Regions of Drosophila Notch That Contribute to Ligand Binding and the Modulatory Influence of Fringe*

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Two glycosyltransferases that transfer sugars to epidermal growth factor (EGF) domains, OFUT1 and Fringe, regulate Notch signaling. To characterize the impact of glycosylation at the 23 consensus O-fucose sites in Drosophila Notch, we conducted deletion mapping and site-specific mutagenesis and then assayed the binding of soluble forms of Notch to cell-surface ligands. Our results support the conclusion that EGFl1 and EGFl2 are essential for ligand binding, but indicate that other EGF domains also make substantial contributions to ligand binding. Characterization of Notch deletion constructs and O-fucose site mutants further revealed that no single site or region can account for the influence of Fringe on Notch-ligand binding. Additionally, we observed an influence of Fringe on a Notch fragment including only 4 of its 36 EGF domains (EGF10–13). Together, our observations imply that glycosylation influences Notch-ligand interactions through a distributive mechanism that involves local interactions with multiple EGF domains and led us to suggest a structural model for how Notch interacts with its ligands.

Notch proteins are transmembrane receptors for an intracellular signaling pathway that affects numerous cell fate decisions throughout the Metazoa (reviewed in Refs. 1 and 2). The extracellular domains of Notch receptors are composed largely of tandemly repeated epidermal growth factor (EGF) domains. The main class of Notch ligands, collectively referred to as DSL (Delta-Serrate-Lag2) ligands, are also transmembrane proteins with multiple EGF domains. In the main Notch signaling pathway, association of Notch with its ligands triggers proteolytic processing of Notch. This liberates the cytoplasmic domain of Notch from the membrane, upon which it travels to the nucleus and forms part of a transcriptional activation complex. The Notch pathway is highly conserved but generally more complex in vertebrates due to the greater number of paralogs for Notch pathway components.

Binding between Notch and its two Drosophila ligands, Delta and Serrate, was first demonstrated by their ability to mediate the aggregation of cultured Drosophila S2 cells (3, 4). Using this assay, 2 of the 36 EGF domains of Notch (EGF11 and EGF12) were identified as necessary and sufficient to mediate binding (4). The remaining 34 EGF domains were identified as having no influence or possibly interfering with optimal binding. Subsequent studies have confirmed the importance of EGFl1 and EGFl2 to DSL ligand binding (5–7); but only a few studies have examined the potential roles for other EGF domains (5, 8–10), and their role in Notch signaling remains poorly understood.

The activation of Notch receptors by DSL ligands is influenced by glycosyltransferases that participate in the synthesis of O-linked fucose glycans attached to EGF domains (reviewed in Refs. 11 and 12). O-Fucosylation of EGF domains is catalyzed by the enzyme O-fucosyltransferase-1 (13, 14). Loss of O-fucosyltransferase-1 activity by RNA interference with or mutation of the Drosophila Ofut1 gene (15–17) or by targeted mutation of the mouse Pofut1 gene (18) results in phenotypes that resemble those observed in the complete absence of Notch signaling. However, studies in Drosophila indicate that OFUT1 has a chaperone activity that is separable from its fucosyltransferase activity (19), which might account for the universal requirement for OFUT1 in Notch signaling. Nonetheless, some aspects of Notch function clearly depend on O-fucosyltransferase-1 activity, as they depend on elongation of the O-linked fucose monosaccharide.

O-Linked fucose is a substrate for β1,3-N-acetylglucomaminyltransferases encoded by fringe genes (20, 21). Glycosylation by Fringe proteins exerts a positive influence on Notch-Delta signaling, but a negative influence on Notch-Serrate signaling (20–27). Genetic and cell culture studies indicate that Fringe (Fng) functions specifically on the receiving side of the Notch pathway (17, 20, 22, 23), and consistent with this, biochemical studies indicate that Notch is a substrate for the glycosyltransferase activities of OFUT1 and Fng (15, 20, 21, 28). Cell-based and in vitro binding studies conducted with Drosophila components of the Notch pathway have consistently supported the conclusion that Fng influences Notch signaling at the ligand binding step (10, 16, 17, 20, 29), although in mammalian cells, the effects of Fng might be more complex (23, 30).

