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Serrate-mediated activation of *Notch* is specifically blocked by the product of the gene *fringe* in the dorsal compartment of the *Drosophila* wing imaginal disc

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SUMMARY

In the developing imaginal wing disc of *Drosophila*, cells at the dorsoventral boundary require localized *Notch* activity for specification of the wing margin. The *Notch* ligands *Serrate* and *Delta* are required on opposite sides of the presumptive wing margin and, even though activated forms of *Notch* generate responses on both sides of the dorsoventral boundary, each ligand generates a compartment-specific response. In this report we demonstrate that *Serrate*, which is expressed in the dorsal compartment, does not signal in the dorsal regions due to the action of the *fringe* gene product. Using ectopic expression, we show that regulation of *Serrate* by *fringe* occurs at the level of protein and not *Serrate* transcription. Furthermore, replacement of the N-terminal region of *Serrate* with the corresponding region of *Delta* abolishes the ability of *fringe* to regulate *Serrate* without altering *Serrate*-specific signaling.

Key words: *Drosophila*, wing development, *Serrate*, *fringe*, Notch activation

INTRODUCTION

The Notch family of transmembrane proteins is a highly conserved group of molecules that are believed to serve as receptors for cell-to-cell communication in both invertebrate and vertebrate organisms (reviewed by Artavanis-Tsakonas et al., 1995). In *Drosophila*, *Notch* function is required for a multitude of developmental processes including neurogenesis, mesoderm formation, germ line and ovarian follicle cell development, larval Malpighian tubule formation, sensory structure differentiation, eye development and limb (wing blade) formation (reviewed by Artavanis-Tsakonas et al., 1991, 1995; Corbin et al., 1991; Ruohola et al., 1991; Xu et al., 1992; Larkin et al., 1996; Hartenstein et al., 1992; Hartenstein and Posakony, 1990; Cagan and Ready, 1989; Welshons, 1965; Rulifson and Blair, 1995; Diaz-Benjumea and Cohen, 1995; De Celis et al., 1996; Neumann and Cohen, 1996). The genes *Delta (Dl)* and *Serrate (Ser)* encode molecules capable of binding with the NOTCH protein and have properties consistent with those expected for NOTCH ligands (Vässin et al., 1987; Kopczynski et al., 1988; Fleming at al., 1990; Thomas et al., 1991; Fehon et al., 1991; Rebay et al., 1991).

Notch function has been most extensively analyzed during neurogenesis where it has been shown that the interaction of the *Dl* product (DL) with NOTCH is required for the proper segregation of neuroblasts from the remaining hypodermal precursors (Heitzler and Simpson, 1991, 1993; Artavanis-Tsakonas and Simpson, 1991; Artavanis-Tsakonas et al., 1991). During embryonic neurogenesis, the *Ser* product (SER) does not normally function as a NOTCH ligand since it is not expressed at this time (Fleming et al., 1990). However, ectopic expression of SER demonstrates that it can function to replace DL as the NOTCH ligand during this developmental stage (Gu et al., 1995). These findings suggest that the SER and DL ligands interact with NOTCH in a developmentally distinct but functionally equivalent manner.

Recent studies have focused on the role of *Notch* signaling during the formation of the adult wing blade in *Drosophila*. *Notch* signals are required for proper establishment of the dorsal-ventral (D/V) wing margin via the activation of margin-specific gene expression and also for cell proliferation within the dorsal and ventral wing compartments mediated in part by activation of the genes *wingless (wg)* and *vestigial (vg)* (Diaz-Benjumea and Cohen, 1995; Couso et al., 1995; De Celis et al., 1996; Doherty et al., 1996; Kim et al., 1996; Neumann and Cohen, 1996). Interestingly, proper specification of the wing margin requires NOTCH interactions with both of its identified ligands. SER is expressed within the dorsal wing compartment and is required for signaling via NOTCH to the ventral compartment while DL is required as the ventral-to-dorsal signal (Diaz-Benjumea and Cohen, 1995; Couso et al., 1995; De Celis et al., 1996; Doherty et al., 1996). Not only are SER and DL differentially expressed within the wing disc but the dorsal and ventral wing compartments respond differently to each ligand. DL can induce expression of the margin-specific gene *cut* only within the dorsal compartment while

