Down-regulation of Delta by proteolytic processing

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Notch signaling regulates cell fate decisions during development through local cell interactions. Signaling is triggered by the interaction of the Notch receptor with its transmembrane ligands expressed on adjacent cells. Recent studies suggest that Delta is cleaved to release an extracellular fragment, DlEC, by a mechanism that involves the activity of the metalloprotease Kuzbanian; however, the functional significance of that cleavage remains controversial.

Introduction

Notch signaling defines a conserved mechanism that controls the acquisition of cell fate in metazoans through local cell interactions (Kimble and Simpson, 1997; Egan et al., 1998; Greenwald, 1998; Weinmaster, 1998; Artavanis-Tsakonas et al., 1999). Signals transmitted into a cell through the Notch surface receptor are activated by ligands expressed in adjacent cells. Two ligands, Serrate and Delta, have been identified with certainty. Both are single-pass transmembrane proteins comprised of a single Delta, Serrate, Lag-2 (DSL)* domain followed by EGF-like repeats, a short juxtamembrane domain, a transmembrane segment, and a short cytoplasmic tail (Fleming et al., 1998; Kopczynski et al., 1990). Molecular genetic evidence suggests that the ligands interact via their DSL domain with a specific region in the extracellular domain of Notch to trigger a series of molecular events that eventually result in the transmission of a signal from the surface to the nucleus (Rebay et al., 1991, 1993a,b; Fitzgerald and Greenwald, 1995). Not much is understood about the molecular mechanisms that regulate Notch activation at the cell surface, but over the past few years, several studies have presented evidence suggesting that the proteolytic processing of both the receptor and the Delta ligand is important for signaling (Pan and Rubin, 1997; Wen et al., 1997; Jarriault et al., 1998; Klueg and Muskavitch, 1999; Qi et al., 1999; Ye and Fortini, 1999; Brou et al., 2000; Klein, 2002; Lieber et al., 2002; Mumm et al., 2000).

Using independent functional assays in vitro and in vivo, we examined the biological activity of purified soluble Delta forms and conclude that Delta cleavage is an important down-regulating event in Notch signaling. The data support a model whereby Delta inactivation is essential for providing the critical ligand/receptor expression differential between neighboring cells in order to distinguish the signaling versus the receiving partner.

*Abbreviations used in this paper: DSL, Delta, Serrate, Lag-2; E(spl)-C, enhancer of split complex.

Key words: Delta; Kuzbanian; Notch; cleavage; down-regulation
inactive, leading to the conclusion that cleavage is a down-regulating event in ligand activity. We discuss the developmental significance of this cleavage and suggest a functional role for Kuzbanian activity in Notch signaling.

Results
Characterization of soluble Delta fragments
Western analysis of extracts from tissues and cultured Drosophila S2 cells show that the ligand Delta is cleaved at an extracellular site close to the transmembrane domain, shedding a fragment that encompasses most of the extracellular domain (DlEC; Qi et al., 1999). Conditioned medium from S2 cells stably expressing Delta (S2-Dl) was used to purify DlEC to homogeneity by affinity chromatography using the 9B monoclonal antibody (C594.9B; Qi et al., 1999; Fig. 1 A).

Resolution of the highly purified product by SDS-PAGE and silver staining demonstrates two species migrating as a doublet of 63 and 65 kD, with the 63 kD species being the predominant form. NH$_2$-terminal sequence analysis revealed a single sequence consistent with the putative NH$_2$ terminus resulting from the signal peptide cleavage (unpublished data; Qi et al., 1999). Direct chemical COOH-terminal sequence analysis determined that the COOH-terminal residue of both isoforms is alanine. These results were corroborated by tryptic digestion followed by mass spectrometry, which revealed the existence of two peptides ending in the sequence LTNA and . . . QYGA. We conclude that Delta is cleaved at two distinct sites: COOH-terminal to Ala581 and Ala593, respectively. Henceforth, we refer to these two isoforms as DlEC$^{581}$ and DlEC$^{593}$.

