O-Linked N-Acetylglucosamine Is Present on the Extracellular Domain of Notch Receptors**§

Received for publication, August 11, 2008, and in revised form, October 21, 2008. Published, JBC Papers in Press, October 23, 2008, DOI 10.1074/jbc.M806202200

Aiko Matsuura†1, Makiko Ito†1, Yuta Sakaidani‡, Tatsuhiko Kondo‡, Kosuke Murakami§, Koichi Furukawa§, Daita Nadano§, Tsukasa Matsuda§, and Tetsuya Okajima†§2

From the †Department of Applied Molecular Biosciences, Nagoya University Graduate School of Bioagricultural Sciences, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan and ‡Department of Biochemistry II, Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-0065, Japan

Rare types of glycosylation often occur in a domain-specific manner and are involved in specific biological processes. In particular, O-fucose glycans are reported to regulate the functions of EGF domain-containing proteins such as Notch receptors. In the course of mass spectrometric analysis of O-glycans displayed on Drosophila Notch receptors expressed in S2 cells, we found an unusual O-linked N-acetylated hexosamine (HexNAc) modification which occurs at a site distinct from those of O-fucose and O-glucose glycosylations. Modification site mapping by mass spectrometry and amino acid substitution studies revealed that O-HexNAc modification occurs on a serine or threonine located between the fifth and sixth cysteines within the EGF domain. This modification occurs simultaneously along with other closely positioned O-glycosylations. This modification was determined to be O-β-GlcNAc by galactosyltransferase labeling and β-N-acetyl-hexosaminidase digestion experiments and by immunoblotting with a specific antibody. O-GlcNAc modification occurs at multiple sites on Notch epidermal growth factor repeats. O-GlcNAc modification was also found on the extracellular domain of Delta, a ligand for Notch receptors. Although the O-GlcNAc modification is known to regulate a wide range of cellular processes, the list of known modified proteins has previously been limited to intracellular proteins in animals. Thus, the finding of O-GlcNAc modification in extracellular environments predicts a distinct glycosylation process that might be associated with a novel regulatory mechanism for Notch receptor activity.

Protein glycosylation is a common co- and post-translational modification that has critical biological functions at both the cellular and organismal levels. In addition to the generally observed types of glycosylation such as N-glycosylation and mucin-type O-glycosylation, several unusual glycans have recently been found to play important roles in specific biological processes. Such unusual glycosylations are often observed as specific modifications on certain protein domains. One such domain is the epidermal growth factor (EGF) domain characterized as a small domain of 30–40 amino acids that is stabilized by 3 disulfide bonds. Three types of unusual post-translational modifications have been found at conserved residues within certain EGF domains. These modifications comprise the β-hydroxylation of Asp or Asn residues, O-linked glucose (O-glucose), and O-linked fucose (O-fucose) (1).

O-Fucose and O-glucose glycans have been reported in many proteins involved in blood coagulation and fibrolysis, which are relatively abundant in blood plasma. The consensus sequence for O-glucosylation is predicted to be C3XXPC2 (C1 and C2 are the first and second conserved cysteine residues; X is any amino acid), and the carbohydrate structure of the fully elongated form is a trisaccharide, Xyl-α1,3-Xyl-α1,3-Glu-O-Ser (1). In contrast, the consensus sequence for O-fucosylation is proposed to be C3XXS(A/G/S)S/TC3 (2), and the fully elongated structure is a tetrasaccharide, NeuAc-α2,6,3-Gal-β1,4-GlcNAc-β1,3-Fuc-O-Ser/Thr (1, 3).

Recent studies have revealed that EGF-specific O-glycans are not limited to plasma glycoproteins and exist in less abundantly expressed cell surface glycoproteins involved in intercellular signal transduction, such as Notch receptors (3). Notch receptors mediate an evolutionarily conserved signaling that regulates a wide range of developmental processes (4). The extracellular domain of Notch receptors is mainly composed of tandemly repeated EGF domains. Drosophila Notch and mammalian Notch1 contain 36 EGF domains in the EGF repeats, many of which are expected to be O-glucosylated and O-fucosylated (2, 5).

The biological significance of these rare O-glycans in EGF domains has been investigated by mutating modifiable residues (6) or, more recently, by manipulating glycosyltransferase genes involved in the glycosylation process. The specific glycosyltransferases include O-fucosyltransferase 1 (Pofut1) in mam-

**This work was supported by grants from the Japanese Ministry of Education, Science, Sports, and Culture and the 21st Century Center of Excellence Program (to T. O., D. N. and T. M.), the Uehara Memorial Foundation, Takeda Science Foundation, and Human Frontier Science Program (to T. O.). 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.

†The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5.

‡These authors contributed equally to this work.

1 To whom correspondence should be addressed. Tel.: 81-52-744-2068; Fax: 81-52-744-2069; E-mail: tokajima@med.nagoya-u.ac.jp.

§The abbreviations used are: EGF, epidermal growth factor; HexNAc, N-acetylhexosamine; NeuAc, N-acetylneuraminic acid; GlcNAc, N-acetyl- glucosamine; PLAP, placental alkaline phosphatase; ECL, E. crista galli lectin; ICN, intracellular Notch; ECN, extracellular Notch; β4GalT-1, β1,4-galactosyltransferase 1; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MS, mass spectrometry; PBS, phosphate-buffered saline; OGT, O-GlcNAc transferase.
mals and Ofut1 in Drosophila) that transfers fucose onto peptides via an α-O-linkage (7) and fringe-related gene products that transfer GlcNAc onto Fuc-O-Ser/Thr (8) (9). The down-regulation or mutation of Pofut1/Ofut1 genes results in the loss of Notch signaling activity, suggesting their essential roles in Notch signaling (10–12). The GlcNAc modification by Fringe was demonstrated in Notch fragments expressed in S2 cells (10, 14, 16–18); however, until very recently the presence of O-glucose glycans had not been experimentally investigated (19). In the course of mass spectrometric analysis to confirm the expression of the assumed O-glycans displayed on Notch EGF repeats, we unexpectedly discovered a novel O-β-GlcNAc modification that modifies a Ser/Thr residue located between the fifth and sixth cysteines within the EGF domain. To the best of our knowledge, this is the first demonstration of extracellularly O-GlcNAcylated proteins in animals.

**EXPERIMENTAL PROCEDURES**

**Materials**—pMT-Bip/EGF20-V5His, pMT-Bip/EGF20-Fuc-V5His, pMT-WB/N:AP, pMT-WB/N:AP-EGF1–10, and pMT-WB/Fc:AP were obtained from Ken Irvine (Rutgers University). A plasmid containing *rumi* cDNA was obtained from Hugo Bellen ( Baylor College of Medicine). The expression vector encoding the intracellular domain of Notch (pMT-N) was obtained from Drosophila Genomics Resource Center. pRmHa3/N-CD2 was obtained from Stephen Cohen (European Molecular Biology Laboratory). The following antibodies were used: mouse anti-Notch intracellular domain (C17.9C6; Developmental Studies Hybridoma Bank (DSHB)), mouse anti-Notch extracellular domain (C458.2H; DSHB), mouse anti-V5 (Invitrogen), goat anti-placental alkaline phosphatase (PLAP; Santa Cruz), mouse anti-α-tubulin (DM1A; Sigma), mouse anti-FLAG (M2; Sigma), mouse RL2 (Abcam), and mouse anti-O-β-GlcNAc (CTD110.6; Abcam).