SER can only induce *cut* expression within the ventral compartment (De Celis et al., 1996; Doherty et al., 1996). The question of how each ligand generates a compartment-specific response is of particular importance because, at least during embryonic neurogenesis, SER and DL are functionally equivalent (Gu et al., 1995). It is unlikely that cells within the dorsal compartment are intrinsically programmed to respond differently from those of the ventral compartment, since the expression of activated forms of NOTCH will induce margin-specific gene expression in both the dorsal and ventral wing compartments (De Celis et al., 1996; Doherty et al., 1996). If each ligand were to transduce an equivalent NOTCH signal, then NOTCH interaction with SER or DL would be expected to produce identical outcomes.

The gene *fringe* (*fng*) displays many characteristic properties that suggest it plays a role in *Notch* regulation during wing development. *fng* encodes a putative secreted molecule (FNG) that is expressed exclusively on the dorsal side of the wing imaginal disc during the second and third larval instar periods (Irvine and Weischaus, 1994). Induction of fng^{-} clones within the dorsal wing compartment produces ectopic margin formation and cellular outgrowths similar to those of activated forms of *Notch*, suggesting that *fng* is involved in processes related to *Notch* function during wing blade formation. Moreover, ectopic expression of *fng* within the wing disc leads to the induction of SER expression where *fng*-expressing cells are juxtaposed to cells not expressing *fng*. Based on these results, the *fng* product (FNG) has been proposed to function as a boundary-determining molecule and is believed to function upstream of *Ser* to regulate its expression (Kim et al., 1995).

We tested the effects of FNG expression on SER function within the context of the developing animal. We find that reduction of *fng* activity in the wing imaginal disc allows SER to signal within the dorsal compartment, indicating that FNG acts as a negative regulator of SER-mediated NOTCH signaling. Furthermore, ectopic expression of both SER and FNG simultaneously during embryonic neurogenesis blocks the ability of ectopic SER to signal through NOTCH but does not significantly interfere with DL-NOTCH interactions at this same developmental stage. These results suggest that FNG acts selectively on the SER ligand and have allowed us to localize a site in the N-terminal region of SER required for selective repression of SER function by FNG.

MATERIALS AND METHODS

Strains

All strains used were raised at 25°C on standard media. Most strains, balancers and mutations have been previously described by Lindsley and Zimm (1992), Fleming et al. (1990) and Irvine and Wiechaus (1994). Additional chromosomes and aberrations used in this work include the *UAS-Ser* line D (p[mini-w^{+} : UAS-*Ser*], Gu et al., 1995); a $Gal4^{ptc}$ driver (Hinz et al., 1994); p[mini-w^{+} : UAS-*Dl*] and p[mini-w^{+} : UAS-$lacZ^{nuc}$] (nuclear-localized *LacZ* reporter) were generated by T. Jacobson; p[mini-w^{+} : UAS-fng^{27}] insert on the third chromosome (gift of K. Irvine); p[mini-w^{+} : vg^{en}] (*vg* wing margin enhancer/reporter construct; Williams et al., 1994) and the p[ry^{+} : ap^{lacZ}] enhancer trap construct (Cohen et al., 1992).

Two copies of the *Ser* promoter (Gu and Fleming, unpublished data), both located on the second chromosome, were used to drive individual UAS-constructs (termed the $Gal4^{Ser2}$ chromosome). For detection of vg^{en} or *ap* expression in $Gal4^{Ser2}$-driven backgrounds, the second chromosome p[mini-w^{+} : vg^{en}] insert or the second chromosome p[ry^{+} : ap^{lacZ}] enhancer trap was placed on the second chromosome with both $Gal4^{Ser2}$ promoters by meiotic recombination. Expression of UAS minigenes in reduced *fng* genetic backgrounds was accomplished by crossing *UAS-minigene/UAS-minigene; mwh jv* fng^{52}*/TM6B* to $Gal4^{Ser2}/Gal4^{Ser2}$*; mwh jv* fng^{80}*/TM6B* animals and selecting for phenotypically non-Tubby (non-*TM6B*) progeny.