In our attempts to explore the functional significance of the two extracellular cleavages in Delta, we generated truncated soluble molecules mimicking the DlEC$^{581}$ and DlEC$^{593}$. In addition, we mutagenized the Ala581 and Ala593 amino acids to serine (henceforth Ala581Ser and Ala593Ser; Fig. 1 B). The constructs were transfected into Drosophila S2 cells, which endogenously express Kuzbanian (Pan and Rubin, 1997) but not Delta. Transfection of the DlEC$^{581}$ and DlEC$^{593}$ constructs effectively generated soluble secreted products, with DlEC$^{593}$ exhibiting a slightly different molecular mass, consistent with the 11-amino-acid difference in their COOH-termini (Fig. 2 A). When expressed in S2 cells, both the Ala581Ser and Ala593Ser mutants were cleaved to generate a product of essentially the same size as DlEC (Fig. 2 B). In addition, we generated an Ala581,593Ser double mutant which was also cleaved to generate a product similar to DlEC (unpublished data). We also assessed the cis or trans requirement of Kuz in Delta cleavage using the S2 cell-culture system. S2 cells stably expressing either WT Kuz or dominant-negative Kuz, when mixed with S2-Dl cells, do not affect Delta cleavage (unpublished data); Kuz effects are seen only when it is cotransfected with Delta into the same cell (unpublished data; Qi et al., 1999).

DlEC–Notch interaction
It has been established that cells expressing Notch aggregate with Delta-expressing cells (Fehon et al., 1990). Although a rigorous, in vivo demonstration that this interaction is direct is still lacking, we do know that specific regions in the extracellular domain of Notch and Delta are necessary and sufficient for aggregation (Rebay et al., 1991; Muskavitch, 1994; Shimizu et al., 1999, 2000). We sought to examine whether WT DlEC, DlEC$^{581}$, and DlEC$^{593}$ interact with Notch and thus inhibit the normal Notch–Delta-mediated cell aggregation.

Preincubation of S2-N cells with concentrated conditioned medium from S2-Dl cells causes a >60% inhibition of aggregation rate (Fig. 3 A; Qi et al., 1999). In contrast, concentrated conditioned medium from S2 cells stably expressing each of the mutant forms of soluble Delta (Fig. 3 A) showed essentially no inhibitory effect in the aggregation assay.

The medium from S2-Dl–expressing cells was fractionated on an anti-Delta (9B) antibody affinity column, and selected fractions were tested for their inhibitory effect in the aggregation assay. We find all the inhibitory activity in the flowthrough of the affinity column. Further, the purified DlEC fractions showed essentially no inhibitory activity in the aggregation assay. We find all the inhibitory activity in the flowthrough of the affinity column. Further, the purified DlEC fractions showed essentially no inhibitory activity in the aggregation assay. Western blot analysis of the different fractions during purification of DlEC showed that the flow through contains full-length Delta, (unpublished data; Fig. 3) suggesting that the inhibitory effect could be either attributed to this Delta protein species or to an un-
DlEC fragment does not compete with the Notch–Delta interaction mediating the cell aggregation.

Assessment of DlEC activity by in vitro activity assays
We extended this analysis by examining the activity of the soluble Delta molecules using three independent in vitro assays of Notch activation.

Cortical neuron neurite retraction assay. It has previously been shown that Notch activation can directly influence neurite morphology by preventing neurite outgrowth from the outset as well as inducing neurite retraction (Qi et al., 1999; Sestan et al., 1999). The presentation of exogenous ligand to neurons, which express the Notch receptor, results in the activation of the receptor and the retraction of the neurites. When primary cortical neurons isolated from E14 mice and cultured in vitro were treated with conditioned medium from S2-Dl, we observed extensive retraction, whereas neurons treated with purified (see above) DlEC, DlEC581, or DlEC593 exhibited minimal or no retraction of neurites, suggesting that these soluble forms cannot activate the Notch signaling pathway (Fig. 5 A). Western analysis of the S2-Dl medium shows the presence of full-length Delta in addition to the DlEC (Fig. 5 B).