**Plasmid Construction**—*In vitro* mutagenesis was performed according to the protocol of the Stratagene QuickChange site-directed mutagenesis kit using pMT-Bip/EGF20-V5His as a template. The details of the mutated constructs are illustrated in supplemental Fig. S1, and the complementary primer sets utilized for mutagenesis are shown in supplemental Fig. S2. Double and triple mutants were generated by repeating the mutagenesis process. The coding sequence of each construct was confirmed by DNA sequencing. pMT-WB/N:AP-EGF6–10 was constructed using the BspEI-EGF6-Fw and BspEI-EGF10-Rv primers, and pMT-WB/N:AP-EGF22–32 was constructed using BspEI-EGF22-Fw and BspEI-EGF32-Rv primers, as described previously (20). The sequences of the primers are shown in supplemental Fig. S2. For constructing pRmHa/ECN:FLAG, fragments encoding a transmembrane domain for PDGFR and 3×FLAG epitopes were amplified by PCR using BspEI-myc-Fw primer (5’-TCCGGAGAACAAAA-ACCTAATCCA-3’), PstI-3×FLAG-TAG primer (5’-CTGCG- AGCTAATTGGCTAATGCTTTTGATGCAGTGTCAT-GATCTTTTATATACCGCTAGTTTGTAGTACTCGACG- TGGCTTCTTCTCTGCA-3’), and pDisplay (Invitrogen) as a template. The PCR products were digested with BspEl/PstI and inserted into BspEl/Sse8387I sites of pRmHa3/N-CD2 (9).

To prepare unglycosylated EGF20-V5His, the EGF20-V5His fragment was inserted into the Xhol/NotI sites of pKLAC1, and a *Kluyveromyces lactis* protein expression kit (New England Biolabs) was used for the production of recombinant proteins.

**Transfection**—S2 cells were cultured in SF-900 II medium (Invitrogen) supplemented with 5% fetal bovine serum. Transfection was performed using Cellfectin (Invitrogen) as described previously (10). To establish stable cell lines for EGF20, the pMT-Bip-EGF20-V5His vector was transfected together with pMK33/hygroMycin in a 10:1 molar ratio. Stably transfected polyclonal cell populations were isolated after 4 weeks of selection with 300 μg/ml hygromycin B. Protein expression was under the control of an inducible metallothio- nein promoter and was induced in SF-900 II supplemented with 0.7 mM CuSO4.

**RNA-mediated Interference in S2 Cells**—S2 cell RNA-mediated interference was performed as described previously (10). For the production of double-stranded RNA, transcription templates were generated by PCR such that they contained T7 promoters on each end of the template. The primer sets used for PCR are described in supplemental Fig. S2.

**Purification of EGF Domains**—Notch EGF domains secreted in the culture media were affinity-purified under native conditions using nickel-magnetic beads (Promega) or anti-His-tag beads (Medical & Biological Laboratories). Ni-magnetic beads were washed twice with 100 mM HEPES-NaOH, pH 7.5, 10 mM imidazole and twice with 30% ethanol, and then the bound proteins were eluted with 100 μl of 0.1% trifluoroacetic acid. For purification with anti-His-tag beads, bound proteins were eluted with 0.1 M NaOH following the manufacturers instruction. The eluates were dried in a SpeedVac concentrator.

**Reduction, S-Carbamidomethylation, and Trypsin Digestion**—For trypsin digestion, nickel affinity-isolated EGF20 was further purified by reverse-phase HPLC with a Cadenza CD-C18 column (2 × 150 mm, Imtakt) at room temperature at a flow rate of 0.2 ml/min. A linear gradient formed between solvent A (H2O, 0.1% trifluoroacetic acid) and solvent B (CH3CN, 0.1% trifluoroacetic acid) was used for the separation. The gradient of solvent A was as follows: 0–2 min, 25%; 2–20 min, 25–45%; 20–25 min, 45–90%; 25–28 min, 90%. Peptide separation was monitored continuously at 220 nm. HPLC-purified Notch EGF20 was neutralized by the addition of 1 mM NH4HCO3 to achieve a final pH of 7.0 and concentrated to a final volume of 10 μl in a SpeedVac concentrator. For reduction and S-carbamidomethylation, 15 μl of 50 mM NH4HCO3 and 1.5 μl of 100 mM dithiothreitol were added to the solution. After incubation for 5 min at 95 °C, 3 μl of 100 mM iodoacetamide was added, and the mixture was incubated for 30 min in the dark. Trypsin digestion was carried out overnight at 37 °C by the addition of 1 μl of 0.1 mg/ml trypsin solution (Trypsin Gold; Sigma). Additional tryp-
sin solution was added after 2 h. Separation of the tryptic digest was performed by reverse-phase HPLC with the following gradient of solvent B: 0–2 min, 15%; 2–30 min, 15–40%; 30–35 min, 40–90%; 35–38 min, 90%.

**Matrix-assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS)** and **MS/MS Analysis**—MALDI-TOF-MS was performed using 4700 Proteomic Analyzer with TOF/TOF optics (Applied Biosystems) or Perseptive Voyager-DE Elite and Voyager DE-Pro spectrometers (Applied Biosystems). For the analysis of intact proteins with the 4700 Proteomic Analyzer, spectra were obtained in the linear positive ion mode using sinapinic acid as a MALDI matrix. Samples were reuspended in 1 μl of 0.1% trifluoroacetic acid and spotted on the sample plate. 1 μl of sinapinic acid (Sigma) in 0.05% trifluoroacetic acid was subsequently spotted on the sample plate and air-dried. MS spectra were recorded as the average of 3000 laser shots with the laser intensity within the range 4700–5000. External calibration was performed using bovine insulin and ubiquitin (Sigma) and human parathyroid hormone (Peptide Institute). The mass range was set at 7500–8500 m/z, with the focus mass at 8500 m/z. For the analysis of intact proteins with the Perseptive Voyager-DE Elite, spectra were obtained in the linear positive ion mode with delayed extraction and an acceleration voltage of 20 kV. The sample preparation and external calibration were performed as described above.

For the analysis of small peptides, spectra were obtained in the positive reflector mode using α-cyano-4-hydroxycinnamic acid as a MALDI matrix. HPLC-purified peptides were concentrated in a SpeedVac concentrator and spotted on the sample plate. Subsequently, 1 μl of α-cyano-4-hydroxycinnamic acid in 0.05% trifluoroacetic acid, 50% CH₃CN was spotted and air-dried. Each MS spectrum comprised an average of 4800 laser shots with a laser intensity of 4400. External calibration was performed using a 4700 Proteomics Analyzer Calibration Mixture (Applied Biosystems). Tandem MS (MS/MS) spectra were recorded using the 1-kV MS-MS positive mode with an average of 6000 laser shots and collision-induced dissociation off.