Embryo collection and heat-shock induction

Embryos for transgene induction experiments were collected for 2 hour intervals on apple juice plates and allowed to age for an additional 3 hours at 25°C until heat shocked. This set the average embryonic age at 4±1 hour at the time of induction. A single heat pulse of 37°C was supplied for 1 hour, after which embryos were returned to 25°C and allowed to age for another 8 hours at which time they were processed for immunohistochemistry.

Immunohistochemistry

Labeling of embryos with anti-β-galactosidase (Promega) and anti-HRP antibodies (directly conjugated to alkaline phosphatase, Jackson Labs) was performed as described in Gu et al. (1995).

Wing discs were dissected in PBS and fixed in 4% paraformaldehyde on ice for 15 minutes followed by 15 minute fixation at room temperature. Discs were washed 4× in PBS, placed for 1-2 hours at 4°C in blocking buffer (1× PBS, 0.2% saponin and 0.3% normal goat serum) then incubated at 4°C overnight with primary antibody [the following primary antibodies were used: mouse anti-β-gal antibody (1:1000 dilution; Promega); anti-cut monoclonal antibody (1:100; provided by K. Blochlinger); Rabbit anti-Ser antibody (1:1000 provided by E. Knust); or mouse anti-αPS2 integrin (1:500; Wilcox et al., 1984]. The wing discs were rinsed 4× in PBS, then washed in blocking buffer for 2-4 hours followed by overnight incubation at 4°C with horseradish peroxidase (HRP)-conjugated, goat anti-mouse (or goat anti-rabbit) secondary antibody (Promega) at 1/250 dilution. HRP detection was performed by standard methodologies and wing discs were mounted in glycerol for observation.

Construction of UAS-NT-DL/SER

The 5′ coding regions of *Dl,* including the signal peptide through the end of the DSL domain (see Fig. 4), were used to replace the equivalent *Ser* domains as follows: A DNA fragment containing the region 55 nucleotides (nt) upstream of translation start and coding for the first 229 amino acids of *Dl* was generated by PCR reactions from a 5′ *Dl* cDNA template using a 5′ primer (5′ ACACAGCCGCGGAATTCTTACACTAAAAGCGAC 3′) and a 3′ primer (5′ GCCTTTGGCGCATATGGGTATGTGACAGTAATC 3′). The 5′ primer contained unique *Sac*II and *Eco*RI sites for subsequent cloning and the 3′ primer contained a unique *Nde*I site situated to place the *Dl* 5′ coding region in frame with the *Nde*I site at nt 1287 of *Ser* (Fleming et al., 1990). The *Ser* cDNA in Bluescript (BT; Stratagene) was cut with *Sac*II (5′ BT polylinker site) and *Nde*I and replaced with the *Sac*II-*Nde*I *Dl* PCR fragment to generate BT-NT-DL/SER. This construct was transferred from BT to the pUAST transformation vector (Brand and Perrimon, 1993) using unique *Eco*RI sites flanking the NT-DL/SER insert. pUAST clones were selected for proper orientation to obtain UAS-NT-DL/SER. The PCR generated 5′ *Dl* region was sequenced by the dideoxy chain-termination technique (Sanger et al., 1977) on double stranded DNA using ABI Prism™ Dye Terminator Cycle Sequencing (Perkin Elmer). Transgenic UAS-NT-DL/SER lines were generated by P element mediated germline transformation (Spradling, 1986) and multiple, independent insert lines were recovered. An X-linked insert (designated G5) was used in this study.

RESULTS

Ectopic *Ser* and *Dl* expression in the developing wing disc

Localized activation of NOTCH along the D/V compartment border during the development of the wing imaginal disc has been shown to be an important step in the establishment of the *Drosophila* wing margin (de Celis et al., 1996; Doherty et al., 1996). A drawing of a third instar imaginal wing disc depicting the location of the presumptive D/V margin is presented in Fig. 1A. NOTCH and DL are found throughout the developing wing disc although DL expression increases along the D/V boundary during the third larval instar period (Fehon et al., 1991; Kooh et al., 1993). SER is initially expressed throughout the dorsal wing compartment during the second larval instar and becomes refined to the dorsal marginal zone during the third instar period (Speicher et al., 1994; Couso et al., 1995). Through the actions of SER and DL, NOTCH becomes activated at the D/V boundary, induces expression of *vg* along the presumptive wing margin (Kim et al., 1996; Neumann and Cohen, 1996) and increases the expression of *wg* (Rulifson and Blair, 1995). Subsequently, the *Notch* and *wg* pathways appear to cooperate to induce *cut* expression along the presumptive margin (Neumann and Cohen, 1996). A second function of *wg* is necessary to produce late expression of *vg* in a gradient with highest levels near the margin (Neumann and Cohen, 1996).