N2A neurite outgrowth assay. An independent neurite outgrowth assay using the Neuro2a mouse neuroblastoma cell line (N2a) confirmed these observations. Withdrawal of serum from the N2a cells triggers neurite formation in ∼40% of the
cells in 16–24 h. The simultaneous activation of the Notch pathway in N2a has been shown to inhibit the outgrowth of neurites (Franklin et al., 1999). Addition of S2-Dl concentrated medium to the N2a cells at the point of serum withdrawal causes a significant inhibition in neurite outgrowth (Fig. 6, A and B). In contrast, concentrated conditioned medium from the DlEC<sup>581</sup> and DlEC<sup>593</sup> cells has no effect.

**E(Spl)m3 expression assay.** Finally, we monitored the effect that soluble Delta forms have on Notch-dependent transcriptional activity. For this we established that the HLH gene m3 of the enhancer of split complex (E[spl]-C) is transcriptionally responsive to Notch activation in S2 cells (Fig. 7 A). In the same cells, the HLH mβ and mγ E(spl)-C transcripts are constitutively expressed or remain unresponsive, respectively, to Notch activation in S2 cells (Fig. 7 A). rp49, which encodes the ribosomal protein L32, was used as an internal control for the RT-PCR (Al-Atia et al., 1985). The effects of full-length and soluble Delta on Notch signaling were analyzed using the up-regulation of m3 expression as a reporter. To distinguish between the juxtacrine (full-length) and paracrine (soluble) effects of Delta, a transwell system was used as has previously been used for studies in EGF signaling (Nakagawa et al., 1996). Although aggregation of S2-N and S2-Dl cells leads to an up-regulation of m3, such an effect is not seen when S2-N cells are cocultured with S2-Dl cells in a transwell. We subjected S2-Dl cells to formalin fixation to stabilize the full-length form of Delta on the cell surface. Aggregation of S2-N with formalin-fixed S2-Dl cells resulted in an induction of m3 expression, which interestingly, does not decline over time as seen with live Delta cells (Fig 7 B). Treatment of S2-N cells with 5× concentrated conditioned medium from S2-Dl cells leads to the expression of m3. However, Western analysis of the conditioned medium shows the presence of full-length Delta in the medium (Fig 7 C).

In conclusion, all of the in vitro assays we employed consistently indicate that the soluble forms of Delta are not active, and support the notion that cleavage of Delta corresponds to an inactivation of the ligand. On the other hand, they also corroborate the existence of a soluble agonist activity in fractions containing small amounts of full length Delta.

**Activity of soluble and mutant Delta constructs in vivo**

The in vitro studies were extended by an assessment of the activity of the mutant constructs in transgenic flies. Flies carrying the various Delta mutants were generated and the effects of expression of the different DlEC isoforms and Delta mutants were analyzed in vivo. Expression was driven by the eye specific glass (pGMR) promoter, which is active in all cells posterior to the morphogenetic furrow. Flies expressing DlEC<sup>581</sup> and DlEC<sup>593</sup> exhibited mild eye phenotypes (Fig. 8, top) similar to those that we reported earlier (Sun and Årta-
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vanis-Tsakonas, 1997). We do not understand the underlying mechanism of the weak effects associated with soluble ligand expression; however, the severity of phenotypes of the various transgenic lines varies from mild to no phenotype, suggesting a link with the level of over expression. This may correlate with the slight inhibition of aggregation we observe with micromolar amounts of purified DIEC in the in vitro aggregation assay (unpublished data).

In contrast, a severe glassy eye phenotype was exhibited with the Ala581Ser and Ala593Ser mutants. Significantly, very similar results are seen with pGMR driven overexpression of WT Delta, consistent with the notion that the cleavage site mutations, in addition to being ineffective at preventing cleavage, do not significantly alter the biological activity of Delta.