Based on a current consensus sequence, C(2-XXX(G/A/S)(T/S)S(11, 31), 23 of the 36 EGF repeats of Drosophila Notch could potentially be O-fucosylated (see Fig. 1). Additional constraints that influence Fng-dependent elongation of O-fucose on particular EGF domains also exist (32), but it is not yet possible to predict which of the potential sites in Drosophila Notch are efficiently modified by Fng. Initial studies of O-fucose sites focused on two regions of interest. The Abruptex region is defined by mutations in EGF24–29 (see Fig. 1) (33), which result in dominant gain-of-function Notch phenotypes (34), and there are some genetic similarities between the effects.
Characterization of Notch O-Fucosylated Domains

of Abruptex mutations and those of Fng (35). However, individual or paired mutations in O-fucose sites within this region do not detectably influence Notch activity in vivo (29), and Abruptex-like mutations do not consistently influence glycosylation of Notch1 by Fng (32). Another region of interest is the ligand-binding domain. The presence of a potential O-fucose site within EGFD12 is highly conserved among Notch receptors from different phyla, and EGFD12 is O-fucosylated in both mammalian and insect cells (see Fig. 1) (29, 32). Mutation of this site in Drosophila indicated that glycosylation of EGFD12 is not essential for Notch signaling, but does contribute to the modulation of Notch signaling by Fng (29). Nonetheless, this site cannot fully account for the influence of Fng, as Fng still influences both Serrate and Delta binding to a Notch receptor that cannot be O-fucosylated on EGFD12 (29).