Ectopic expression of activated forms of NOTCH during wing development leads to cell proliferation and the activation of margin-specific genes such as *wg, cut* and *vg* in both dorsal and ventral wing compartments (De Celis et al., 1996; Doherty et al., 1996). Since NOTCH activation in the marginal zone is normally mediated by its ligands, SER and DL, we examined compartment-specific responses for these ligands within the imaginal wing disc. To identify compartment-specific effects, we used the dorsal-specific expression of an *ap*-lacZ reporter construct (Fig. 1B; Cohen et al., 1992) or the ventral-specific staining of αPS2 integrin (Fig. 1D) to identify each compartment. Using the Gal4/UAS system (Brand and Perrimon, 1993), we ectopically expressed SER or DL under the control of the *patched (ptc)* promoter (termed $Gal4^{ptc}$), which is active along the anterior-posterior compartment boundary in both the dorsal and ventral wing compartments (Fig. 1C). When $Gal4^{ptc}$ was used to express DL, cell proliferation was induced in both the dorsal and ventral wing compartments (Fig. 1D). This suggests that DL can function in both dorsal and ventral wing compartments. Interestingly, however, ectopic DL production induces significant expression of margin-specific genes such as *vg* and *cut* only within the dorsal compartment (Fig. 1E and 1F, respectively). In contrast to the effects of DL expression, when SER is expressed under the control of $Gal4^{ptc}$, both cell proliferation (Fig. 1G) and the expression of the margin-specific genes *vg* and *cut* are limited to the ventral wing compartment (Fig. 1 H and 1I, respectively). It appears, therefore, that SER can only activate NOTCH in the ventral compartment and is inhibited from activating NOTCH dorsally.

We further explored possible differences in NOTCH activation by SER and DL using a *Ser* promoter (termed $Gal4^{Ser2}$; Gu and Fleming, unpublished data) to ectopically express either *Ser* or *Dl* cDNAs in a normal *Ser* pattern during wing development. As shown in Fig. 2A, the $Gal4^{Ser2}$ promoter expresses primarily along the presumptive margin in the dorsal wing compartment in late third instar imaginal discs. When $Gal4^{Ser2}$ is used to express a UAS-*Ser* cDNA in an otherwise wild-type background, nearly normal wing discs and blades are produced (Fig. 2B and 2D respectively). $Gal4^{Ser2}$/UAS-*Ser* wings exhibit slight overgrowth in the ventral regions and minor double margin formation along the distal edge (Fig. 2D). The disruptions in these wings are likely to result from SER activity along the margin and within the ventral compartment as a consequence of SER expression across the D/V margin by the $Gal4^{Ser2}$ promoter (see Fig. 2A). Expression of margin-specific genes, such as *vg* and *cut*, appear indistinguishable from wild type under these conditions (data not shown). In contrast to these results, when DL is expressed under the same conditions, extensive cell proliferation is induced in the dorsal compartment (Fig. 2C,E). Even though DL normally signals to the dorsal compartment and can induce *cut* and *vg* gene expression in that region when ectopically expressed under the $Gal4^{ptc}$ promoter (see Fig. 1E,F), expression of DL under the $Gal4^{Ser2}$ promoter only weakly induces *vg* gene expression and does not induce *cut* along the presumptive margin (Fig. 2H,K). We suspect that the inability of DL to induce high levels of margin-specific gene expression under these circumstances results from the presence of the DL signal on both the dorsal (ectopic DL) and ventral side (endogenous DL) of the presumptive boundary. The high levels of DL on dorsal cells that are expressing SER may effectively out-compete SER for NOTCH activation, resulting in the failure of margin specification. The differential effects associated with ectopic DL versus SER expression by the $Gal4^{Ser2}$ promoter confirm the hypothesis that SER signaling is blocked or drastically reduced in the dorsal compartment relative to the signaling ability of DL.

