation value than 0.05 and rank 1 was accepted. Among them, peptides having the consensus sequence of N-glycosylation—NX[STC], where X is any except proline, and IGOT (stable isotope tag on Asn)—were assigned as formerly glycosylated peptides.

**Supplementary data**
Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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**Conflict of interest**
None declared.

**Abbreviations**
AAL, *Aleuria aurantia* lectin; Asn, asparagine; BSA, bovine serum albumin; Cys, cysteine; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; FKP, L-fucokinase/L-fucosyltransferase 9; G418, neomycin; GDP, guanosine-5′-diphosphate; GlcNAc, N-acetylg glucosamine; Gu-HCl, guanidine-HCl; HRP, horseradish peroxidase; IGOT, stable isotope-labeling of streptavidin-captured glycopeptides; IGOT (deamidation and incorporation of stable isotope $^{18}$O) of Asn, maximum miss cleavage; Lea, Lewis a; Lys-C, endoproteinase Lys-C; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PBBT, phosphate-buffered saline Tween-20; PNGase F, peptide N-glycosidase F; PVDF, polyvinylidene fluoride; SDS, sodium dodecyl sulfate; RT, room temperature.

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