Because the effects of Notch signals are highly dependent on the developmental context, we sought to corroborate the effect of the mutants in another tissue and assayed expression of the mutants in the developing wing using the UAS-Gal4 system. The two drivers used were Vg-Gal4, which drives expression along the D/V boundary of the developing wing disc and the A9-Gal4, which drives expression predominantly in the dorsal wing compartment. The severity of phenotypes associated with the different mutants is similar to those observed in the eye. Expression of DIEC^{581} and DIEC^{593} with the vestigial driver did not affect wing development, whereas the A9-gal4 driver resulted in the formation of mild wing deltas (Fig. 8, bottom). Transgenic flies expressing either Ala581Ser, Ala593Ser, or WT Delta with the vestigial or A9 driver could not eclose and pharate adults exhibited a severe wing phenotype. Raising the flies at 18°C helped in obtaining a few escapers all of which exhibited rudimentary wings.

In summary, the in vivo activity of the mutant Delta molecules corroborates the results of the cell-based analysis. It is important to note that unlike the strong phenotypes associated with the overexpression of the WT ligand, or the full-length mutant ligands that are normally cleaved in the aforementioned cell based assays, the soluble forms have mild effects. The expression of the soluble Delta isoforms in every context examined could at best elicit only mild phenotypes consistent with the notion that these molecules are inactive.

Discussion

The past few years have brought an increasing appreciation of the importance of proteolysis in the Notch signaling pathway, but the exact mechanistic role of these events is not well established and is often contradictory (Pan and Rubin, 1997; Sotillos et al., 1997; Jarriault et al., 1998; Klueg et al.,
The finding that the proteolytic processing of Delta releases soluble DlEC raised the obvious question of the functional significance of this cleavage. Even though several studies have addressed this question, either directly or indirectly, in flies, nematodes and vertebrates, it is unclear whether this is an antagonistic or agonistic event in Notch signaling (Fitzgerald and Greenwald, 1995; Hukriede et al., 1997; Sun and Artavanis-Tsakonas, 1997; Qi et al., 1999). Our initial characterization of soluble fractions of Delta suggested an agonistic function for the DlEC. More rigorous biochemical characterization presented here clearly shows Delta proteolysis yields more than one DlEC (DlEC581 and DlEC593), neither of which exhibit significant biological activity. Furthermore, the previously reported soluble activity is most likely attributed to trace levels of full-length Delta in the cell culture media. Our conclusion is that the proteolytic processing of Delta is a step that renders this Notch ligand inactive.

Based on the results we report here, we suggest that the agonistic activity, previously reported by us to be associated with the medium from Delta expressing cells, was not due to the activity of DlEC. However, we note that the present study detected the presence of a “soluble” activity in the medium, raising the possibility that such an activity may after all exist in vivo. Formally at least, this activity can be attributed to the detectable quantities of full length Delta in the medium or to another yet-to-be-determined molecule. It is not inconceivable that soluble, full-length, membrane-associated Delta may in fact be secreted into the medium even if only to act on a neighbor rather than over long distances.

![Image of Figure 6](https://example.com/image.png)

Figure 6. **DlEC does not inhibit neurite outgrowth in neuroblastoma N2A cells.**

(A) Withdrawal of serum from actively growing N2A cells results in neurite outgrowth. This response is associated with Notch activation and shown in representative cultures in A. Addition of 5× concentrated conditioned medium from S2-Dl cells at the time of serum withdrawal inhibits neurite outgrowth. (B) The number of neurite presenting cells relative to the total number of cells in the plate is represented in the graph. Conditioned medium from S2, S2-DlEC581, and DlEC593 had a very mild effect on neurite outgrowth as compared with the effect of conditioned medium from S2-Dl cells.
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For instance, in the case of wingless, the existence of membrane exovesicles as a vehicle for wingless delivery has been documented (Greco and Eaton, 2001). Whether a soluble, biologically significant Delta activity can be generated by exocytic events remains to be tested, but we suggest that it is worth considering.