Ser function is restricted by *fng*

The ability of *fng* to induce SER production at the borders of *fng*-expressing and non-*fng*-expressing cells has suggested that the FNG product functions upstream of *Ser* to regulate SER expression (Kim et al., 1995). What remained unclear from these studies is the mechanism by which such regulation might occur.

To examine possible effects of *fng* on *Ser* expression, we expressed *Ser* or *Dl* cDNAs in the dorsal wing compartment under the $Gal4^{Ser2}$ promoter in a genetic background with reduced *fng* expression. The mutation fng^{80} results in homozygous larval lethality and is believed to be a null allele of the *fng* locus. A second allele, fng^{52}, is believed to be a hypomorphic variant and produces viable adults that show reductions in wing margin formation (Irvine and Wieschaus, 1994). fng^{52}/fng^{80} animals are weakly viable and display a severe wing margin loss phenotype. Examination of fng^{52}/fng^{80} animals also reveals weak or lost margin expression of *vg* and *cut* (data not shown). Ectopic expression of DL by $Gal4^{Ser2}$ in the fng^{52}/fng^{80} background resulted in cell proliferation in the dorsal wing compartment comparable to that of DL under the same promoter in a wild-type background (data not shown). Similarly, expression of *cut* and *vg* are not induced by DL at the presumptive wing margin in the fng^{52}/fng^{80} background (data not shown). More significantly, in the dorsal wing compartment, which is normally unresponsive to SER expression, ectopic expression of SER by $Gal4^{Ser2}$ in the fng^{52}/fng^{80} background produces excessive cell proliferation (Fig. 2F). Furthermore, in contrast to the effects of DL expressed under $Gal4^{Ser2}$, expression of