**Glycosyltransferase Assay**—Nickle-magnetic beads loaded with acceptor substrates were equilibrated with glycosylation buffer (200 mM HEPES-NaOH, pH 7.0, 1 mM MnCl₂, 1 mg/ml bovine serum albumin). For the β₄GalT-1 assay, the beads were incubated in glycosylation buffer containing 1 mM UDP-Gal and 0.4 units/ml bovine β₄GalT-1 (Sigma). After incubation overnight at 30 °C, the reaction was terminated by washing the beads 3 times with 100 mM Heps, pH 7.5, 10 μM imidazole.

A GlcNAC transferase assay was performed in the glycosylation buffer containing 1.6 μM UDP-[³H]GlcNAC (60 Ci mmol⁻¹; American Radiolabeled Chemicals), 2 μg of EGF20-V5His produced in *K. lactis*, and 0.2 μg of membrane fraction proteins isolated from S2 cells as described previously (21). After incubation overnight at 25 °C, the beads were washed 3 times with phosphate-buffered saline (PBS), and radioactivity was measured by liquid scintillation counting.

**Glycosidase Treatment**—Glycosidase digestion of glycopeptides was performed in 50 mM sodium citrate, pH 4.5, containing 2 units/ml bovine β-N-acetylgalactosaminidase (Sigma). After 1 h at 37 °C, the reaction solution was desalted with a C18 microcolumn (Zip Tip; Millipore) and subjected to MALDI-TOF-MS analysis. Glycosidase digestion for N-EGF:AP was performed for 3 h at 37 °C under denaturing conditions according to the protocol of the Enzymatic Protein Deglycosylation kit (Sigma) using recombinant β-N-acetylgalactosaminidase (0.1 units/μl; New England Biolabs) or α-N-acetylgalactosaminidase (0.4 units/μl; New England Biolabs).

**Lectin Blotting and Western Blotting**—After electroblotting to a polyvinylidene difluoride membrane and blocking with Tris-buffered saline containing 0.1% Triton X-100 (TBST: 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20), the membrane was incubated with TBST containing 5 μg/ml *Erythrina crista galli* lectin (ECL; Vector Laboratories) for 1 h. After washing 3 times with TBST, the blot was incubated with Vectastain ABC reagent (Vector) for 30 min. After washing 3 times with PBS, the bound lectin was visualized in PBS containing 0.05% 3,3’-diaminobenzidine (Sigma), 0.03% H₂O₂, and 2 mM NiCl₂. Western blotting with anti-O-GlcNAc antibody was performed as described previously (22). For immunoblotting with antibodies other than the anti-O-GlcNAc antibody, the membrane was incubated with blocking buffer (NETG solution: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 0.05% Triton X-100 containing 0.25% gelatin) and then with the primary antibodies followed by peroxidase-conjugated secondary antibodies diluted in the blocking buffer. For immunoblotting with an anti-O-GlcNAc antibody, 4% bovine serum albumin in TBST (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) was used as the blocking buffer. The bound antibody was visualized using a chemiluminescent substrate (ECL or ECL plus; GE Healthcare). The image was captured using a densitometer (ATTO Cool Saver) or by exposing to x-ray films.

**Immunoprecipitation and Glycopeptidase F Treatment**—For the immunoprecipitation of PLAP-fused proteins, the spent culture media was incubated with anti-PLAP antibody-conjugated agarose (Sigma). After washing with PBS, the bound proteins were eluted by boiling in 0.5% SDS supplemented with 90 mM 2-mercaptoethanol. For peptide glycopeptidase F treatment, the denatured proteins were incubated at 37 °C for 2 h in 100 mM Tris-HCl, pH 8.6, 0.1% SDS, and 1% Nonidet P-40 containing 40 milliunits/ml glycopeptidase F (Takara).

For the immunoprecipitation of endogenous Notch receptors, Kc cells were lysed in Nonidet P-40 buffer containing 40 mM Heps, pH 7.0, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 0.5% Nonidet P-40, and complete protease inhibitor mixture (Roche Applied Science). After centrifugation, the cleared supernatant was immunoprecipitated at 4 °C for 2 h using Dynabeads Protein G (Invitrogen), which were preincubated with 2 μg of anti-Notch antibodies.

**Trypsin Treatment**—Kc cells were washed twice with Hanks’ balanced salt solution and incubated with 0.05% trypsin, 0.53 mM EDTA solution (Invitrogen) for 3 min. After rinsing with Sf-900 II containing 0.1% sodium azide, the cells were washed twice with Hanks’ balanced salt solution containing 0.1% sodium azide and lysed in Nonidet P-40 buffer as described above.

**EDTA Treatment**—Kc cells were washed twice with Hanks’ balanced salt solution and incubated in PBS containing 0.5 mM EDTA for 30 min at room temperature. After sequential cen-
A Novel O-GlcNAc Modification on Notch Receptors

Identification of a Novel O-HexNAc Modification on Notch EGF20—To perform the mass spectrometric analysis of glycans displayed on the EGF repeats of Notch receptors, the 20th EGF domain (Notch EGF20) was selected based on the observation that its amino acid sequence conforms to the consensus sequences of both O-fucose and O-glucose modifications (Fig. 1A). Notch EGF20 was transiently expressed in S2 cells, purified from the culture media using nickel-affinity beads, and subjected to MALDI-TOF-MS analysis (Fig. 1B). Six major peaks were observed. Peak 1 at m/z 8438 corresponds to the unglycosylated EGF20 peptide with three disulfide bonds. The mass increment by 146 from peak 1 to peak 2 (m/z 8584; +146) indicated the presence of deoxyhexose. This peak was interpreted as that corresponding to the peptide with O-fucose modification site (Thr38) are underlined. B, MALDI-TOF-MS spectrum of Notch EGF20. Notch EGF20 was transiently expressed in S2 cells, affinity-purified, and analyzed using 4700 Proteome Analyzer in the linear positive mode. Major peaks in the spectrum are numbered in bold letters. Peak-to-peak mass increments corresponding to sugar modification are indicated by double-headed arrows. The theoretical average mass for a singly charged and unglycosylated EGF20 peptide with three disulfide bonds is 8438. C, MALDI-TOF-MS spectrum of the tryptic digest of Notch EGF20, which is stably expressed in S2 cells. The ions at m/z 2065 are derived from heterogeneous peptide fragments. dHex, deoxyhexose; Pen, pentose; Hex, hexose.

