In Vitro Reconstitution of the Modulation of Drosophila Notch-Ligand Binding by Fringe

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Notch signaling plays critical roles in animal development and physiology. The activation of Notch receptors by their ligands is modulated by Fringe-dependent glycosylation. Fringe catalyzes the addition of N-acetylgalactosamine in a β1,3 linkage onto O-fucose on epidermal growth factor-like domains. This modification of Notch by Fringe influences the binding of Notch ligands to Notch receptors. However, prior studies have relied on in vivo glycosylation, leaving unresolved the question of whether addition of N-acetylgalactosamine is sufficient to modulate Notch-ligand interactions on its own, or whether instead it serves as a precursor to subsequent post-translational modifications. Here, we describe the results of in vitro assays using purified components of the Drosophila Notch signaling pathway. In vitro glycosylation and ligand binding studies establish that the addition of N-acetylgalactosamine onto O-fucose in vitro is sufficient both to enhance Notch binding to the Delta ligand and to inhibit Notch binding to the Serrate ligand. Further elongation by galactose does not detectably influence Notch-ligand binding in vitro. Consistent with these observations, carbohydrate compositional analysis and mass spectrometry on Notch isolated from cells identified only N-acetylgalactosamine added onto Notch in the presence of Fringe. These observations argue against models in which Fringe-dependent glycosylation modulates Notch signaling by acting as a precursor to subsequent modifications and instead establish the simple addition of N-acetylgalactosamine as a basis for the effects of Fringe on Drosophila Notch-ligand binding.

Notch proteins are transmembrane receptors for a conserved signaling pathway that mediates a wide range of cell fate decisions during development (1, 2). Notch receptors are activated by binding to transmembrane ligands expressed on adjacent cells. In a subset of Notch signaling events, such as occurs along the dorsal-ventral boundary of the wing disc in Drosophila or in developing somites in vertebrates, the interaction of ligands with the Notch receptor is modulated by differential glycosylation of Notch (3, 4). This is effected by Fringes, β1,3-N-acetylgalactosaminyltransferases that extend O-fucose glycans attached to epidermal growth factor-like (EGF) domains (5, 6).

Drosophila has single fringe (fng) and Notch genes, and two Notch ligands, called Delta and Serrate (SER). Expression of fng potentiates the activation of Notch by Delta while inhibiting the activation of Notch by SER (7, 8). Mammals have four Notches, three Delta-related ligands, two Serrate-related ligands (called Jaggeds), and three Fringes. Although only some of the many possible Fringe-ligand-Notch combinations have been examined, mammalian Fringes can also modulate Notch signaling, both in vivo and in cell-based assays (6, 9–14). Binding assays in which soluble forms of Notch or its ligands are bound to cells expressing a ligand or Notch have, in most cases, found that Fringes influence Notch-ligand binding, identifying this as a critical step modulated by Fringe (5, 9, 11, 15). However, because these studies all relied on in vivo glycosylation, they could not reveal whether glycosylation per se is sufficient to modulate Notch-ligand interactions, or instead whether it acts as a precursor to subsequent events.

In Chinese hamster ovary (CHO) cells, the N-acetylgalactosamine-fucose (GlCNac-β1,3-Fuc) disaccharide that is the product of Fringes is further elongated by other glycosyltransferases to yield a tetrasaccharide, Sia-α2,3-Gal-β1,4-GlcNAc-β1,3-Fuc (6, 16). Investigations of the glycan structures that mediate Fringe-dependent modulation of Notch signaling have been carried out in CHO cells by using mutants deficient in specific steps of glycosylation. The ability of Manic fringe (Mfng) or Lunatic fringe (Lfng) to inhibit Jagged1 to Notch1 signaling in these cells requires the action of β1,4-galactosyltransferase 1 (β4GalT-1), which is also required for the elongation of the GlcNAc-β1,3-Fuc disaccharide to a Gal-β1,4-GlcNAc-β1,3-Fuc trisaccharide (14). These observations, together with the lack of requirement for sialylation, suggested that the relevant glycan structure for Fringe-dependent modulation of Notch signaling in mammalian cells is the trisaccharide Gal-β1,4-GlcNAc-β1,3-Fuc (14).

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The abbreviations used are: EGF, epidermal growth factor-like; β4GalT, β1,4-galactosyltransferase; AP, alkaline phosphatase; ββGalNAcT, β1,4-N-acetyl-galactosaminyltransferase; FNG, Fringe; SER, Serrate; MS/MS, tandem mass spectrometry; CID, collision-induced dissociation; LC, liquid chromatography; CHO, Chinese hamster ovary; HBSS, Hanks’ balanced salt solution.

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Gene-targeted mutations in murine β4GalT-1 are viable and do not exhibit the defects in Notch signaling observed in Lfng mutants (17, 18), although subtle defects in the expression of Notch pathway targets in developing somites have recently been identified (19). The absence of a Lfng phenotype in β4GalT-1 mutant mice might be due to genetic redundancy, because there are six mammalian members of the β4GalT family with similar amino acid sequences that can catalyze the transfer of Gal in a β1,4 linkage to GlcNAc acceptors (20–22).

However, Drosophila encode only two members of the β4GalT family, and animals that are doubly mutant for both genes are actually required for the influence of FNG on Notch signaling, at least in Drosophila.

To determine whether any post-translational events subsequent to the addition of GlcNac by FNG are required for its influence on Notch-ligand binding, we employed an in vitro glycosylation and ligand binding assay. The influence of FNG on Notch–SER and Notch–Delta binding can be reconstituted in vitro with purified components. Our results show that the simple addition of GlcNac by FNG is sufficient to dramatically alter the interaction of Notch with its ligands, in a manner that suffices to explain the biological effects of fng on Notch-signaling in vivo. Conversely, further elongation of the GlcNac-Fuc disaccharide by Gal has no noticeable effect on Notch-ligand binding in vitro. These observations enhance our mechanistic understanding of the regulation of Notch signaling and confirm a striking example of the direct modulation of protein–protein interactions through glycosylation.