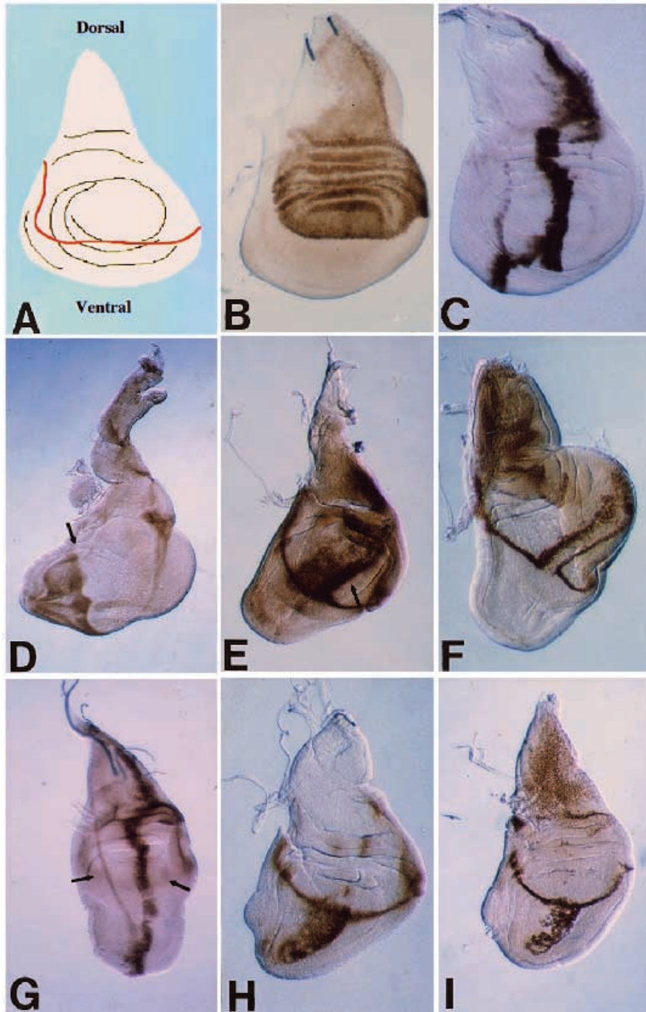


Fig. 1. Compartmental restriction of NOTCH signaling by SER and DL. For all panels, late third instar wing imaginal discs were isolated and processed to detect expression of the gene products indicated. In these panels, anterior is left and the dorsal compartment is at the top. The $Gal4^{ptc}$ promoter was used to drive the UAS-controlled reporter constructs indicated. (A) Drawing showing approximate location of the D/V boundary (red line) in the wild-type disc. (B) Wild-type wing disc stained for dorsal compartment-specific expression of an *ap*-lacZ reporter construct. Note that the D/V compartment boundary runs perpendicular to the long axis of the disc. (C) UAS-nuc^{lacZ} construct showing *ptc* expression pattern in wild-type disc. (D) Ectopic UAS-*Dl* expression under *ptc* control showing cell proliferation in both dorsal (unstained) and ventral compartments (stained for ventral-specific expression of the αPS2 integrin). Note displacement of D/V margin (arrow) indicating cell proliferation in the dorsal compartment. (E) Wing disc from UAS-*Dl* driven by *ptc* shows strong expression of a *vg*-lacZ reporter construct only along the *ptc*-expressing region in the dorsal compartment (arrow). (F) UAS-*Dl* expression under *ptc* produces stripes of *cut* product adjacent to the *ptc*-expressing region in the dorsal but not in the ventral compartment. (G) Ectopic expression of UAS-*Ser* under *ptc* control (stained for expression of SER protein). Note light staining for endogenous SER expression along the presumptive wing margin (arrows) is located at the normal position, demonstrating that cell proliferation is restricted to the ventral compartment. (H) UAS-*Ser* under *ptc* control produces *vg*-lacZ staining limited to ventral regions of the disc (compare to *Dl* expression in 1E). (I) UAS-*Ser* under *ptc* control shows ectopic *cut* expression is limited to ventral wing compartment.

SER in the fng^{52}/fng^{80} background does induce expression of *vg* and *cut* at the margin (Fig. 2I,L, respectively). These findings suggest strongly that normal levels of *fng* expression in the dorsal compartment act to restrict or prevent SER from signaling within that region but have little or no inhibitory effect on DL signaling. These results also suggest that the quality of the SER-mediated signal must differ from that of the DL-mediated signal to account for the differences in these molecules' ability to induce margin-specific gene expression.

fng restricts *Ser* activity at the post-transcriptional level

Since we had produced ectopic SER using sequences found within its own promoter regions, it remained unclear whether *fng* was restricting dorsal SER activity at the transcriptional or post-transcriptional levels. We therefore tested whether *fng* could regulate *Ser* activity under conditions where SER was expressed under a foreign promoter. We have previously demonstrated that SER expression can functionally replace DL during neurogenesis, indicating that these molecules function in an equivalent manner at that time (Gu et al., 1995). As previously described, however, during wing blade formation, SER function is compartmentally restricted. One notable distinction between neurogenesis and wing margin specification that might serve to explain these differences is that *fng* is expressed in the dorsal wing compartment during wing formation but is not expressed detectably during early neurogenesis (K. Irvine, personal communication). We reasoned that if *fng* were able to affect *Ser* activity in a post-transcriptional manner, then simultaneous expression of SER and FNG should affect the ability of SER to function during neurogenesis and alter the expected phenotype.

Using a $Gal4^{hsp70}$ heat-shock promoter, we ectopically expressed SER at 4 hours after egg laying (AEL). Animals treated in this manner die prior to the end of embryogenesis and display severely reduced neuronal differentiation resulting from inappropriate NOTCH activation during the lateral inhibition process of neurogenesis (Gu et al., 1995; see Table 1; compare Fig. 3A and B). In contrast, simultaneous expression of *UAS-Ser* and *UAS-fng* minigene constructs using the $Gal4^{hsp70}$ promoter at 4 hours AEL, greatly reduced the lethality associated with ectopic SER expression alone (Table 1) and restored normal neuronal differentiation (Fig. 3C). More