Despite the uncertainty of the role of ligand processing, several studies have attempted to use soluble forms of the ligand as an agonist of the receptor with variable success. However, a common element in these studies is that the soluble forms display activity only if they are forced into an oligomeric state either via Fc fusions (Wang et al., 1998; Shimizu et al., 1999) or by immobilization on a matrix (Varnum-Finney et al., 2000). We find that the biologically inactive DIEC fragment secreted in the medium does not have a natural tendency to oligomerize because it exists in a monomeric state (as judged by gel filtration and centrifugal/sedimentation studies [unpublished data]). Furthermore, the inactivity of DIEC expressed in vivo indicates that a biologically relevant mechanism for immobilization of the DIEC so as to make it active is nonexistent. Therefore, it is of utmost importance to consider the physiological relevance of continued attempts to employ soluble ligands as Notch agonists.

Irrespective of the potential requirement for oligomerization or immobilization as an essential activation step for the ligand, Muskavitch (1994) has proposed that endocytosis of the dissociated Notch extracellular domain bound to Delta into the Delta-expressing cells (transendocytosis) is a critical part of the Notch signaling mechanism (Parks et al., 2000). If such a mechanism is essential for Notch activation, then the blocking of an endocytic event may result in inhibition of signaling. This notion is also compatible with the in vivo analysis which demonstrates that membrane-tethered forms of either Delta or Serrate lacking the intracellular domain cannot undergo effective endocytosis, and hence behave as antagonists of Notch signaling (Sun and Artavanis-Tsakonas, 1996; Klueg et al., 1998). On the other hand, the present analysis shows that Delta molecules fixed on the cells, similar to a molecule immobilized on a matrix, is still capable of activating the Notch receptor. This observation...
would then favor the hypothesis that endocytosis of Delta may be a facilitating but not necessarily an essential part of Notch signaling.

In assessing the developmental significance of Delta cleavage, the activity of Kuzbanian needs to be examined more closely. Although the initial link between Notch signaling and Kuzbanian was reported to involve Notch processing, genetic data show that multiple copies of Delta can suppress the phenotypes associated with dominant-negative Kuzbanian (KuzDN) expression. This observation is compatible with the notion that Delta cleavage produces an active soluble ligand (Qi et al., 1999). However the mechanism of action of KuzDN is not known and it may be equally plausible to consider that KuzDN acts by sequestering Delta, such that the addition of more WT Delta molecules suppress the KuzDN phenotype. It is also worth emphasizing that whereas the dominant-negative forms of Kuzbanian inhibit Delta cleavage, and that Delta cleavage products are not detected in loss of function kuz embryos (Qi et al., 1999), it is quite possible that the Kuzbanian–Delta interaction is indirect. The original proposal that Kuzbanian is involved in the proteolytic processing of Notch has been challenged by subsequent experimentation (Qi et al., 1999; Brou et al., 2000; Mumm et al., 2000). Indeed, recent reports documenting Kuzbanian cleavage of Notch rely on deletion mutants of the receptor that are susceptible to cleavage, bringing further uncertainty to the physiological relevance of Kuzbanian acting on Notch directly (Lieber et al., 2002).

A model to explain the role of Delta down-regulation by proteolysis must consider the mechanism of action of the