EXPERIMENTAL PROCEDURES

Expression Constructs—Notch (N), Delta (DL), and Serrate (SER) constructs were all expressed under the control of the metallothionein promoter. The alkaline phosphatase (AP) fusion proteins N:AP, DL:AP, and SER:AP, cloned into the pRMHa-3 vector (N:AP/pRMHa-3, DL:AP/pRMHa-3, and SER:AP/pRMHa-3), were gifts from S. Cohen and have been described previously (5). N:AP includes amino acids 1–1467 of Notch; DL:AP includes amino acids 1–592 of DL; and SER:AP includes amino acids 1–1213 of SER (see Fig. 1). N-EGF:FLAG includes amino acids 66–1452 of Notch and has been described previously (15) (see Fig. 1). Fc:AP includes the Fc domain from human IgG and has been described previously (15).

To facilitate purification of larger quantities of DL:AP and SER:AP, these transgenes were cloned from pRMHa-3 into pMT(WB) (23), which includes the blasticidin resistance gene as a selectable marker for making stable cell lines. The plasmids Delta:AP/pMT(WB) and Serrate:AP/pMT(WB) were constructed by ligating 3.5- or 5.3-kb, respectively, EcoRI-XbaI fragments from Delta-AP/pRMHa3 or Serrate-AP/pRMHa3, respectively (5) into pMT(WB).

To facilitate purification of ligand-AP fusion proteins, hexahistidine tags were inserted into DL:AP and SER:AP by amplifying across Stul-Xbal for (DL) or Stul-Xhol for (SER) fragments including the C terminus of AP from Delta:AP/pMT(WB) or Serrate:AP/pMT(WB) using as primers (forward) TGATGTGATCCTAGGTGGAGG and (reverse, Delta) GCTCTAGAGCATGGTGATGGTGATGACCCGGGTGCGCGGCGT or (reverse, Serrate) AACCGCTCGAGGCATGGTGATGGTGATGACCCGGGTGCGCGGCGT-CGGTG (underlined nucleotides encode His6 tag) and cloning into Stul-XbaI-cut Delta:AP/pMT(WB) or Stul-XhoI-cut Serrate:AP/pMT(WB) to create DL:AP:His6/pMT(WB) or SER:AP:His6/pMT(WB), respectively.

Full-length, His-tagged FNG was expressed in S2 cells from a pMTHy vector construct that has been described previously (6). Full-length β4GalNAcTA and β4GalNAcTB were expressed from pMT(WB) constructs that have been described previously and are active on a pNP-GlcNac substrate (23).

Protein Purification—For purification of hexahistidine-tagged proteins, stably transfected S2 cells were cultured to 40 ml in Schneider’s Drosophila medium (Invitrogen), and expression was induced by addition of 0.7 mM CuSO4 for 2 days. The cells were then pelleted by centrifugation, and the conditioned medium was mixed with 50 μl of His-Select nickel affinity gel (Sigma) with gentle agitation on an orbital shaker overnight at 4 °C. The beads were then pelleted by centrifugation at 5000 × g for 5 min and washed three times in 50 mM sodium phosphate, pH 8.0, 0.3 M sodium chloride, 10 mM imidazole. The ligands were eluted from beads in 100 μl of 50 mM sodium phosphate, pH 8.0, 0.3 M sodium chloride, 250 mM imidazole, and the eluate was dialyzed overnight at 4 °C in HBSS (1.26 mM CaCl2, 5.33 mM KCl, 0.44 mM Na2HPO4, 0.34 mM NaHCO3, 0.41 mM MgCl2, 0.41 mM MgSO4, 138 mM NaCl, 4 mM NaHCO3, 0.3 mM Na2PO4, 5.6 mM glucose).

Cell-based Binding Assays—Cell-based binding assays were conducted as described previously (5, 15), using 4 μg of N:AP DNA, 2 μg each of glycosyltransferase DNA (pMTHy-FNG: His6, pMT(WB)-β4GalNAcTA, or pMT(WB)-β4GalNAcTB), and empty vector DNA (pRMHa-3) to bring the total transfected DNA to 8 μg in all cases. Knockdown of β4GalNAcTA or β4GalNAcTB by RNA interference was performed as described previously (15), using 40 μg of double-stranded RNA added 6 h after transfection of expression constructs. This procedure reduces but does not eliminate β4GalNAcT expression as monitored by reverse transcription-PCR. The cells were then cultured for 4 days, transgene expression was induced for 2 days using 0.7 mM CuSO4, and conditioned medium was collected, centrifuged 10 min at 14,000 × g to remove cell debris, and assayed for AP activity.

In Vitro Binding Assays—In vitro binding assays were performed as described (15), except that in some assays, affinity-purified ligands were used. Briefly, N-EGF:FLAG (~1 μg) was loaded onto –FLAG beads (Sigma) and incubated with ligand-AP fusion proteins (affinity-purified or in conditioned medium). After washing, binding was quantified by assaying for alkaline phosphatase activity. N-EGF:FLAG was modified by FNG either in vivo (by coexpressing FNG in S2 cells as described above) or in vitro. In vitro glycosylation of N-EGF:
FLAG by FNG was conducted as described previously (24). For \textit{in vitro} glycosylation of N-EGF:FLAG by \( \beta \)-GalNAcT-1, anti-FLAG beads (Sigma) loaded with Notch (N-EGF:FLAG or N-EGF:FLAG from cells co-transfected with FNG) or S2 conditioned medium were equilibrated with glycosylation buffer (50 mM Hepes, pH 7.7, 150 mM NaCl, 50 mM MnCl\(_2\)), and 5 \( \mu \)l of beads were incubated with 100 milliunits of \( \beta \)-GalNAcT-1 (Sigma) and 2.5 \( \mu \)M UDP-Gal (Sigma) for 4 h at 28 °C. The beads were then washed four times in HBSS and used for \textit{in vitro} binding (15).