We report here on a series of mutations that collectively encompass all of the 23 potential O-fucose sites in Notch. This site-specific mutagenesis has been complemented by analysis of a series of Notch deletion constructs. Our results support the conclusion that EGFD11 and EGFD12 are the most critical EGF domains for ligand binding. However, we indicate that other EGF domains also make substantial contributions to ligand binding. Our results also show that Fng glycosylation influences Notch-ligand binding by a distributive mechanism: there is no single site whose glycosylation can fully account for the effects of Fng; rather, Fng can influence binding through multiple distinct O-fucose sites. This suggests that an influence on local interactions between individual EGF domains of Notch and its ligands is most likely responsible for the influence of Fng glycosylation, rather than glycosylation of a unique site or region or a global influence on Notch structure. Consistent with this, an effect of Fng did not require a complete Notch protein, but could be detected on a fragment of Notch including only four EGF domains.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Plasmids expressing truncated versions of the extracellular domain of Notch fused to alkaline phosphatase (AP; referred to as N:AP) were created using a PCR-based strategy. PCR plasmid pMT-WBN-N-AP was constructed by cloning a 6.3-kb EcoRI-XbaI fragment including Notch EGF domains fused to AP from pMtHA3N-AP (20) into pMT-WB. A 4.5-kb EcoRI-BspEl fragment including most of the Notch sequences was then excised from pMT-WBN-AP and replaced with a PCR product (cut with EcoRI and BspEl) encoding amino acids 1–58 and a PCR product (cut with BspEl) encoding a subset of Notch EGF domains. The addition of the BspEl site adds two amino acids (Ser and Gly) between amino acids 58 and 59 of Notch. For the N-EGFD1–10,13–36 construct, the N-terminal EcoRI-BspEl PCR fragment extended instead through EGFD10. The junction sequences and the orientation of the BspEl fragment were determined by DNA sequencing. The primers and junction amino acids encoded by these PCR products are as follows. For amino acids 1–58, the forward primer was gtgaattccgagctgcctgaacagtcatttgctgtagttt (reverse primer); C-terminal of EGFD3 (ends at Gly1465), ggtactattcggagctgcctgaacagtcatttgctgtagttt (reverse primer); C-terminal of EGFD50 (ends at Asn1522), aaggatttcggagctgcctgaacagtcatttgctgtagttt (reverse primer); and C-terminal of EGFD36 (ends at Gly1465), ggtactattcggagctgcctgaacagtcatttgctgtagttt (reverse primer). Site-specific mutagenesis was conducted as described previously (29) using the QuickChange multi-site-directed mutagenesis kit (Strategene). Mutagenesis was conducted within pBS-N or O-fucose site mutant derivatives. Mutagenesis was designed to change the O-fucosylation site from Ser or Thr to Ala. The primers used for the mutagenesis of each EGF domain were as follows: EGFD1, cagtaaggggagctgcctgaacagtcatttgctgtagttt (forward primer); EGFD2, cagtaaggggagctgcctgaacagtcatttgctgtagttt (reverse primer); EGFD4, eggatcggagctgcctgaacagtcatttgctgtagttt (forward primer); EGFD5, cagtaagggagctgcctgaacagtcatttgctgtagttt (reverse primer); EGFD6, cagtaagggagctgcctgaacagtcatttgctgtagttt (reverse primer); EGFD8, cagtaaggggagctgcctgaacagtcatttgctgtagttt (reverse primer); EGFD9, cagtaagggagctgcctgaacagtcatttgctgtagttt (reverse primer); and C-terminal of EGFD36 (ends at Gly1465), ggtactattcggagctgcctgaacagtcatttgctgtagttt (reverse primer).
identified ligand-binding region is eliminated suggests that other EGF domains can actually make a substantial contribution to Serrate and Delta binding (29). This prompted us to re-examine the contributions of different EGF domains. This was accomplished by employing a cell-based reverse binding assay in which the extracellular domain of Notch was fused to alkaline phosphatase (N:AP), and this soluble form of the receptor was bound to S2 cells transfected to express a Notch ligand (20). An AP-tagged immunoglobulin protein domain (Fc:AP) and S2 cells transfected with empty vector served as negative controls. This assay has been employed previously to characterize the influences of Fng, OFUT1, and Notch mutations on ligand binding (10, 16, 17, 20, 29). It appears to be more sensitive to differences in binding affinity than the cell aggregation assay, as the influence of Fng is readily detectable with this assay, but cannot be detected by cell aggregation (36). It facilitates quantitation of input amounts of Notch (a critical issue when evaluating mutant proteins) as well as amounts of bound Notch, and assays of AP activity were used to normalize the amounts of fusion protein in each experiment. The assay is also roughly linear over a range of concentrations (17), permitting comparisons of relative binding strength to be made.

Binding studies on fragments of Notch including different EGF domains (see Fig. 2A) are summarized in Table I. No constructs lacking EGF11 and EGF12 exhibited binding significantly above background levels, even when 34 of the 36 EGF domains of Notch were included (N:AP-EGF1–10,13–36), consistent with a prior study indicating a critical requirement for these EGF domains in ligand binding (4). However, truncation of Notch EGF domains from either the N terminus (N:AP-EGF10–36) or the C terminus (N:AP-EGF1–24) substantially impaired ligand binding. This suggests that EGF1–9 and EGF25–36 contribute to ligand binding. An intermediate deletion from the N terminus (N:AP-EGF6–36) bound to ligands as well as or slightly better than full-length Notch, indicating that EGF1–5 are dispensable for ligand binding. A more severe deletion from the C terminus (N:AP-EGF1–13) bound detectably to Serrate-expressing cells, but not to Delta-expressing cells. Intermediate deletions from the C terminus (N:AP-EGF1–27, N:AP-EGF1–30, and N:AP-EGF6–30) were not extensively characterized because they were secreted very poorly, presumably due to misfolding.