Because the O-fucose glycans appeared to be terminated with monosaccharides, all the other peaks were subjected to MS/MS analysis; however, none were identified as peptides with HexNAc modification, with the exception of the m/z 2065.1 ions, which generated complicated MS/MS spectra due to the presence of multiple ions derived from different peptides. To isolate the peptide corresponding to the m/z 2065.1 ion, the tryptic peptides were separated by reverse-phase HPLC (supplemental Fig. S4); all the fractionated peptides were subjected to MALDI-TOF-MS/MS analysis. We isolated the peptide P2 that showed a major peak at m/z 2064.8 (Fig. 2A). The minor peak at m/z 1861.7 matches the calculated mass of the peptide EGF20(26–41) (monoisotopic mass, 1861.8), the sequence of which was further confirmed by subsequent MS/MS analysis (not shown). Thus, the mass difference of 203.1 atomic mass units is consistent with the monoisotopic mass of HexNAc. MS/MS analysis of the ion at m/z 2064.8 yielded a major product ion at m/z 1862.0, which corresponds to the unglycosylated peptide mass (Fig. 2B). The neutral loss of the sugar moiety upon MS/MS is a characteristic feature of labile sugar modifications such as O-GlcNAc and O-fucose (22, 23); this further suggests the presence of HexNAc modification on the EGF20(26–41).

Determination of a Modification Site—To locate the HexNAc modification site, the MS/MS spectrum of the P2 peptide was further analyzed. In the spectrum, fragment ions corresponding to both glycosylated and deglycosylated peptides were observed (Fig. 2A; supplemental Fig. S5). For example, the y4 fragment occurs as deglycosylated (m/z 433.3) and glycosylated (m/z 636.4) ions. In contrast, the b12 fragment only based on the observation that RNA-mediated interference for Ofut1 significantly decreased the intensity of the peak (supplemental Fig. S3). The mass increments from peak 2 to peak 3 (m/z 8788; +204), peak 3 to peak 5 (m/z 8950; 162), and peak 5 to peak 6 (m/z 9082; +132) indicated the presence of HexNAc, hexose, and pentose, respectively. The presence of HexNAc could be interpreted as a GlcNAc modification on O-fucose. To test this possibility, a stable cell line for EGF20 was established to obtain large amounts of recombinant proteins. Notch EGF20 in the media was purified from the culture media using Ni-affinity beads followed by reverse phase HPLC. After reduction and 5-carboxymethylation, EGF20 was enzymatically digested with trypsin. The tryptic digest was analyzed by mass spectrometry (Fig. 1C). MS/MS analysis of ions at m/z 3125.4 and 3419.5 revealed that both ions were derived from the same tryptic peptide EGF20(1–25). The theoretical mass of the peptide is 2979.2. Thus, the ion at m/z 3125.4 (+146.2) corresponds to the peptide with O-fucose. The ion at m/z 3419.5 (+440.3) can be interpreted as the O-fucosylated peptide further modified with hexose and pentose (+294.1). In the MS/MS spectrum glycosylated fragment ions with additional mass corresponding to hexose and pentose were observed (not shown). As expected, those glycosylated fragment ions always include an O-glycosylation site but not an O-fucosylation site. Thus, the ion at m/z 3419.5 was interpreted to be glycans containing both O-fucose monosaccharides and O-glucose disaccharides based on the proposed O-glycan structure in mammals.
A Novel O-GlcNAc Modification on Notch Receptors

FIGURE 2. Identification of the O-HexNAc modification site by MS/MS analysis. Tryptic digest of Notch EGF20 was separated by HPLC, and the isolated P2 peptide was analyzed by MALDI-TOF-MS and MS/MS. The spectrum of P2 peptide showed dominant peaks at m/z 1861.7 and m/z 2064.8, which corresponds to the unglycosylated and glycosylated EGF20 (26–41) peptide (+203), respectively. Because of oxidation of the methionyl residue, +16 peaks were detected (denoted as M+16). An asterisk marks the metastable ions, which are a characteristic feature of labile post-translational modification. B, MS/MS spectrum of the precursor ion at m/z 2064.8. The neutral loss of the sugar group yielded a major product ion at m/z 1862.0. The mass difference relative to the precursor ion was 203, indicating the presence of O-HexNAc. Asterisks indicate the glycosylated fragment ion peaks. The expanded product ion spectrum is shown in Supplemental Fig. S4.

occurs as deglycosylated ions (m/z 1429.6). This result narrowed the HexNAc modification site to TGQK.

In the course of HPLC purification of the tryptic digest, a derivative of the EGF20 (26–41) peptide was obtained (P1 peptide in supplemental Fig. S4). MS/MS analysis of the P1 peptide indicated that this peptide contains a decomposed derivate of S-carbamidomethylated methionine; this decreased the mass of the peptide by 48 atomic mass units (24). The P1 peptide generated a distinct pattern in the MS/MS spectrum (supplemental Fig. S5), providing additional information useful for mapping the HexNAc modification site. The presence of glycosylated b13 ions and the absence of glycosylated y3 ions showed that threonine at position 38 is the modified amino acid. These results suggest that the HexNAc moiety is attached to Thr via an O-linkage.

To verify the O-HexNAc modification site, Thr was substituted with Ala (ΔHexNAc) by in vitro mutagenesis. The MALDI-TOF-MS spectrum of the ΔHexNAc mutants displayed three major peaks (Fig. 3A). The mass difference of 146 between Peak 1 at m/z 8554 and the theoretical mass of the unglycosylated peptide (M+H)+ = 8408) indicated O-fucose modification. Similarly, the mass differences of 161 and 133 between Peaks 1 and 2 and between Peaks 2 and 3, respectively, indicated modification with hexose and pentose, respectively. On comparing the spectra of these mutants with that of wild-type EGF20 (Fig. 1B), the absence of a mass increment corresponding to HexNAc modification was clearly suggested. In contrast, the spectrum of the O-fucosylation site mutant (ΔFuc; Fig. 3B) detected no mass increment corresponding to O-fucose, whereas the mass increment (+204) corresponding to O-HexNAc was still observed. These data further confirmed that HexNAc is not derived from the extended structure of the O-fucose glycan (i.e., GlcNAc-B1,3-Fuc-O).

We further mutated the O-glucosylation site either singly (ΔGlc) or together with other O-glucosylation sites (ΔGlc/ΔFuc and ΔGlc/ΔFuc/ΔHexNAc). The spectrum of ΔGlc showed the absence of mass increments corresponding to hexose and pentose (Fig. 3C), and in the ΔFuc/ΔGlc spectrum, only a mass increment corresponding to HexNAc was observed (Fig. 3D). The spectrum of the triple glycosylation mutant showed a single prominent peak that appears to correspond to unglycosylated peptides (Fig. 3E). Taken together, these results established that Notch EGF20 is modified simultaneously with 3 different O-glycans that are attached to distinct modification sites at Ser14, Thr21, and Thr38 (Fig. 1A).