Sugars used in competition binding assays were purchased from Sigma, except for GlcNAc-\( \beta \)-fucose, which was a gift from Dr. Kushi Matta (Roswell Park Memorial Institute, Buffalo, NY). The sugars were premixed with ligand:AP fusion proteins, and these solutions were then mixed with beads.

For quantitation of labeling with FNG \( \beta \)-GalNAcTA, or \( \beta \)-GalNAcC, 1 \( \mu \)g of N-EGF:FLAG on anti-FLAG beads was incubated with purified FNG (6), \( \beta \)-GalNAcTA, or \( \beta \)-GalNAcTB (23), and 3.6 \( \mu \)M UDP-[\(^{14}C\)]GlcNAc (266 mCi/mmol, Amersham Biosciences), or 2.5 \( \mu \)M UDP-[\(^{14}C\)]GalNAc (266 mCi/mmol, Amersham Biosciences) in 50 \( \mu \)l of glycosylation buffer for 4 h at 28 °C. The beads were then washed four times in HBSS and subjected to scintillation counting. The counts on mock loaded (S2 conditioned medium) beads were taken as background. To normalize counts to the amount of labeled sugar, 2 \( \mu \)l of labeled UDP-sugar was counted directly in scintillation fluid.

Quantitation of galactose added to GlcNAc on N-EGF:FLAG was performed by \textit{in vitro} radiolabeling with UDP-[\(^{3}H\)]galactose (60 Ci/mm; American Radiolabeled Chemicals, Inc.) and saturating levels of bovine \( \beta \)-GalT-1 (Sigma) as described (25). Removal of N-glycans by digestion with peptide N-glycosidase F, release of O-glycans by alkali-induced \( \beta \)-elimination, and characterization of the released glycans by gel filtration chromatography on a Superdex peptide column were all performed as described (26).

**Carbohydrate Analysis**—Quantitative compositional analysis of carbohydrates was performed by acid hydrolysis and Dionex high pressure anion exchange chromatography as described (27). N-EGF:FLAG was eluted from anti-FLAG beads using 3\( \times \) FLAG peptide (Sigma) by mixing 0.5–ml beads with 0.5 ml of 3\( \times \) FLAG peptide (150 ng/ml in Tris-buffered saline) and incubating 30 min at 4 °C. The beads were then pelleted for 30 s at 8200 \( \times \) g, and the supernatant was dialyzed in HBSS to remove 3\( \times \) FLAG peptide. Coomassie staining of SDS-PAGE gels (not shown) confirmed that N-EGF:FLAG was purified to apparent homogeneity. Approximately 10 \( \mu \)g of N-EGF:FLAG purified from S2 cells with or without exogenous FNG was concentrated by acetone precipitation and hydrolyzed in 2 \( m \) trifluoroacetic acid (Pierce) for 4 h at 100 °C. The samples were dried in a SpeedVac, resuspended in water, passed through a C\(_{18}\) ZipTip (Millipore), and dried again. The pellets were dissolved in 100 \( \mu \)l of water and analyzed by high pH anion exchange chromatography on a CarboPac PA-1 column (20 \( \mu \)l/run) on a Dionex DX-300 system with pulsed amperometric detection. All of the experiments were performed in duplicate.

Mass spectral analysis of O-fucose glycosylation sites was performed essentially as described (28). Briefly, \( \approx \)1 \( \mu \)g of N-EGF:FLAG expressed in S2 cells with exogenous FNG was reduced and alkylated, separated by SDS-PAGE, and subjected to in-gel tryptic digestion. The resulting peptides were analyzed by LC-MS/MS on an Agilent XCT Ion Trap mass spectrometer. Glycosylated peptides were identified by searching MS/MS data for neutral losses of the GlcNAc-fucose disaccharide (349.1 Da). Loss of the disaccharide gave a characteristic fragmentation pattern allowing rapid identification of glycopeptides (see Fig. 6). The mass of the unglycosylated peptide was then matched to predicted masses of tryptic peptides from Notch containing the O-fucose consensus sequence: C\( ^2 \)XXX(S/T)C\( ^3 \), where C\( ^2 \) and C\( ^3 \) are the second and third conserved cysteines of an EGF domain (29) (see Table 1). Once glycopeptides were identified, additional searches of the MS/MS data for the unmodified peptides were performed (Extracted Ion Searches). The extracted ion searches take advantage of the fact that glycosidic linkages are more labile than peptide bonds upon collision-induced dissociation (CID), and hence the major product ion from fragmentation of a glycopeptide is the unglycosylated peptide. Using this method, peptides bearing different forms of the O-fucose glycans (e.g. mono- or disaccharide) were found (see Fig. 6, Table 1, and supplemental Fig. S3). No glycopeptides with tri- or tetrasaccharide forms of O-fucose were identified, nor, aside from O-glucose glycans (to be reported elsewhere) were any other modifications of O-fucose bearing peptides identified.