Fringe Influences Notch-Ligand Binding through Different Regions of Notch—To investigate the contributions of O-fucosylated sites within different regions of Notches to ligand binding, studies were conducted on Notch fragments produced in S2 cells cotransfected to express Fng. In the case of Serrate, we also examined binding by fragments cotransfected to overexpress OFUT1. Although OFUT1 is expressed endogenously by S2 cells (15, 21), and thus, Notch produced in S2 cells is modified by OFUT1, overexpression of OFUT1 enhances Notch-Serrate binding (17). All fragments that exhibited detectable binding remained sensitive to coexpression with these glycosyltransferases (Table I). In some cases, the -fold increases in Delta or Serrate binding for full-length N:AP and truncated N:AP fragments that retained the ability to bind ligands were comparable. However, binding of N:AP-EGF10–36 to Delta-expressing cells was stimulated ~8-fold by Fng, which contrasted with the 26-fold stimulation by Fng of full-length N:AP-EGF binding. Additionally, binding of N:AP-EGF10–36 to Serrate-expressing cells was inhibited only 2-fold by Fng, whereas binding of N:AP to Serrate-expressing cells was inhibited ~13-fold. Also, binding of N:AP-EGF1–13 to Serrate-expressing cells was not detectably enhanced by coexpression with OFUT1, but coexpression with Fng demonstrated that N:AP-EGF1–13 retained the ability to bind to Delta. The only overlap between N- and C-terminally truncated Notch molecules that exhibited Fng-sensitive binding (i.e. N:AP-EGF1–13 and N:AP-EGF10–36) was EGF10–13. The site in EGF12 cannot fully account for the influence of Fng (29), and the site in EGF13 is not well conserved (Fig. 1). Thus, these results suggest that multiple sites through which Fng can act to modulate binding are located in distinct regions of Notch. The reduced sensitivity of N:AP-EGF10–36 to Fringe as compared with that of N:AP-EGF6–36 further suggests that functionally important sites of glycosylation by Fng are located in EGF6–9.

Multiple O-Fucosyl Sites Contribute to the Modulation of Notch-Ligand Binding by Fringe—As a complementary approach to evaluating the influence of glycosylation of different EGF domains on Notch-ligand binding, we created point mutations in the predicted sites of fucose attachment in the context of full-length N:AP-EGF, changing the Ser or Thr residue to which fucose could be added to Ala. These mutations are unlikely to disrupt the structure of individual EGF domains because Ala occurs at this site in some EGF domains of Notch or its ligands, and full-length Notch proteins that include individual O-fucose site mutations can function in vivo (29).
Because a prior study indicated that individual site mutations in EGF24, EGF26, or EGF31 have no detectable influence on Notch activity (29), we focused on making larger arrays of mutations. Initially, we made three arrays: an N-terminal array encompassing the eight O-fucosic sites from EGF1–9 (N:AP-EGF1–9f), a central array encompassing the four O-fucosic sites from EGF13–21 (N:AP-EGF13–21f), and a C-terminal array encompassing the 10 O-fucosic sites from EGF23–32 (N:AP-EGF23–32f). Together with the previously characterized N:AP-EGF12f construct (29), these collectively encompass all 23 of the potential O-fucosic sites in Notch (Fig. 2B). Interestingly, each of the larger arrays of O-fucosic site mutations interferes to some degree with the secretion of N:AP, implying that they compromise Notch folding (19) (data not shown). The observations that OFUT1 has a chaperone activity and that the OFUT1-binding site presumably includes the mutated amino acid (the normal site of O-fucosylation) suggest that multiple O-fucosic site point mutations might impair Notch folding by decreasing OFUT1 binding. Nonetheless, enough secreted N:AP mutants could be collected to conduct binding studies, and equal amounts were employed for all comparisons. Each of the three arrays of mutants retained some ability to bind to ligands, although at a reduced level (Table II). The reduction in binding most likely results from some degree of misfolding, as it can be partially corrected by OFUT1R245A (19) (data not shown). Notably, the binding of each of these O-fucosic site mutants of Notch was nonetheless sensitive to coexpression with OFUT1 or Fng (Table II), reinforcing the conclusion that OFUT1 and Fng can act through multiple non-overlapping sites to modulate Notch-ligand binding. However, although the -fold stimulation of Notch-Delta binding for wild-type N:AP (27-fold) and N:AP-EGF23–32f (36-fold) in these experiments was comparable, stimulation of N:AP-EGF1–9f (11-fold) and N:AP-EGF13–21f (10-fold) appeared to be reduced.