To determine whether a Thr to Ser substitution permits the 4GalT-1 reaction, elongation with galactose was detected by the galactose-specific lectin ECL. EGF20 incubated with β4GalT-1 was detected by lectin blotting with ECL. The specificity of this method was confirmed by the following criteria; 1) the signal was observed only after the β4GalT-1 reaction, and 2) the signal was eliminated by the

FIGURE 3. MALDI-TOF-MS analysis of mutated Notch EGF20 defective in glycosylation. Mutated EGF20 was transiently expressed in S2 cells and affinity-purified, and MALDI-TOF-MS spectra were recorded using a 4700 Proteomic Analyzer in the linear positive mode. Major peaks in each spectrum are numbered in bold. Peak-to-peak mass increments characteristic to sugar modification are indicated by double-headed arrows. The [M+H]+ value represents the theoretical average mass of the nonglycosylated and disulfide-bonded peptides. The spectra shown correspond to ΔHexNAc (A), ΔFuc (B), ΔGlc (C), ΔGlc/ΔFuc (D), ΔGlc/ΔFuc/ΔHexNAc (E), and ΔGlc/ΔFuc/T38S (F). In panels A–D, the positions corresponding to the nonglycosylated peptide are indicated by dashed lines.

Identification of O-β-GlcNAc Modification—To determine the structure of this novel O-glycan, we performed a β4GalT-1 assay (22). This assay exploited the specific action of β4GalT-1 toward the terminal GlcNAc, thereby generating a Gal-B1,4-GlcNAc structure. After the β4GalT-1 reaction, elongation with galactose was detected by the galactose-specific lectin ECL. EGF20 incubated with β4GalT-1 was detected by lectin blotting with ECL. The specificity of this method was confirmed by the following criteria; 1) the signal was observed only after the β4GalT-1 reaction, and 2) the signal was eliminated by the
A Novel O-GlcNAc Modification on Notch Receptors

A Novel O-GlcNAc Modification on Notch Receptors

online-oxidized EGF20-(26–41) with HexNAc modification. This methionine-oxidation occurred spontaneously during sample preparation. Upon glycosidase digestion, the ion at m/z 2080.9 was almost absent, and the ion corresponding to the unglycosylated peptide (m/z 1877.85) was prominently observed (Fig. 4D). These data suggest the β-anomeric configuration of the O-HexNAc modification, which is clearly different from mucin-type O-glycosylation that is initiated with GlcNAc-α-O-Thr/Ser.

Although the O-β-GlcNAc modification on Notch EGF20 is an unprecedented extracellular modification, it is known to occur intracellularly on a number of cytoplasmic and nuclear proteins. As a specific antibody toward the β-O-linked serine or threonine GlcNAc modification, the CTD110.6 antibody has been widely accepted (22). To verify whether O-β-GlcNAc modification on Notch EGF20 can be detected by the CTD110.6 antibody, wild-type EGF20 and ΔHexNAc were subjected to immunoblotting. Although no signal was detected in the ΔHexNAc mutant, a signal was readily detected in the wild-type EGF 20, confirming that this antibody indeed detects O-β-GlcNAc modification on Notch EGF20 (Fig. 4E). As an independent test to confirm O-GlcNAc modification on EGF20, we asked if O-GlcNAcylolation on EGF20 could be recapitulated in vitro. Membrane fraction proteins were prepared from S2 cells, and in vitro glycosyltransferase assay was performed using unglycosylated EGF20-V5His prepared from yeast as an acceptor substrate. As shown in Fig. 4F, EGF20 was apparently radiolabeled with GlcNAc by the membrane fraction proteins. Interestingly, unlike cytoplasmic and nuclear O-GlcNAc transferase (OGT) (25), the O-GlcNAc transferase-mediating EGF domain modification required Mn2+ for its activity. Thus, O-GlcNAc modification on EGF domains is likely to be catalyzed by an as yet unidentified glycosyltransferase.

Indeed, the O-GlcNAc modification did not appear to be influenced by down-regulation of known glycosyltransferase genes involved in EGF-domain specific O-glycosylation and by treatment with O-(2-Acetamido-2-deoxy-D-glucopyranosylidamino)-N-phenylcarbamate, an inhibitor of cytoplasmic O-GlcNAcase, which elevates the intracellular O-GlcNAc level (supplemental Fig. S3) (26).

O-β-GlcNAc Modification Occurs at Multiple Sites on Notch EGF Repeats—Next, we asked whether O-β-GlcNAc modification occurs on the intact EGF repeats of Notch receptors. Notch EGF repeats C-terminally fused with PLAP (N-EGF:AP) were expressed in S2 cells and analyzed by Western blotting with the CTD110.6 antibody. Specific reactivity was observed in N-EGF:AP but not in the control Fc:AP construct. This signal is not derived from the N-glycans on the Notch receptors as similar results were obtained after peptide glycopeptidase treatment (Fig. 5A). The signal was nearly eliminated after the treatment with recombinant β-N-acetylhexosaminidase but not with α-N-acetylgalactosaminidase, verifying that O-β-GlcNAc modification indeed occurs on EGF repeats of Notch receptors (Fig. 5B). To determine whether O-GlcNAc modification occurs on other EGF domains, several deletion mutants of the N-EGF:AP construct were analyzed. The signal was observed in EGF1–10 and EGF22–31 but not in EGF6–10 (Fig. 5C).

addition of lactose (Fig. 4A). Consistently, MALDI-TOF-MS analysis of the reaction products detected the mass shift corresponding to the modification with galactose (not shown). These observations suggested that EGF20 is modified with O-GlcNAc. To verify that O-GlcNAc modification indeed occurs on Thr38, a series of mutants with a modified O-glycosylation site(s) was analyzed (Fig. 4B). As expected, ΔGlc/ΔFuc and ΔGlc/ΔFuc/T38S still reacted with the ECL lectin, whereas in the case of ΔHexNAc no signals were observed. Thus, we concluded that Thr38 is modified with O-GlcNAc.

To determine the anomeric configuration of the glycosidic bond between HexNAc and threonine, HPLC-purified EGF20-(26–41) was digested with linkage-specific β-N-acetylhexosaminidase and subjected to MALDI-TOF-MS analysis. In the spectrum from the untreated control sample (Fig. 4C), a major peak was observed at m/z 2080.9 that corresponds to methionine-oxidation EGF20-(26–41) with HexNAc modification. This methionine-oxidation occurred spontaneously during sample preparation. Upon glycosidase digestion, the ion at m/z 2080.9 was almost absent, and the ion corresponding to the unglycosylated peptide (m/z 1877.85) was prominently observed (Fig. 4D). These data suggest the β-anomeric configuration of the O-HexNAc modification, which is clearly different from mucin-type O-glycosylation that is initiated with GlcNAc-α-O-Thr/Ser.