**RESULTS**

\( \text{fng} \) null mutant flies die as first instar larvae, whereas hypomorphic alleles are viable but exhibit defects in wing and eye development (30–32). By contrast, flies doubly mutant for \( \beta \)-GalNAcTA and \( \beta \)-GalNAcTB are viable and appear morphologically normal (23). Because these are the only \textit{Drosophila} homologues of mammalian \( \beta \)-GalTs that act on GlcNAc acceptors, the absence of developmental phenotypes indicated that this gene family does not influence Notch signaling during \textit{Drosophila} development and suggested that further elongation of the GlcNAc-\( \beta \)-Fuc disaccharide might not be required for modulation of Notch signaling in \textit{Drosophila}. Nonetheless, we examined the influence of these glycosyltransferases in Notch-ligand binding assays. A secreted fragment of Notch including most of the extracellular domain fused to alkaline phosphatase (N:AP, Fig. 1) can bind specifically to ligand-expressing cells. This assay has been used previously to demonstrate that FNG can influence Notch-ligand binding (5, 15). To examine their potential influence, we overexpressed \( \beta \)-GalNAcTA or \( \beta \)-GalNAcTB in cells producing N:AP and FNG or down-regulated their expression by RNA interference. However, neither elevated nor decreased expression of these glycosyltransferases detectably influenced Notch-ligand binding (Fig. 2), consistent with the absence of Notch or \( \text{fng} \) phenotypes in \( \beta \)-GalNAcTA and \( \beta \)-GalNAcTB null mutant animals (23).

**The Influence of Fringe Is Detectable in an \textit{in Vitro} Binding System**—The above observations argued against the possibility that \( \beta \)-GalNAcTA or \( \beta \)-GalNAcTB are required for the modulation of Notch signaling by FNG but left open the possibility that some other as yet unidentified glycosyltransferases effect a functionally important modification of GlcNAc on Notch, be it addition of Gal or some other sugar. Moreover, prior studies
investigating the influence of FNG on Notch-ligand binding have all relied on in vivo glycosylation by FNG and have been conducted with conditioned medium rather than purified proteins. Thus, they could not address the potential importance of modifications subsequent to glycosylation or of additional, accessory factors in FNG-dependent modulation of Notch signaling.

We established an in vitro binding assay using a Notch construct (N-EGF:FLAG; Fig. 1) comprising all 36 EGF domains of Drosophila Notch fused to a triple FLAG epitope tag (15). This Notch polypeptide was then purified on anti-FLAG agarose beads, and the beads were mixed with ligands fused to AP (Fig. 1). This in vitro binding system provides a means for determining whether or not additional factors or modifications are required for the influence of FNG on Notch. Importantly, both the positive modulation of Notch-Delta binding and the negative modulation of Notch-Serrate can be detected with this assay when N-EGF:FLAG expressed in the presence of FNG is compared with N-EGF:FLAG expressed in the absence of FNG (Fig. 3A). Thus, the influence of FNG can be detected using an in vitro binding assay.

The GlcNAc-Fuc Disaccharide Is Sufficient to Modulate Notch-Ligand Binding—To evaluate the potential significance of post-translational modifications subsequent to FNG, we modified this in vitro assay by first purifying N-EGF:FLAG expressed from S2 cells without FNG and then glycosylating it in vitro with FNG. When N-EGF:FLAG is purified on anti-FLAG beads, only a single prominent band is detected on Coomassie-stained gels (supplemental Fig. S1A). Similarly, when Fringe:His6 was purified using agarose-Ni2+ beads, only a single prominent band was detected by Coomassie staining (supplemental Fig. S1B). We then conducted in vitro glycosylation experiments, using N-EGF:FLAG attached to beads, soluble Fringe:His6 and UDP-[14C]GlcNAc. The addition of ∼4.5 mol of GlcNAc/mol of N-EGF:FLAG was catalyzed by FNG in this reaction. Although there are 23 potential sites of O-fucosylation on Drosophila Notch, it is not known whether all of them can be modified by FNG. In addition, because FNG requires a properly folded EGF domain (29), if any Notch on the beads was misfolded or aggregated, the calculated value of ∼5 sites/Notch could be an underestimate. Regardless, these results suggest that purified Notch can be substantially modified by purified FNG in vitro.

To investigate whether factors in the conditioned medium of S2 cells were required for the influence of glycosylation on Notch-ligand binding, we also constructed new versions of the SER:AP and DL:AP transgenes that included a C-terminal His6 tag. This enabled us to purify soluble forms of these tagged ligands to apparent homogeneity on Ni2+-agarose beads (supplemental Fig. S1C).

Importantly, the influence of FNG on ligand binding was readily detectable when in vitro binding assays were conducted with these purified ligands, using purified N-EGF:FLAG glycosylated in vitro by FNG (Fig. 4). Thus, the effect of FNG can be reproduced in vitro with purified components. Although the fold enhancement in Delta binding was
less with in vitro glycosylated Notch (Fig. 4) than with in vivo glycosylated Notch (Fig. 3A), these results clearly demonstrate that the simple addition of GlcNAc is sufficient to dramatically alter the interactions of Notch with its ligands. They further indicate that this effect does not require the presence of detectable levels of other proteins or of subsequent in vivo modifications.

Galactose Does Not Modify Drosophila Notch-Ligand Binding in Vitro—Although the above experiments indicate that simple addition of GlcNAc by FNG is sufficient to modulate Notch-ligand binding, it remained possible that further elongation could nonetheless alter this binding, either enhancing the effects of FNG, as suggested by studies in CHO cells (14), or counteracting its effects, if a terminal GlcNAc were critical to Notch modulation. Thus, in a complementary approach to evaluating the impact of glycosylation on Notch-ligand binding, we investigated the consequences of in vitro elongation of the GlcNAc/1,3-Fuc disaccharide. Purified forms of GalNAcTA or GalNAcTB have some activity on a simple pNp-GlcNAc acceptor (23) but did not detectably transfer GalNAc to FNG-modified N-EGF:FLAG in vitro (supplemental Fig. S2). Thus, Notch is not a good substrate for these glycosyltransferases, consistent with recent reports that they participate in glycolipid biosynthesis (33–35) and the absence of fng or Notch.
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phenotypes in mutants (23). To elongate GlcNAc on Notch in vitro, we therefore turned to mammalian β4GalT-1, which is known to modify the GlcNAc-Fuc disaccharide in vivo and has been implicated in modulation of Notch signaling in mammalian cells (14, 19).