To investigate further the requirements for different sites, we created additional combinations of site mutants (Fig. 2B). Some of these additional constructs were secreted so poorly that it was difficult to get enough material for binding studies (data not shown). Thus, we resorted to coexpression with the chaperone-only form of OFUT1 (OFUT1R245A), which facilitates secretion and folding of mutant forms of Notch, but cannot fucosylate it (19). Most of the smaller arrays of mutations retained some ability to bind to Notch, and in every case where Notch binding could be detected, coexpression with Fng influenced binding (Table III). However, in most cases, the -fold change was reduced. Thus, although we detected an average of 15-fold stimulation of Delta binding by Fng for wild-type N:AP in this set of experiments, N:AP-EGF7–9–20–21f (9-fold), N:AP-EGF12+20–21f (3-fold), and N:AP-EGF12f (6-fold) exhibited reduced stimulation. Similarly, the -fold decrease in Notch-Serrate binding effected by Fng (5-fold for N:AP in this set of experiments) was also affected by these mutations, being reduced to 1.7-fold (N:AP-EGF7–9–20–21f), 2.4-fold (N:AP-EGF12+20–21f), and 2-fold (N:AP-EGF12f). The largest arrays (N:AP-EGF12–32f, N:AP-EGF1–21f, and N:AP-EGF7–21f) were secreted very poorly even with OFUT1R245A, and little or no binding could be detected above background levels. It is not clear whether this reflects an absolute requirement for some degree of fucosylation of Notch or a degree of Notch misfolding that could not be completely offset by expression of OFUT1R245A. Similarly, binding of N:AP-EGF7–12f to Serrate-expressing cells was inhibited 4-fold, whereas binding to Delta-expressing cells was unaffected; but as this mutant was secreted poorly and exhibited relatively little binding above background levels, these results have to be treated with caution. Nonetheless, the combined analysis of all mutants identified at least three regions that contribute to the influence of Fng on Notch-Delta and Notch-Serrate binding: EGFl−9, EGF12, and EGF20–21. None of these regions is sufficient to modulate Notch-ligand binding. However, the observation that EGFls contribute to Notch-ligand binding suggested a model in which multiple EGF domains contribute to Notch-ligand binding, and fucosylation of an individual EGF domain by Fng
Characterization of Notch O-Fucose Sites

The results are from binding assays conducted with conditioned medium including AP activity (800 milli-absorbance units (mAU)/min) of the indicated fusion proteins ("Media"); see Fig. 2B) displayed as described in the legend to Table I. For the results shown here, all of the AP fusion proteins were isolated from cells cotransfected with OFUT1.

### Table II

**Binding of Notch point mutations**

| Control S2 cells | Delta-expressing S2 cells | Ser-expressing S2 cells |
|------------------|---------------------------|------------------------|
|                  | None | OFUT1 | Fng | None | OFUT1 | Fng |
|                  | mAU/min | S.E. | mAU/min | S.E. | mAU/min | S.E. | -fold increase |
| N:AP             | 0.13 | 0.08 | 12.64 | 0.76 | 5.92 | 0.79 | 337.67 | 50.59 | 26.7 |
| N:AP-EGF1–9f     | 0.78 | 0.38 | 5.31 | 1.13 | 1.77 | 0.75 | 60.23 | 10.20 | 11.4 |
| N:AP-EGF13–21f   | 0.14 | 0.33 | 4.46 | 1.75 | 1.08 | 0.50 | 46.07 | 6.63 | 10.3 |
| N:AP-EGF23–32f   | 0.34 | 0.19 | 6.31 | 1.35 | 1.29 | 0.61 | 163.00 | 23.64 | 25.8 |
| F:AP             | 0.02 | 0.06 | 0.62 | 0.50 | 1.12 | 0.40 | 39.29 | 6.50 | 2.18 |