Although the O-β-GlcNAc modification on Notch EGF20 is an unprecedented extracellular modification, it is known to occur intracellularly on a number of cytoplasmic and nuclear proteins. As a specific antibody toward the β-O-linked serine or threonine GlcNAc modification, the CTD110.6 antibody has been widely accepted (22). To verify whether O-β-GlcNAc modification on Notch EGF20 can be detected by the CTD110.6 antibody, wild-type EGF20 and ΔHexNAc were subjected to immunoblotting. Although no signal was detected in the ΔHexNAc mutant, a signal was readily detected in the wild-type EGF 20, confirming that this antibody indeed detects O-β-GlcNAc modification on Notch EGF20 (Fig. 4E). As an independent test to confirm O-GlcNAc modification on EGF20, we asked if O-GlcNAcylolation on EGF20 could be recapitulated in vitro. Membrane fraction proteins were prepared from S2 cells, and in vitro glycosyltransferase assay was performed using unglycosylated EGF20-V5His prepared from yeast as an acceptor substrate. As shown in Fig. 4F, EGF20 was apparently radiolabeled with GlcNAc by the membrane fraction proteins. Interestingly, unlike cytoplasmic and nuclear O-GlcNAc transferase (OGT) (25), the O-GlcNAc transferase-mediating EGF domain modification required Mn2+ for its activity. Thus, O-GlcNAc modification on EGF domains is likely to be catalyzed by an as yet unidentified glycosyltransferase. Indeed, the O-GlcNAc modification did not appear to be influenced by down-regulation of known glycosyltransferase genes involved in EGF-domain specific O-glycosylation and by treatment with O-(2-Acetamido-2-deoxy-D-glucopyranosylidamino)-N-phenylcarbamate, an inhibitor of cytoplasmic O-GlcNAcase, which elevates the intracellular O-GlcNAc level (supplemental Fig. S3) (26).

O-β-GlcNAc Modification Occurs at Multiple Sites on Notch EGF Repeats—Next, we asked whether O-β-GlcNAc modification occurs on the intact EGF repeats of Notch receptors. Notch EGF repeats C-terminally fused with PLAP (N-EGF:AP) were expressed in S2 cells and analyzed by Western blotting with the CTD110.6 antibody. Specific reactivity was observed in N-EGF:AP but not in the control Fc:AP construct. This signal is not derived from the N-glycans on the Notch receptors as similar results were obtained after peptide glycopeptidase treatment (Fig. 5A). The signal was nearly eliminated after the treatment with recombinant β-N-acetylhexosaminidase but not with α-N-acetylgalactosaminidase, verifying that O-β-GlcNAc modification indeed occurs on EGF repeats of Notch receptors (Fig. 5B). To determine whether O-GlcNAc modification occurs on other EGF domains, several deletion mutants of the N-EGF:AP construct were analyzed. The signal was observed in EGF1–10 and EGF22–31 but not in EGF6–10 (Fig. 5C).

addition of lactose (Fig. 4A). Consistently, MALDI-TOF-MS analysis of the reaction products detected the mass shift corresponding to the modification with galactose (not shown). These observations suggested that EGF20 is modified with O-GlcNAc. To verify that O-GlcNAc modification indeed occurs on Thr38, a series of mutants with a modified O-glycosylation site(s) was analyzed (Fig. 4B). As expected, ΔGlc/ΔFuc and ΔGlc/ΔFuc/T38S still reacted with the ECL lectin, whereas in the case of ΔHexNAc no signals were observed. Thus, we concluded that Thr38 is modified with O-GlcNAc.

To determine the anomeric configuration of the glycosidic bond between HexNAc and threonine, HPLC-purified EGF20-(26–41) was digested with linkage-specific β-N-acetylhexosaminidase and subjected to MALDI-TOF-MS analysis. In the spectrum from the untreated control sample (Fig. 4C), a major peak was observed at m/z 2080.9 that corresponds to methi-
Although the lack of reactivity with CTD110.6 does not necessarily indicate the absence of O-GlcNAc modifications, these results clearly demonstrate that O-GlcNAcylation occurs at multiple sites on Notch EGF repeats such as EGF20, EGF1–5, and EGF22–31, which contain at least one Thr/Ser residue at the position corresponding to the O-GlcNAcylation site on EGF20 (Fig. 6). O-GlcNAc modification was also detected on Delta, a ligand for Notch receptors, which contains multiple Thr/Ser residues at the corresponding position (Fig. 5D and data not shown).

Detection of O-GlcNAc Modifications on Endogenous Notch Receptors—To demonstrate the presence of O-GlcNAc modification on the wild-type Notch, the constructs encoding full-length Notch receptor or the intracellular fragment (ICN) were expressed in S2 cells, immunoprecipitated by anti-ICN antibody, and subjected to immunoblotting (WB) with CTD110.6 (arrow). Immunoblotting with the anti-PLAP antibody is shown below. Where indicated, peptide glycopeptidase F (PNGase) treatment was performed before SDS-PAGE. B, N-EGF:AP was digested with or without β-N-acetylgalactosaminidase (β-HexNAcase) or α-N-acetylhexosaminidase (α-HexNAcase) and analyzed as described above. IP, immunoprecipitate; Cont, control. C and D, the deletion mutants of the N-EGF:AP construct (EGF1–10, EGF6–10, and EGF22–32) (C) and the extracellular domain of Delta fused with PLAP (Delta:AP (14)) (D) were analyzed as described above.

FIGURE 5. Detection of O-GlcNAc modifications on the extracellular domain of Notch receptor and its ligand. A, Notch EGF repeats C-terminally fused with PLAP (N-EGF:AP) and Fc:AP as a control (14) were expressed in S2 cells, affinity-purified with the anti-PLAP antibody, and subjected to immunoblotting (WB) with CTD110.6 (arrow). Immunoblotting with the anti-PLAP antibody is shown below. Where indicated, peptide glycopeptidase F (PNGase) treatment was performed before SDS-PAGE. B, N-EGF:AP was digested with or without β-N-acetylgalactosaminidase (β-HexNAcase) or α-N-acetylhexosaminidase (α-HexNAcase) and analyzed as described above. IP, immunoprecipitate; Cont, control. C and D, the deletion mutants of the N-EGF:AP construct (EGF1–10, EGF6–10, and EGF22–32) (C) and the extracellular domain of Delta fused with PLAP (Delta:AP (14)) (D) were analyzed as described above.

FIGURE 6. Amino acid sequence alignment of the O-GlcNAc modification region of Drosophila and human Notch receptors. The amino acid sequence located between the fifth and sixth conserved cysteines of 36 EGF domains from Drosophila Notch and human Notch1 are aligned. The O-GlcNAc modification site is indicated by an arrowhead. Gray boxes indicate conserved residues, and identical residues are boxed in black.
A Novel O-GlcNAc Modification on Notch Receptors

were treated with trypsin/EDTA. As expected, cleaved Notch fragments were hardly detected by O-GlcNAc antibody (Fig. 7B), indicating that Notch O-GlcNAcylation is predominantly extracellular. To isolate the Notch extracellular domain (ECN), we took advantage of the fact that metal ion chelators induce the shedding of ECN from the cells (27). A small number of O-GlcNAcylated proteins were identified in the bulk proteins released by EDTA treatment. The immunoprecipitation by anti-ECN antibody revealed that one of the major O-GlcNAcylated proteins migrated at ~220 kDa, corresponding to Notch ECN (Fig. 7C). Finally, removal of intracellular O-GlcNAc transferase activity by treatment with double-stranded RNA for OGT did not affect O-GlcNAc modification on Notch receptors (Fig. 7D). Taken together, these results demonstrated that the O-GlcNAcylation on the extracellular domain of Notch receptors occurs under physiological conditions.