Figure 8. **Mild eye and wing phenotypes associated with DlEC in transgenic flies.** (top) Scanning electron micrograph (SEM) of adult eyes. Expression of DlEC581 and DlEC593 driven by the GMR promoter yields a phenotype similar to a WT eye with occasional miss positioning or duplication of bristles (arrows). Expression of Ala581Ser, Ala593Ser, and WT Delta with the same driver results in duplication of bristles and melting and smoothing of lens material. Tangential sections show extra photoreceptors (arrowheads) as well as extra interommatidial pigment cells (*) in the Ala581Ser, Ala593Ser, and WT Delta eyes. (bottom) Adult wing phenotypes resulting from expression of Delta mutants: DlEC581 and DlEC593 exhibited WT wings with the Vg-Gal4 driver, whereas mild wing deltas and occasional extra vein material was seen with the A9-Gal4 driver. Expression of WT Delta under either the Vg- or A9-driver resulted in rudimentary wings. The Ala581Ser or Ala593Ser mutants resulted in phenotypes identical to those seen with WT Delta (not depicted).
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Figure 9. A functional model for Delta cleavage. Notch and Delta are initially expressed equivalently on the cell surface. Delta (Dl) is cleaved and consequently inactivated either directly or indirectly by Kuzbanian (Kuz). The ligand is down-regulated so as to no longer interact with the Notch receptor in the same or adjacent cells; Delta removal is reinforcing the “signal receiving status” of that cell. Thus, the Notch receptor (N) can interact with the ligands in the adjacent, “signaling” cell. Although the model implies that Kuz is differentially regulated between critical neighbors, this hypothesis is yet to be tested. Furthermore, the model allows for the possibility of a positive interaction between Kuz and Notch, which has been postulated by some studies.

Notch ligands. Delta can influence Notch through two modes of action: in trans, where Notch and Delta are presented on adjacent cells and Delta can act as agonist (Jarriault et al., 1998, Heitzler and Simpson, 1991), or in cis, where Notch and Delta are presented on the same cell and Delta (and Serrate) can act as a dominant-negative antagonist (de Celis and Bray, 1997, Klein et al., 1997, Micchelli et al., 1997; Jacobsen et al., 1998). It is well established that cells in tissues undergoing Notch signaling can express Notch and Delta simultaneously. For instance, in the early Drosophila embryo, all cells in the proneural clusters, the group of cells which will eventually segregate into epidermal and neuronal lineages via Notch–Delta signaling, express both Notch and Delta (Muskavitch, 1994; Artavanis-Tsakonas et al., 1995; Kimble and Simpson, 1997; Weinmaster, 1997). However, in order for proper signaling to occur, there must be a distinction between a signaling versus a receiving cell. The accumulated studies to date suggest that the critical parameter for a cell to be a receiving or signaling cell is the ratio rather than the absolute expression levels of Delta and Notch (Simpson, 1990, 1997a,b; Kimble and Simpson, 1997; Greenwald, 1998). Moreover, feedback loops may be responsible for consolidating and amplifying a given state (ratio) (Muskavitch, 1994; Huppert et al., 1997; Kimble and Simpson, 1997; Greenwald, 1998). Thus, a mechanism that inactivates Delta in a given cell may contribute to the feedbacks that are necessary to establish a critical expression differential between two neighbors.

Mosaic analysis in Drosophila during cell fate acquisition in the neuroectoderm demonstrated that Kuz is required in cells to receive signals that inhibit the neural fate (Rooke et al., 1996; Rooke and Xu, 1998). These signals are known to be transmitted through the Notch receptor and this cell autonomous effect of Kuz is consistent with studies in nematodes (Wen et al., 1997). Similarly, using a cell-culture system, we find that dominant-negative or WT forms of Kuzbanian can affect Delta only when cotransfected in the same cell, and have no effect when transfected into adjacent cells (unpublished data). Hence, we suggest that Kuz acts on Delta in the same cell, although it is not clear whether the cleavage occurs at the cell surface or inside the cell. In either case, we propose that this proteolysis renders Delta incapable of interacting with Notch either on an adjacent cell or on the same cell.