### Table III

**Binding of additional Notch point mutations**

| Media | Control S2 cells | Delta-expressing S2 cells | Ser-expressing S2 cells |
|-------|------------------|---------------------------|------------------------|
|       | mAU/min | S.E. | mAU/min | S.E. | mAU/min | S.E. | -fold increase |
| N:AP  | 0.69 | 0.39 | 17.68 | 1.92 | 261.88 | 73.34 | 14.8 |
| N:AP-EGF7–9+20–21f | 1.00 | 1.03 | 9.27 | 2.73 | 82.42 | 47.03 | 8.9 |
| N:AP-EGF12–13f | 1.03 | 1.90 | 1.52 | 0.48 | 2.52 | 0.40 | 1.67 |
| N:AP-EGF12–32f | 0.33 | 0.70 | -0.05 | 0.37 | 1.38 | 1.52 | 1.90 |
| N:AP-EGF7–12f | 0.89 | 0.30 | 4.31 | 2.41 | 5.19 | 1.34 | 1.2 |
| N:AP-EGF12+20–21f | 1.16 | 0.57 | 22.74 | 5.84 | 66.34 | 15.06 | 2.9 |
| N:AP-EGF7–21f | 1.12 | 0.58 | 3.81 | 0.62 | 0.34 | 1.76 | 3.87 |
| N:AP-EGF12f | -0.18 | 0.85 | 21.13 | 5.90 | 127.58 | 15.58 | 0.6 |
| F:AP  | 0.84 | 0.28 | 1.12 | 0.30 | 0.52 | 0.30 | 0.52 |

**DISCUSSION**

Multiple EGF Domains Contribute to Notch-Ligand Binding—Of the 36 EGF repeats in the extracellular domain of Notch, only two (EGF11 and EGF12) are essential for binding (4). This gave rise to the suggestion that Notch might be a multifunctional receptor in which different EGF domains would interact with different ligands, possibly eliciting different outcomes. An alternative although not mutually exclusive possibility is that many EGF domains actually influence binding to the well established Notch ligands, Delta and Serrate, even though the contributions of some EGF domains are greater than others. Our results indicate that optimal binding between Notch and its ligands requires many of the 36 EGF domains of Notch. First, deletion of other EGF domains decreases ligand binding. Although some deletions appear to impair Notch binding completely, others produce a range of outcomes. An alternative although not mutually exclusive possibility is that many EGF domains actually influence binding to the well established Notch ligands, Delta and Serrate.

**Multiple O-Fucosylation Site Mutations Can Be Dominant-negative in Vivo**—To assay the activity of altered Notch proteins in vivo, we created transgenic flies in which full-length Notch proteins including the EGF1–9f, EGF13–21f, or EGF23–32f site-specific mutation were expressed under UAS-Gal4 control. When ptc-Gal4 was used to drive their expression in a stripe of cells across the wing imaginal disc, these constructs either had no activity or appeared to inhibit Notch function to varying degrees (data not shown). The appearance of these Notch loss-of-function phenotypes suggests that these mutant forms of Notch can, to some degree, act as dominant-negative proteins. As each of these mutants is secreted relatively poorly in S2 cells, they evidently have a tendency to misfold, and the dominant-negative effect might result from mutant Notch interfering with the folding, secretion, or activity of wild-type Notch.
Influence of Glycosylation on Notch-Ligand Binding—The determination that multiple EGF domains influence Notch-ligand binding provides a simple explanation for the observation that Fng influences the binding of a Notch receptor that cannot be O-fucosylated on EGF11 or EGF12 (29). Indeed, the observation that Fng can modulate the binding of ligands to a Notch receptor that it cannot glycosylate on EGF11 or EGF12 (N-EGF12f) clearly indicates that glycosylation of other EGF domains has a major impact on Notch-ligand binding. However, analysis of Notch mutants that cannot be glycosylated on other EGF domains indicated that there is no specific site or even region of Notch that acts as an essential focal point for the action of Fng. Instead, glycosylation of disparate repeats in different areas of the extracellular domain is sufficient to modulate binding. Among these, EGF7–9, EGF12, and EGF20–21 appear to exert the greatest effect. Finally, we note that, even though EGF12 or EGF13 is not essential for an influence of Fng within the context of full-length Notch, both can apparently become essential within the context of a mini-Notch composed of EGF10–13. Altogether, these observations imply that glycosylation of different EGF domains can independently and locally influence Notch-ligand interactions.