**DISCUSSION**

Our attempts to confirm the proposed structure of O-glycans on Notch receptors led to the unexpected discovery of an extracellular O-β-GlcNAc modification in Drosophila S2 cells. Back in the 1980s it was reported that a small percentage of O-GlcNAc-containing proteins was detected on the surface of lymphocytes (28). However, subsequent re-examination using improved methods failed to detect O-GlcNAc-bearing proteins on the cell surface of lymphocytes (29). Although there is another report that suggested the presence of O-GlcNAc at the luminal faces of the endoplasmic reticulum (30), such O-GlcNAcylated proteins have not been molecularly identified, and O-GlcNAc is believed to be highly restricted to the cytoplasmic and nuclear compartments. Thus, our present study provides the first report of O-GlcNAcylated proteins in the animal extracellular environment.

Intracellular O-GlcNAc modification is catalyzed by the cytoplasmic enzyme OGT (31). This OGT is not appeared to be responsible for the O-GlcNAc modification identified in this study as the O-linked glycan modification on EGF domains typically occurs during secretion by the action of endoplasmic reticulum- or Golgi-localized glycosyltransferases. This view is supported by the fact that RNA-mediated interference for OGT did not affect O-GlcNAc level on the extracellular domain of Notch receptors. We cannot formally exclude the possibility that residual OGT activity in OGT-depleted cells might contribute to the O-GlcNAcylation on Notch receptors at the extracellular domain. For example, OGT might translocate from cytosol to the luminal side of endoplasmic reticulum or Golgi by an unknown mechanism. However, O-GlcNAc transferase mediating EGF domain modification requires Mn2+ for its activity unlike OGT, suggesting that this new modification occurs in an OGT-independent manner. Although Golgi O-GlcNAc transferases have been reported in microbial eukaryotes such as Dictyostelium (32) and Trypanosoma cruzi (33), the GlcNAc modification of Thr and Ser residues mediated by these enzymes occurs via an α-linkage. Thus, we predict that the O-GlcNAc transferase responsible for the O-GlcNAc modification of the Notch EGF repeats is distinct from the Golgi O-GlcNAc transferases in microbial eukaryotes. This view is consistent with the fact that no homologous genes for

---

**FIGURE 7. Detection of O-GlcNAc modifications on endogenous Notch receptors.** A, full-length Notch (Full N) or the ICN was expressed in S2 cells, immunoprecipitated (IP) with anti-ICN antibody, and subjected to Western blotting (WB) with CTD110.6 (anti-O-GlcNAc antibody) or anti-ICN antibody. The arrow indicates the full-length Notch protein, and the arrowhead shows the intracellular Notch fragments. B, Kc cells were incubated with (+) or without (−) trypsin. Total cell lysates were immunoprecipitated with anti-ICN antibody and subjected to Western blotting with CTD110.6 or anti-ICN antibody. The arrow indicates the intact full-length Notch protein, and the arrowhead shows a trypsin-digested fragment. C, Kc cells were incubated in PBS containing 0.5 mM EDTA. The conditioned media were subjected to immunoprecipitation with anti-ECN or with no antibody (−). Western blotting was performed with CTD110.6 or anti-ECN antibody. Input represents the conditioned media before immunoprecipitation. D, full-length Notch (Full N) or membrane-tethered Notch EGF repeats C-terminally tagged with 3×FLAG epitopes (ECN:FLAG) were expressed in S2 treated with or without double-stranded RNA (dsRNA) for OGT. Total cell lysates were immunoprecipitated with anti-ICN or FLAG antibody, and the immunoprecipitants were analyzed with antibodies against O-GlcNAc and ECN. Aliquot of total cell lysates were probed with RL2 antibody that recognizes O-GlcNAc modification on nucleopore complex and with anti-α-tubulin antibody to confirm equal protein loading.

digenously express Notch receptors (27). Endogenous Notch receptors were immunoprecipitated, and the presence of O-GlcNAc modification was confirmed by immunoblotting with O-GlcNAc antibody (Fig. 7B). To determine whether the O-GlcNAc modification is present on the cell surface, Kc cells
A Novel O-GlcNAc Modification on Notch Receptors

Dictyostelium O-GlcNAc transferase have been identified in the genomic sequences of Drosophila, mice, or humans (32). Although it is tempting to speculate that novel glycosyltransferases homologous to OGT might contribute to the O-GlcNAc modification in the secretory pathway, BLAST analysis of the catalytic domain of OGT did not lead to the identification of the homologous proteins with the topology characteristic of endoplasmic reticulum – or Golgi-localized glycosyltransferases.

The fact that extracellularly O-β-GlcNAc-acylated proteins have not been formally identified until now suggests that such modification is particularly unusual. Consistent with this supposition, a recent study designed to characterize the [3H]galactosylated products of the β4GalT-1–treated EGF repeats prepared from S2 cells suggested that, although O-linked monosaccharides labeled by β4GalT-1 were present on EGF repeats, the amount of labeled products were significantly lower than that derived from GlcNAc-Fuc disaccharide (16). However, this observation is somewhat contradictory to our finding that many EGF domains in Drosophila are possibly modified with O-GlcNAc as the Ser/Thr residues at the position corresponding to the O-β-GlcNAc modification site are relatively conserved (Fig. 6). Thus, although our data suggest that Notch EGF20 is efficiently O-GlcNAcylated in S2 cells, modification on other sites would occur substoichiometrically, or alternatively, only a limited number of EGF domains are O-GlcNAcylated under the experimental conditions used in these studies. Identification of other O-GlcNAcylated peptides and determination of the consensus sequence for O-GlcNAc modification on EGF domains will be required to address these questions. In addition to Notch receptors, Factors VII, IX, and XII, plasminogen activators, and Protein Z are reported to be O-fucosylated or O-glucosylated (34). Interestingly, with the exception of Factor XII, these plasma glycoproteins do not contain potentially O-GlcNAcylated Ser/Thr residues. In contrast, the Ser/Thr residues are present in both Notch receptors and Notch ligands from various animals. Thus, O-GlcNAc modification might be specifically employed to regulate Notch-dependent biological processes during animal development.

Although the O-GlcNAc modification was identified as a monosaccharide in S2 cells, the structure might be elongated in other cellular contexts. The fact that β4GalT-1 can extend the sugar structure indicates that similar glycosylation events could occur where β4GalT-1 or other glycosyltransferases with similar substrate specificity are available. In mammals, β4GalT-1 is required for the elongation of GlcNAc-Fuc disaccharide on EGF domains into NeuAc-Gal-GlcNAc-Fuc tetrasaccharide (15). Accordingly, if the O-GlcNAc modification occurs in mammals, it would be elongated into NeuAc-Gal-GlcNAc.