Therefore, we favor a model (Fig. 9) whereby proteolytic processing of Delta on a Notch/Delta-expressing cell has the overall effect of rendering that cell the signal receiving cell by (a) alleviating the dominant-negative activity of Delta toward Notch on that cell; and (b) down-regulating the Delta available to signal Notch on adjacent cells. Consistent with this model, Lai et al. (2001) propose a similar role for the neuralized ubiquitin ligase in Delta down-regulation (Pavlopoulos et al., 2001). Interestingly, because neuralized is not required in all Notch dependent developmental contexts, these authors emphasize that multiple mechanisms must exist to clear Delta from the plasma membrane. The above model is also compatible with the possibility that Kuzbanian may play more than one role in Notch signaling. For example, if Kuz is somehow involved in the activation of the Notch receptor (Pan and Rubin, 1997; Lieber et al., 2002), the existence of an activity such as Kuzbanian in a particular cell, which is able to simultaneously enhance receptor function and inactivate the ligand, is a hypothesis worth testing. One of the predictions of the proposed scheme is that Kuzbanian activity must be differentially regulated between critical neighbors. More experimentation will be necessary to confirm or discount this hypothesis and indeed this model.

Materials and methods

Preparation of Delta conditioned media and purification of DIEC

Medium concentrates from Drosophila Schneider 2 cells (S2) and Delta-expressing S2 cells (S2-Dl) were prepared, and the extracellular domain of Delta was purified as described previously (Qi et al., 1999). For the present study, cultures of S2-Dl cells (2–4 × 10^6 cells/ml) were scaled up to 2–4 liter. The cells were centrifuged at 700 g for 10 min and then resuspended in Sang’s M3 medium without serum or bactopeptone or yeastolate supplement at ~10^6 cells/ml. Delta expression was induced with the addition of CuSO4 to 0.7 mM and cultured for an additional 48 h. The conditioned medium was collected after centrifugation at 2000 g and clarified by centrifugation at 10,000 g for 10 min. Proteins were precipitated with 70% saturation of (NH4)2SO4 and centrifuged at 20,000 g for 40 min. The pellet was resuspended in 20 ml of 20 mM Hepes, 150 mM NaCl, 2 mM CaCl2, pH 7.4 (HBS, Ca^2+), at which point it was dialyzed 3× against 2 l of HBS, Ca^2+. The dialyzed sample containing DIEC was used for immunoaffinity purification and size exclusion chromatography.

Immunopurification of DIEC was performed by passing the S2-Dl cell medium protein concentrate over a 1.5–ml anti-Delta (9B, Qi, Rand et al., 1999) Sepharose column, followed by washing with 10 column volumes each of HBS, Ca^2+, and HBS, 1 M NaCl, Ca^2+. The DIEC was eluted with 0.1 M glycine, pH 2.8, and neutralized immediately with 1 M Tris HCl, pH 8.0. The peak fractions were pooled and dialyzed extensively against HBS, Ca^2+ and stored at 4°C. The flowthrough fraction was collected as a pool and was stored at 4°C or frozen at ~20°C. Fractions were analyzed by SDS-PAGE and Western blotting with anti-Delta (9B) monoclonal antibody and with the aggregation assay described below.
Size exclusion chromatography was performed in HBS, Ca\(^{2+}\) on a Superdex HR200 column using an AKTA FPLC system (Amersham Biosciences). Fractions were collected and analyzed by SDS-PAGE and Western blotting with anti-Delta (9B) monoclonal antibody and with the aggregation assay described below.

**COOH-terminal amino acid sequencing and mass spectrometry**

COOH-terminal amino acid sequencing and mass spectrometry was performed at the Harvard Microchemistry Facility (Boston, MA). Samples were prepared from Coomassie blue-stained SDS-PAGE gel slices of immunopurified DlEC. COOH-terminal sequencing was performed with an HP GI1005A protein sequence with online 1090HPLC-MS/MS peptide sequence was performed on tryptic digests of DlEC followed by microcapillary reverse-phase HPLC nanoelectrospray tandem mass spectrometry on a Finnigan LCQ quadrupole ion trap mass spectrometer. The spectra were then correlated with known sequences using the algorithm Sequest (Eng et al., 1994; Yates, 2000) and programs developed at the Harvard Microchemistry Facility (Chittum et al., 1998).