For these experiments, N-EGF:FLAG was isolated from wild-type or FNG-expressing S2 cells and then glycosylated in vitro with saturating amounts of bovine β4GalT-1 and UDP-[3H]Gal. When N-EGF:FLAG was produced in the absence of FNG, a maximum of ~3.6 mol of Gal were added to each mol of protein. When produced in FNG-expressing S2 cells, ~15.9 mol of Gal were added to each mol of N-EGF:FLAG. Thus, FNG caused an increase in incorporation of Gal of ~12.3 mol/mol, suggesting that ~12 GlcNAc residues were added to each molecule of Notch by FNG in vivo, which were then substrates for β4GalT-1 in vitro. This estimate is higher than the 4.5 mol of...
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GlcNAc added by FNG in vitro, implying that in vivo modification by FNG was more efficient. This might explain why there was a larger effect of FNG on the binding of in vivo modified N-EGF:FLAG (Fig. 3A) than the in vitro modified protein (Fig. 4).

To characterize the galactosylated products of the β4GalT-1 treated Notch, peptide N-glycosidase F-treated material was subjected to alkali-induced β-elimination to release the O-glycans, which were then separated by size on a Superdex peptide column (Fig. 3C). The radiolabeled O-glycans from the N-EGF:FLAG + FNG sample migrated at the size of Gal-β1,4-GlcNAc-β1,3-fucitol, the expected product of the β4GalT-1 radiolabeling of the GlcNAc-β1,3-Fuc disaccharide. No tri saccharide product could be detected in the N-EGF:FLAG sample expressed in cells without FNG, suggesting that little or no GlcNAc-β1,3-fucose exists in this sample, consistent with the absence of FNG. A small amount of a disaccharide did appear in this sample, possibly the result of a small amount of galactose incorporated into a monosaccharide (possibly O-GalNAc or O-GlcNAc), although the structure of this disaccharide has not been established. These results show that N-EGF:FLAG made in FNG-expressing cells is heavily decorated with the GlcNAc-β1,3-fucose disaccharide, whereas that made in the absence of FNG is not. Importantly, binding studies indicated that Gal addition did not significantly enhance or suppress the effects of FNG on Notch-ER or Notch-Delta binding (Fig. 3B).

No Extension beyond GlcNAc Occurs on Notch Produced in S2 Cells with Exogenous Fringe—Although the in vitro experiments described above indicate that the GlcNAc-β1,3-Fuc disaccharide is sufficient to modulate Notch-ligand binding, they left open the possibility that subsequent modifications nonetheless occur in vivo in Drosophila cells. To investigate this possibility, we performed carbohydrate compositional analysis on N-EGF:FLAG produced in S2 cells in the presence or absence of exogenous FNG. Fig. 5 shows that FNG causes a significant increase in the level of glucosamine (hydrolyzed form of GlcNAc) on N-EGF:FLAG but that no other significant changes are observed. Neither galactose nor galactosamine (hydrolyzed form of GalNAc) were detected in significant amounts in the presence or absence of FNG. Mannose and glucosamine in the absence of FNG are presumably derived from N-glycans on Notch (16), and glucose and xylose are derived from O-glucose glycans (16). Fucose, mannos, glucose, and xylose (and an unidentified species) were unaffected by the presence of FNG. These results indicate that in contrast to mammals (6, 16), no additional sugars are added to GlcNAc following the action of FNG in Drosophila S2 cells.

As an additional means of addressing this question, we analyzed tryptic peptides derived from N-EGF:FLAG by tandem mass spectrometry (LC-MS/MS). We and others have used this approach to identify the structures of O-fucose and O-glucose glycans at specific sites on a protein (28, 36). Using N-EGF:FLAG isolated from S2 cells with exogenous FNG, we have identified several peptides modified with O-fucose and/or O-glucose glycans (a complete description of these results, including identification of peptides modified with O-glucose, will be reported elsewhere). Because the data in Figs. 3 and 5 suggest the presence of the O-fucose disaccharide, GlcNAc-β1,3-fucose, on N-EGF:FLAG made in S2 cells with FNG, we first searched the MS/MS data for ions that lost masses corresponding to this disaccharide upon CID (28). Fig. 6 shows several traces of the chromatogram resulting from analysis of tryptic peptides from N-EGF:FLAG made in S2 cells with FNG. The top trace in Fig. 6A is the base peak chromatogram, showing the elution of the most abundant ions at any given time. Fig. 6B shows a constant neutral loss search of the MS/MS data from the same experiment. The constant neutral loss search queries the data for ions that lose a specific mass (in this case, 174.5, the mass of the GlcNAc-fucose disaccharide on a doubly charged peptide) upon CID fragmentation. One major ion was identified in this search eluting at ~36 min (the ion was fragmented several times during elution, resulting in several closely spaced peaks). Both the MS (top panel) and MS/MS (bottom panel) spectra of this ion are shown in Fig. 6E. The MS spectrum contains an ion ([m/z 872.9] that corresponds to the doubly charged form of a peptide modified with a GlcNAc-fucose (HexNAc-