MALDI-TOF-MS analyses of the intact Notch EGF20 peptide clearly demonstrate that the O-GlcNAc modification occurs simultaneously with other O-glycosylation events (Fig. 1B). Mutations in the O-GlcNAc modification site do not appear to influence other glycosylation patterns. In reciprocal experiments, mutations in O-fucose and/or O-glucose modification sites did not significantly affect O-GlcNAcylated levels (Fig. 3). Thus, the O-GlcNAc modification and other O-glycosylations neither depend on nor compete with each other.

It has been established that GlcNAc modification onto the O-fucose of EGF domains plays an essential role in the modulation of physical interactions between Notch receptors and their ligands (8, 9). Furthermore, Fringe proteins, which catalyze the transfer of GlcNAc onto O-fucose, are shown to be involved in many developmental processes both in mice and Drosophila (2, 13). Although the physiological importance of extracellular O-GlcNAc modification is currently unknown, it might be associated with a novel GlcNAc-dependent regulation of Notch receptor activity. It is reported that fringe-connection mutants in which global GlcNAc modifications on Notch receptors are impaired due to a defect in the specific sugar transporter activity displayed more severe Notch phenotypes compared with the fringe mutant (35, 36). Although not all rare types of glycosylation are essential for protein function (37), it is likely that O-GlcNAc modification on Notch receptors is associated with a subset of the phenotypes observed in fringe-connection mutants. The isolation of the glycosyltransferase genes responsible for O-GlcNAc modification and reverse genetic studies in Drosophila and mice would provide greater insight into the biological contributions of this novel post-translational modification.

Acknowledgments—We thank the Developmental Studies Hybridoma Bank for antibodies, Y. Sakagami and R. Haltiwanger for helpful discussions, and K. Irvine for comments on the manuscript.

REFERENCES

1. Harris, R. J., and Spellman, M. W. (1993) Glycobiology 3, 219–224
2. Haines, N., and Irvine, K. D. (2003) Nat. Rev. Mol. Cell Biol. 4, 786–797
3. 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
4. Artavanis-Tsakonas, S., Rand, M. D., and Lake, R. J. (1999) Science 284, 770–776
5. Haltiwanger, R. S., and Stanley, P. (2002) Biochim. Biophys. Acta 1573, 328–335
6. Bjoern, S., Foster, D. C., Thim, L., Wiberg, F. C., Christensen, M., Komiyama, Y., Pedersen, A. H., and Kisiel, W. (1991) J. Biol. Chem. 266, 11051–11057
7. Wang, Y., Shao, L., Shi, S., Harris, R. J., Spellman, M. W., Stanley, P., and Haltiwanger, R. S. (2001) J. Biol. Chem. 276, 40338–40345
8. 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
9. Bruckner, K., Perez, L., Clausen, H., and Cohen, S. (2000) Nature 406, 411–415
10. Okajima, T., and Irvine, K. D. (2002) Cell 111, 893–904
11. Sasamura, T., Sasaki, N., Miyashita, F., Nakao, S., Ishikawa, H. O., Ito, M., Kiragawa, M., Harigaya, K., Spana, E., Bilder, D., Perrimon, N., and Matsumoto, K. (2003) Development 130, 4785–4795
12. Shi, S., and Stanley, P. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 5234–5239
13. Stanley, P. (2007) Curr. Opin. Struct. Biol. 17, 530–535
14. Okajima, T., Xu, A., and Irvine, K. D. (2003) J. Biol. Chem. 278, 42340–42345
15. Chen, J., Moloney, D. J., and Stanley, P. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 13716–13721
16. 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
17. Lei, L., Xu, A., Panin, V. M., and Irvine, K. D. (2003) Development 130, 6411–6421
18. Panin, V. M., Shao, L., Lei, L., Moloney, D. J., Irvine, K. D., and Haltiwanger, R. S. (2002) J. Biol. Chem. 277, 29945–29952
19. Acar, M., Jafar-Nejad, H., Takeuchi, H., Rajan, A., Ibrani, D., Rana, N. A., Pan, H., Haltiwanger, R. S., and Bellen, H. J. (2008) Cell 132, 247–258
20. Xu, A., Lei, L., and Irvine, K. D. (2005) J. Biol. Chem. 280, 30158–30165
21. Okajima, T., Nakamura, Y., Uchikawa, M., Haslam, D. B., Numata, S. I., Furukawa, K., Urano, T., and Furukawa, K. (2000) J. Biol. Chem. 275, 40498–40503
22. Whelan, S. A., and Hart, G. W. (2006) Methods Enzymol. 415, 113–133
23. Nita-Lazar, A., and Haltiwanger, R. S. (2006) Methods Enzymol. 417, 93–111
24. Jones, M. D., Merewether, L. A., Clogston, C. L., and Lu, H. S. (1994) Anal. Biochem. 216, 135–146
25. Haltiwanger, R. S., Blomberg, M. A., and Hart, G. W. (1992) J. Biol. Chem. 267, 9005–9013
26. Haltiwanger, R. S., Grove, K., and Philipsberg, G. A. (1998) J. Biol. Chem. 273, 3611–3617
27. Rand, M. D., Grimm, L. M., Artavanis-Tsakonas, S., Patriub, V., Blacklow, S. C., Sklar, J., and Aster, J. C. (2000) Mol. Cell. Biol. 20, 1825–1835
28. Torres, C. R., and Hart, G. W. (1984) J. Biol. Chem. 259, 3308–3317
29. Kearse, K. P., and Hart, G. W. (1991) Arch. Biochem. Biophys. 290, 543–548
30. Abeijon, C., and Hirschberg, C. B. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 1010–1014
31. Kreppel, L. K., Blomberg, M. A., and Hart, G. W. (1997) J. Biol. Chem. 272, 9308–9315
32. Wang, F., Metcalf, T., van der Wel, H., and West, C. M. (2003) J. Biol. Chem. 278, 51395–51407
33. Previato, J. O., Sola-Penna, M., Agrellos, O. A., Jones, C., Oeltmann, T., Travassos, L. R., and Mendonca-Previato, L. (1998) J. Biol. Chem. 273, 14982–14988
34. Okajima, T., Matsuura, A., and Matsuda, T. (2008) J. Biochem. 144, 1–6
35. Goto, S., Taniguchi, M., Muraoka, M., Toyoda, H., Sado, Y., Kawakita, M., and Hayashi, S. (2001) Nat. Cell Biol. 3, 816–822
36. Selva, E. M., Hong, K., Baeg, G. H., Beverley, S. M., Turco, S. J., Perrimon, N., and Hacker, U. (2001) Nat. Cell Biol. 3, 809–815
37. Shi, S., Ge, C., Luo, Y., Hou, X., Haltiwanger, R. S., and Stanley, P. (2007) J. Biol. Chem. 282, 20133–20141