This conclusion has two important implications for our understanding of Notch-ligand binding. First, the conclusion that glycosylation of an individual EGF domain can directly modify binding of that EGF domain to Delta or Serrate argues against the hypothesis that glycosylation influences binding solely by effecting a global conformational change in Notch. This conclusion is reinforced by the observation that even binding of a minimal Notch fragment can be modulated by Fng. The conclusion that glycosylation of many distinct individual EGF domains can modulate binding in turn suggests that multiple EGF domains of Notch normally participate in interactions with ligands. In this regard, analysis of the influence of glycosylation on Notch-ligand interactions provides independent support for the conclusion from our deletion studies, i.e. that multiple EGF domains participate in Notch-ligand interactions, not just EGF11 and EGF12.

Model for Notch-Ligand Interactions—The conclusion that multiple EGF domains of Notch participate in interactions with ligands and the pattern of predicted Ca\(^{2+}\)-binding EGF do-

| Media       | Control S2 cells | Delta-expressing S2 cells | Ser-expressing S2 cells |
|-------------|------------------|---------------------------|-------------------------|
|             | mAU/min S.D.     | mAU/min S.D.              | mAU/min S.D.           |
| None        | 0.87 0.13        | 28.80 5.14                | 480.62 51.85           |
| N:AP        | 3.24 0.98        | 69.78 10.30               | 7.07 4.20              |
| N:AP-EGF10–13| 1.20 0.71       | 1.20 0.71                 | 1.57 0.19              |
| N:AP-EGF10–13f| 1.72 0.92       | 1.38 0.31                 | 2.43 1.04              |
| N:AP-EGF10–13f| 1.20 0.71       | 1.20 0.71                 | 1.57 0.19              |
| N:AP-EGF10–13f| 1.72 0.92       | 1.38 0.31                 | 2.43 1.04              |
| N:AP-EGF10–13,13f| 0.85 0.73     | 1.18 0.68                 | 1.28 1.25              |
| N:AP-EGF10–13,13f| 0.85 0.73     | 1.18 0.68                 | 1.28 1.25              |
| None        | 1.18 0.68        | 0.18 0.73                 | 0.68 0.49              |
| OFUT1       | 0.87 0.24        | 0.52 0.26                 | 1.37 0.47              |
| Fng         | 0.87 0.24        | 0.52 0.26                 | 1.37 0.47              |

The results are from binding assays conducted with conditioned medium including AP activity (1600 milli-absorbance units (mAU)/min) of the indicated fusion proteins (“Media”; see Fig. 2C) displayed as described in the legend to Table I, except that S.D. is indicated instead of S.E. Even region of Notch that acts as an essential focal point for the action of Fng within the context of full-length Notch, both can apparently become essential within the context of a mini-Notch composed of EGF10–13. Altogether, these observations imply that glycosylation of different EGF domains can independently and locally influence Notch-ligand interactions.

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Model for Notch-Ligand Interactions—The conclusion that multiple EGF domains of Notch participate in interactions with ligands and the pattern of predicted Ca\(^{2+}\)-binding EGF do-
linker between tandem Ca$^{2+}$-binding EGF domains in multiple proteins has been found to be stiff, with the EGF domains packed end-to-end, forming an elongated structure (38–40). Recently, a structure for three Ca$^{2+}$-binding EGF domains of Notch1 has been reported, and these, too, form an extended structure with “stiff” linkers (40). Extending these structural observations to other EGF domains of Notch, one can predict that EGF10–21 will form an extended stiff chain, with EGF6–9 and EGF22–26 folded back against them (Fig. 3D) (40).