![FIGURE 6. No elongation past the GlcNAc-β1,3-fucose disaccharide is detected on glycopeptides from Notch produced in S2 cells with FNG. N-EGF:FLAG produced in S2 cells with exogenous FNG was subjected to reduction, alkylation, and in gel trypsin digestion as described under “Experimental Procedures.” The resulting tryptic peptides were analyzed by LC-MS/MS. A, base peak chromatogram showing elution of the most abundant ions in each MS scan. B, constant neutral loss scan for ions losing 174.5 Da (mass of the GlcNAc-fucose disaccharide on doubly charged peptides) upon CID fragmentation. E indicates elution position of the ion [m/z 872.9], which loses 174.5 upon fragmentation (to m/z 698.5). This ion was fragmented several times as it eluted, resulting in multiple closely eluting peaks (identification of the same ion at slightly different elution times is indicated by an asterisk). The spectra associated with this ion are shown in E. C, extracted ion chromatogram (EIC) scan for m/z 698.5 in the MS/MS data. This ion matches the unglycosylated peptide identified in the constant neutral loss scan in B. F indicates elution position of the ion [m/z 771.4], which loses 72.9 Da (mass of Fucose monosaccharide on a doubly charged peptide) upon fragmentation. The spectra are shown in F. E is same as the ion described for B, D, constant neutral loss scan for ions losing 116.3 Da (mass of the GlcNAc-fucose disaccharide on triply charged peptides) upon CID fragmentation. 20, 5, 12, 17, 7, and 3 all represent the elution positions of triply charged ions modified with the GlcNAc-fucose disaccharide. The numbers refer to the EGF domain from which the corresponding tryptic peptide is derived. Spectra for several of these (3, 5, and 7) are shown in Fig. 53, and the data are summarized in Table 1. E, top panel, MS spectra showing the major ions eluting at 36.0 min. M corresponds to the mass of the peptide (sequence shown above spectra) plus HexNAc (GlcNAc and dHex (fucose)). The other ions present in the MS spectrum represent other peptides eluting from the LC at the same time. The red diamonds indicated ions selected for CID fragmentation. Bottom panel, MS/MS spectra of m/z 771.4 ion ([M + 2H]10) from the MS spectrum. The blue diamond shows the position of the parent ion prior to fragmentation. The major fragments show the sequential loss of a HexNAc ([M + 2H- HexNAc]10), a HexNAc ([M + 2H- HexNAc-dHex]10), and a Hex ([M + 2H- HexNAc-dHex]10) from the parent ion. [M + 2H- HexNAc-dHex]10 corresponds to the doubly charged mass of a tryptic peptide from EGF domain 23 of Notch that contains an O-fucose consensus site as for E. Further confirmation of this assignment comes from peptide fragment ions (b and/or y ions), several of which are indicated.)
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**TABLE 1**

O-Fucosylated peptides from N-EGF:FLAG produced in S2 cells with FNG

| EGF | Sequence | Parent ion (M+H+) | Deglycosylated product (M+H+) | Mass Δ | Predicted mass (M+H+) |
|-----|----------|------------------|------------------------------|--------|----------------------|
| 3   | 23: CPLGFDLSCLEIAVPAVCIDTVCLMGOCTCLQK104 | 3986.2 | 3636.7 | 349.5 | 3638.1 |
| 7   | 177: TTLGGVTVPGFVGCCCTSV878 | 3782.2 | 3636.7 | 145.5 | 3638.1 |
| 5   | 177: TTLGGVTVPGFVGCCCTSV878 | 2949.1 | 2600.5 | 348.6 | 2600.9 |
| 23  | 23:66NGASCLNVPGSYR878 | 3945.4 | 3697.7 | 347.7 | 3697.8 |
| 23  | 866:66NGASCLNVPGSYR878 | 1744.8 | 1396.0 | 348.8 | 1395.5 |

DHex) disaccharide. The MS/MS spectrum of the CID-induced fragmentation of this ion is shown in the bottom panel. Fragmentation results in sequential loss of a HexNAc (resulting in m/z 771.4 ion) and a dHex (resulting in m/z 698.5 ion). Because the glycosidic linkages are significantly more susceptible to CID than peptide bonds, the major product of fragmentation is the deglycosylated peptide. The ion at m/z 698.5 corresponds to the predicted mass for the doubly charged form of a tryptic peptide containing an O-fucose consensus sequence from EGF domain 23: 866:NGASCLNVPGSYR878 (see Table 1 for predicted mass). Additional fragment ions in the MS/MS spectrum confirm this assignment (Fig. 6E), because they correspond to N- or C-terminal fragments of the tryptic peptide.

To examine whether this same peptide exists with other modifications, we searched the MS/MS data for the m/z 698.5 ion (extracted ion chromatogram; Fig. 6C). Analysis of these spectra revealed the presence of this peptide modified with the O-fucose disaccharide (Fig. 6E) and the O-fucose monosaccharide (Fig. 6F). These data demonstrate that this analysis is capable of identifying the peptide with different forms of O-fucosylation. No other forms of O-fucose were found using this analysis, suggesting that the peptide is modified by only one of the mono- and disaccharide forms of O-fucose but not by more elongated forms of O-fucose. Additionally, because no other ions that fragmented to the m/z 698.5 ion were detected, we conclude that no other modifications of this peptide exist in S2 cells.

Several other peptides modified with the O-fucose disaccharide were identified by performing a neutral loss search of the MS/MS data for ions losing 116.3 Da (mass of the GlcNAc-fucose disaccharide on a triply charged peptide) upon CID fragmentation (Fig. 6D). Spectra confirming the presence of the O-fucose disaccharide on peptides from EGF domains 3, 5, and 7 are shown in supplemental Fig. S3 and summarized in Table 1. The other ions identified in Fig. 6D (from EGF domains 20, 12, and 17) correspond to peptides that are also modified with O-glucose glycans. Details of their analysis will be reported elsewhere. Extracted ion chromatograms of the deglycosylated peptides revealed that the peptide from EGF domain 3 exists as both the mono- and disaccharide form (Table 1 and supplemental Fig. S3B), whereas those from EGF domains 5 and 7 bear only disaccharide (Table 1 and supplemental Fig. S3, C and D). No additional elongation beyond the disaccharide was found on any of these peptides. Moreover, aside from the O-glucose modification, no other modifications of these peptides were observed. Together with the compositional analysis described above, these data indicate that the GlcNAc added by FNG is not further elongated in Drosophila S2 cells.