**Delta mutant construction**

Various Delta mutant forms were generated using the Stratagene site-directed mutagenesis kit and primers with the specific amino acid codons altered. For DlEC\(^{527}\) and DlEC\(^{531}\) a stop codon was added after the GCG at position 1885 or after GCC at position 2192, respectively. The ~2-kb EcoR1 fragment encoding the soluble Delta was then cloned into the pMT vector (Invitrogen). For the point mutants, the GCG at position 1881 (encoding Ala581) was changed to CGA for serine and the GCC at position 1913 (encoding Ala593) to CTG for CTT for serine. The mutant forms were cloned into the EcoR1 site of either pRmHa-3 (Bunch et al., 1988) or pUAST (Brand and Perrimon, 1993) vectors.

**Cell culture**

*Drosophila* S2 cells were routinely cultured in Sango M3 medium with 19% fetal bovine serum and bactopeptone (2.5 g/L) and ascorbate (1 g/L). S2 cells were plated 1:30 and plated for 1 to 2 h in D10 to allow Notch activity modulation (Franklin et al., 1999). The medium was harvested by spin-coupled harvests of S2-N and S2-Dl cells in the upper chamber. Twice the amount of S2-N cells were mixed with the other aggregation assays to compensate for the RNA from DlEC. The plates were incubated for various time intervals and then RNA was harvested from the S2-N cells. As a control, RNA was harvested from S2-N and S2-Dl cells incubated alone. 750 ng of RNA was used for the RT reaction using a poly-dT24 primer. The same RT was then used to perform PCR for m\(\bar{f}\), my, m3, and rp49. The following primers were used for the PCR reactions: rp49, AGT ATCTGATGCTCCAACATCG and TTCCGACAGGTTCAGAAGAC; m3, AACAGCAGCAACCAGCAG and GGACTCGTCCGGAGAAT; m\(\bar{f}\), ATACGTTCATGCTCGACATG and ATTCAGAGGTGGTGGAATGCTG; and my, GCTAATGAGGTCTCCGGTCT and GGTCAACAGGAATGCACTGG.

**Fly strains**

All transgenic flies were generated in a w\(^{1118}\) background. The fly strains used in genetic interaction studies were Df(2R)Dll. The gal4 lines used were: A9-gal4, a gift from Dr. K. Wierdinger (Brown University, Providence, RI); Vg-gal4, a gift from Dr. K. Vijayraghavan (TIFR, Bangalore, India); and GMR-gal4, a gift from Dr. G.M. Rubin (Howard Hughes Medical Institute, Chevy Chase, MD).

**Construction of transgenic flies, scanning EM, and sectioning of adult eyes**

Germeline transformation was performed using standard procedures described by Spradling (1986). Each construct was injected with the \(\Delta 2\)-helper plasmid into w\(^{1118}\) embryos. For scanning EM, adult flies were dehydrated sequentially in 25, 50, 75, and 100% ethanol, for at least 12 h in each step. The 100% dehydration was repeated three times. The scanning EM was performed at the Electron Microscopy facility at Northeastern University (Boston, MA). Plastic sections were prepared and observed as described previously (Carthew and Rubin, 1990).

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**Supplementary Material**

The Neuro 2a (N2a) cell line (ATCC CCL-131) was used to assay neurite outgrowth. This cell line is well characterized for its ability to differentiate upon withdrawal of serum. This differentiation to produce extensive neurite outgrowth, has been previously documented to be associated with Notch activity modulation (Franklin et al., 1999). N2a cells were grown in DME with 10% fetal calf serum and penicillin and streptomycin (D10). Confluent cultures were split 1:30 and plated for 1 to 2 h in D10 to allow the cells to adhere. Differentiation was induced by switching the cells to serum free DME. After 16–20 h of culture the degree of neurite outgrowth was quantified by counting the number of cells expressing a neurite of greater than one cell body diameter in length. The effect of DlEC containing medium and purified DlEC was assayed by the addition of a 20 \(\mu\)l sample to the 1 ml of culture medium in each well. Assays were performed in quadruplicate.
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