This predicted structure is appealing because it provides an immediate and logical explanation for how other EGF domains participate in ligand binding. Notch ligands contain an N-terminal DSL motif, followed by a series of tandemly repeated EGF domains. If a Notch ligand is packed against the proposed structure for EGF6–26 of Notch, then it would have opportunities to interact with many different EGF domains (Fig. 3C), as indicated by our binding studies. Although this structural model must be considered speculative at this point, it provides a simple and logical explanation for how different EGF domains of Notch contribute to ligand binding. According to this model, the central feature of a Notch-ligand complex is a triple-stranded structure. Additional features of these proteins are consistent with this suggestion. The structure of EGF10–12 of Notch1 revealed that, although the linkers are stiff, there is a 120° twist between adjacent EGF domains (40). Within the context of a triple-stranded structure, this would allow for alternating yet repeated interactions along the length of the strands. Intriguingly, in some Notch receptors, most noticeably mammalian Notch1 receptors, there is a tendency to have a three-EGF repeat in the pattern of predicted O-fucose sites (e.g., EGF2–10 and EGF20–28) (Fig. 1).

Another appealing feature of the proposed model is that extension of the suggested Notch structure beyond EGF6–26 might explain why certain Notch mutations are associated with elevated ligand binding and how ligand binding triggers proteolytic cleavage of Notch. We propose that, in the absence of ligand, more N- and C-terminal EGF domains could pack against the central region, forming a Notch triple-stranded structure (Fig. 3D). Ligand binding would thus involve a strand displacement process (Fig. 3E). This model would explain the slight elevation in binding of N:AP-EGF6–36 relative to wild-type Notch (Table I) as resulting from the lack of need to displace EGF2–5. Similarly, the enhanced ligand binding of EGF12f mutations (Table III) (29) could result from weakened interactions with EGF2–5. The possibility of strand displacement at the C terminus and the characterization of the Abrupect alleles of Drosophila Notch further suggest a possible explanation for how ligand binding makes Notch a substrate for metalloproteases, which is the critical step in Notch activation. Strand displacement would effect a conformational change at the C terminus of the extracellular domain and hence could act as the critical conformational trigger that makes this region accessible to a protease. In support of this hypothesis, we note that the Abrupect alleles behave genetically as though they confer increased sensitivity to ligands, and molecularly, they are all single amino acid substitutions that map to EGF24, EGF25, EGF27, or EGF29 of Notch. Their location within the proposed triple-stranded structure suggests that they could enhance the ability of ligands to activate Notch by decreasing the stability of the proposed Notch triple-stranded structure (Fig. 3, D and E). Although other explanations for the Abrupect alleles have been suggested (10, 35, 41), the proposed model, although speculative, provides a good explanation for the gain-of-function aspect of Abrupect mutations and is not excluded by prior studies.

Delta Versus Serrate—One of the most intriguing features of Fng is its opposite influences on Serrate versus Delta signaling. Thus, although glycosylation clearly could have effects on intramolecular Notch EGF domain interactions, the differential influence of Fng suggests that intermolecular Notch-ligand interactions are most critically affected by glycosylation. Notably then, despite some slight differences, overall, our results indicate that many of the same sites that contribute to the ability of Fng to enhance Delta binding also contribute to the ability of Fng to inhibit Serrate binding. How can the same glycosylation event have such different effects on the two Notch ligands? Within the context of our proposed model for Notch-ligand interactions, we suggest that Delta and Serrate pack against Notch in different phases (Fig. 3E) such that, in the case of Serrate, binding to Notch is sterically hindered by the addition of GlcNAc to EGF domains by Fng. Conversely, in the case of Delta, the GlcNAc residue added by Fng would form favorable contacts with Delta. The phases of binding would presumably be set by regions that participate in the strongest binding interactions, which, in the case of the ligands, is thought to be the N-terminal region, including the DSL motif (reviewed in Ref. 42). Although admittedly speculative, this phase-positioning model would make it possible to reconcile the observations that swapping the N terminus of Serrate for that of Delta is sufficient to stop Fng from inhibiting Serrate signaling (25), even though, as we have shown here, Fng can act through disparate regions of Notch to modulate ligand binding.

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