**Notch-Ligand Binding Is Not Competed by Exogenous Sugars**—

The observation that Notch-ligand binding is directly modulated by the glycan structures on Notch raised the possibility that Notch ligands might recognize Notch by acting as lectins. In this case, one might expect that Notch ligand binding could be competed by exogenous sugars. To evaluate this possibility, we conducted both cell-based and in vitro ligand binding assays, using both Delta and SER in the presence of the monosaccharides fucose, galactose, glucose, xylose, mannose, GalNAc, and GlcNAc (supplemental Fig. S4 and data not shown). We also examined the influence of the disaccharides GlcNAcβ1,3-Fuc, lactose (Galβ1,4-Glc), and LacNAc (Galβ1,4-GlcNAc) on Notch-Delta binding (supplemental Fig. S4). Although some decrease in ligand binding occurs at very high saccharide concentrations, the effect is nonspecific and thus does not represent specific competition of Notch-ligand interactions by a particular sugar.

**DISCUSSION**

Expression of Fringe glycosyltransferases in Notch-expressing cells has dramatic effects, both enhancing Delta-Notch signaling and suppressing Serrate-Notch signaling (3, 4). Left unclear, however, has been the question of whether the GlcNAc added by FNG needs to be elongated by other glycosyltransferases and whether differential glycosylation per se is sufficient to alter Notch signaling. Indeed, because prior binding and signaling studies all relied on in vivo glycosylation, it had not actually been formally proven that Notch was the biologically relevant FNG substrate. By glycosylating Notch with FNG in vitro and demonstrating that this glycosylation alters the interaction of Notch with its ligands in in vitro binding assays with purified components, we have provided powerful evidence that Notch is the critical substrate for FNG, that glycosylation exerts a direct influence on Notch–ligand interactions, and that the addition of GlcNAc alone to O-fucose on EGF domains of Drosophila Notch is sufficient to enhance the binding of Notch to Delta and to inhibit the binding of Notch to SER. Importantly, the influence of FNG on Notch-ligand binding in vitro is in principle sufficient to account for its biological effects in Drosophila. In addition, our results demonstrate that no further elongation of...
the GlcNAc on O-fucose occurs in FNG-expressing S2 cells. The ability to reproduce the influence of FNG in vitro with purified components further implies that accessory lectins or other co-factors are not essential for glycosylation of Notch to modulate its interactions with ligands.

Two classes of models can be considered for how addition of GlcNAc to EGF domains of Notch alters its binding interactions with ligands. In indirect models, the addition of GlcNAc onto Notch might alter the conformation of the Notch extracellular domain. Such models presume that different conformations of Notch would then have different affinities for SER versus Delta. In direct models, the binding of Delta or SER to Notch would involve the sites of glycan attachment. The lack of detectable inhibition of Notch-ligand binding upon the addition of exogenous sugars argues against a pure lectin model, in which binding interactions are mediated solely by sugars. However, if sugars formed only part of a composite binding site, it is conceivable that exogenous sugars might not act as effective competitors. Thus, it remains possible that GlcNAc is directly involved in ligand binding, forming favorable contacts with Delta, and preventing favorable Notch-SER contacts.

The evidence presented here in favor of action by the GlcNAc-β1,3-Fuc disaccharide in modulating Notch signaling in Drosophila contrasts with the reported requirement for a Gal β1,4-GlcNAc β1,3-Fuc trisaccharide for the inhibition of Jagged1 to Notch1 signaling by mammalian Fringes in CHO cells (14) and a mild influence of murine β4GalT-1 on Notch signaling in vivo (19). A requirement for further elongation in Drosophila is not supported by genetic analysis of the closest Drosophila homologues of mammalian β4GalT-1, β4GalNAcTA, and β4GalNAcTB, because flies doubly mutant for null mutations in these genes do not exhibit fng phenotypes (23).

Moreover, in vitro glycosylation with bovine β4GalT-1 confirms that addition of Gal does not influence Drosophila Notch-ligand binding in vitro.

We offer three possible explanations for the discrepancy regarding the requirement for elongation of the GlcNAc-β1,3-Fuc disaccharide. First, analysis of Notch signaling in Lec1 CHO cells has excluded roles for N-glycans (6), but it remains possible that the reported requirement for β4GalT-1 actually reflects an influence on some other O-glycan or a glycolipid that impinges on the ability of Fringes to modulate the Notch pathway, rather than a requirement for the trisaccharide on Notch. This explanation would presume that even though the influence we observe on binding in vitro is sufficient to account for the affects of FNG, additional factors can nonetheless come into play in vivo.

It is also conceivable that glycosylation of Notch in mammals has additional effects. Indeed, although the effects of FNG on Notch signaling have been consistently found to correlate with its effects on ligand binding in Drosophila (5, 15, 37, 38), the influence of Fringes on Notch signaling in mammalian cells has been reported to correlate with effects on Notch ligand binding in some cases but not in others (9–11). It should be emphasized in this regard that the conclusion of Chen et al. (14) that β4GalT-1 is required for the influence of Fringe was based on a signaling assay rather than a binding assay. Thus, the requirement for Gal might reflect an influence on Notch activation that is separate from an effect on ligand binding, and this additional mechanism might be specific to mammalian cells. Finally, it is conceivable that the effects of glycans on Notch-ligand binding are simply different in mammals, and a Gal influences ligand binding of mammalian Notch1, whereas it does not for Drosophila Notch. Given the general conservation of components of the Notch pathway, it would be surprising if structural differences between mammalian and Drosophila components were great enough for the glycan requirements to actually be distinct, but this possibility cannot be excluded. Resolution of which, if any, of these possibilities accounts for the discrepancy between our observations and those of Chen et al. (14) will require establishment of in vitro glycosylation and ligand binding assays with mammalian components of Notch signaling analogous to what we have achieved here with Drosophila components.

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REFERENCES

1. Schweisguth, F. (2004) Curr. Biol. 14, R129–R138
2. Lai, E. C. (2004) Development 131, 965–973
3. Haines, N., and Irvine, K. D. (2003) Nat. Rev. Mol. Cell. Biol. 4, 786–797
4. Haltiwanger, R. S., and Stanley, P. (2002) Biochim. Biophys. Acta 1573, 328–335
5. Bruckner, K., Perez, L., Clausen, H., and Cohen, S. (2000) Nature 406, 411–415
6. 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
7. Panin, V. M., Papayannopoulos, V., Wilson, R., and Irvine, K. D. (1997) Nature 387, 908–912
8. Fleming, R. I., Gu, Y., and Hukriede, N. A. (1997) Development 124, 2973–2981
9. Hicks, C., Johnston, S. H., diSibio, G., Collazo, A., Vogt, T. F., and Weinmaster, G. (2000) Nat. Cell Biol. 2, 515–520
10. Shimaizu, K., Chiba, S., Saito, T., Kumanu, K., Takahashi, T., and Hirai, H. (2001) J. Biol. Chem. 276, 25753–25758
11. Yang, L. T., Nichols, J. T., Yao, C., Manilay, J. O., Robey, E. A., and Weinmaster, G. (2005) Mol. Biol. Cell 16, 927–942
12. Zhang, N., and Gridley, T. (1998) Nature 394, 374–377
13. Evrard, Y. A., Lun, Y., Aulehla, A., Gan, L., and Johnson, R. L. (1998) Nature 394, 377–381
14. Chen, J., Moloney, D. J., and Stanley, P. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 13716–13721
15. Okajima, T., Xu, A., and Irvine, K. D. (2003) J. Biol. Chem. 278, 42340–42345
16. 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
17. Asano, M., Furukawa, K., Kido, M., Matsumoto, S., Umekoshi, Y., Kobice, N., and Ikawa, Y. (1997) EMBO J. 16, 1850–1857
18. Lu, Q., Hasty, P., and Shur, B. D. (1997) Dev. Biol. 181, 257–267
19. Chen, J., Lu, L., Shi, S., and Stanley, P. (2006) Gene Expr. Patterns 6, 376–382
20. Amado, M., Almeida, R., Schwientek, T., and Clausen, H. (1999) Biochim. Biophys. Acta 1473, 35–53
21. Guo, S., Sato, T., Shirane, K., and Furukawa, K. (2001) Glycobiology 11, 813–820
22. Furukawa, K., and Sato, T. (1999) Biochim. Biophys. Acta 1473, 54–66
23. Haines, N., and Irvine, K. D. (2005) Glycobiology 15, 335–346
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24. Okajima, T., and Irvine, K. D. (2002) Cell 111, 893–904
25. Haltiwanger, R. S., and Philipsberg, G. A. (1997) J. Biol. Chem. 272, 8752–8758
26. Moloney, D. J., Lin, A. L., and Haltiwanger, R. S. (1997) J. Biol. Chem. 272, 19046–19050
27. Hardy, M. R., and Townsend, R. R. (1994) Methods Enzymol. 230, 208–225
28. Nita-Lazar, A., and Haltiwanger, R. S. (2006) Methods Enzymol. 417, 93–111
29. Shao, L., Moloney, D. J., and Haltiwanger, R. (2003) J. Biol. Chem. 278, 7775–7782
30. Irvine, K. D., and Wieschaus, E. (1994) Cell 79, 595–606
31. Correia, T., Papayannopoulos, V., Panin, V., Woronoff, P., Jiang, J., Vogt, T. F., and Irvine, K. D. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 6404–6409
32. Papayannopoulos, V., Tomlinson, A., Panin, V. M., Rauskolb, C., and Irvine, K. D. (1998) Science 281, 2031–2034
33. Sasaki, N., Yoshida, H., Fuwa, T. J., Kinoshita-Toyoda, A., Toyoda, H., Hirabayashi, Y., Ishida, H., Ueda, R., and Nishihara, S. (2007) Biochem. Biophys. Res. Commun. 354, 522–527
34. Chen, Y. W., Pedersen, J. W., Wandall, H. H., Levery, S. B., Pizette, S., Clausen, H., and Cohen, S. M. (2007) Dev. Biol. 306, 736–749
35. Stolz, A., Haines, N., Pich, A., Irvine, K. D., Hokke, C. H., Deelder, A. M., Gerardy-Schahn, R., Wuhrer, M., and Bakker, H. (September 18, 2007) Glycoconj. J., in press (DOI 10.1007/s10719-007-9069-5)
36. 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
37. Sasamura, T., Sasaki, N., Miyashita, F., Nako, S., Ishikawa, H. O., Ito, M., Kitagawa, M., Harigaya, K., Spana, E., Bilder, D., Perrimon, N., and Matsuno, K. (2003) Development 130, 4785–4795
38. Perez, L., Milan, M., Bray, S., and Cohen, S. M. (2005) Mech. Dev. 122, 479–486