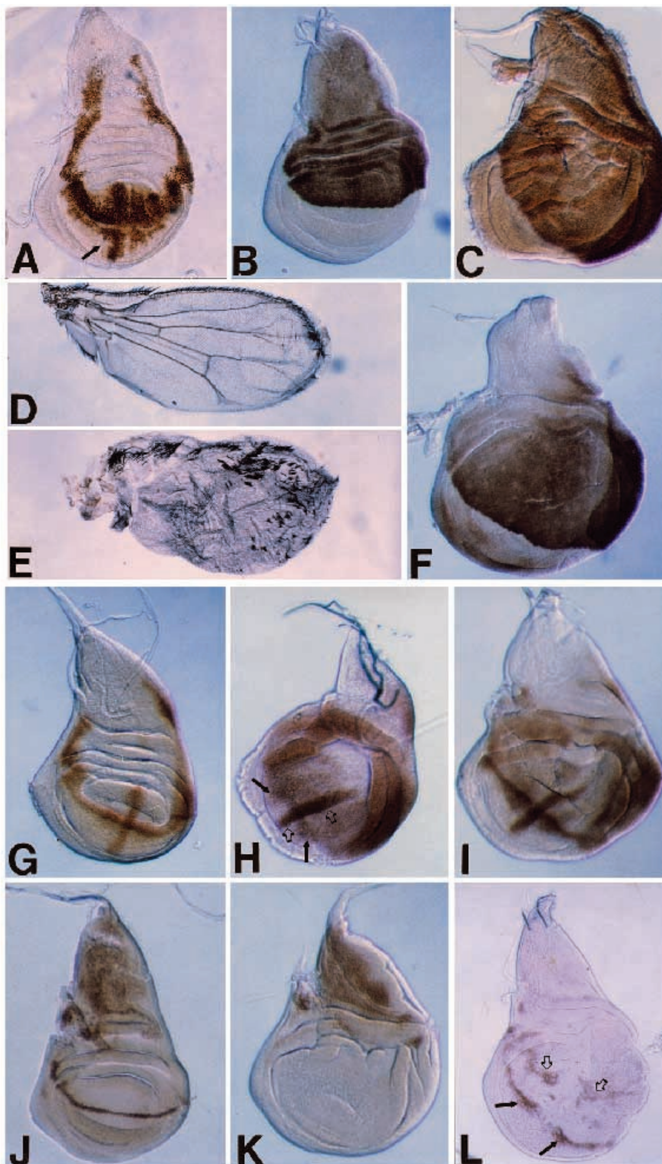
Table 1. Viability of animals ectopically expressing SER and FNG during embryonic neurogenesis

	No heat induction		37°C induced	
Genotype	*Hsp70Gal4/TM3*	% viability	*Hsp70Gal4/TM3*	% viability
UASSer	216 / 209	103	15 / 304	5
UASSer;UASfng	219 / 211	104	260 / 433	60
UASfng	212 / 210	101	355 / 557	64

Individual groups of sibling animals having the partial genotypes: Row 1 [*UAS-Ser/+; $Gal4^{Hsp70}$/+*], Row 2 [*UAS-Ser/+; $Gal4^{Hsp70}$/UAS-fng^{27}*] or Row 3 [*$Gal4^{Hsp70}$/UAS-fng^{27}*] (experimental) were reared at 25°C to adulthood (no heat induction) or subjected to a 1 hour heat pulse at 37°C at 4 hours AEL and returned to 25°C to complete development (heat induction). The viability of these animals is compared to their siblings, which have the partial genotypes, respectively: Row 1 [*UAS-Ser/+; TM3, β-gal, Sb/+*], Row 2 [*UAS-Ser/+; TM3, β-gal, Sb /UAS-fng^{27}*] or Row 3 [*TM3, β-gal, Sb /UAS-fng^{27}*] (controls). Following eclosion, animals of each type were counted and the survival index calculated as percentage survival for experimental animals/control animals. A minimum of 200 control animals were scored for each time point.

significantly, co-expression of SER and FNG at this time rescues significant numbers of animals that go on to develop into phenotypically wild-type adults. This suggests strongly that FNG is capable of specifically blocking activation of NOTCH by SER while still allowing normal DL-NOTCH activation to occur. Importantly, in these experiments, both SER and FNG are being produced ectopically under the Gal4^{Hsp70} promoter and anti-SER antibody staining of these embryos demonstrates that SER is detectable at levels comparable to Gal4^{Hsp70} expression of SER alone (data not shown). Additionally, ectopic expression of FNG alone under these conditions did not appear to significantly affect SER expression in the embryo (data not shown). These results suggest that FNG is altering SER activity at the protein level and not at the level of transcription.

Ectopic expression of FNG during early embryogenesis is not without deleterious consequences. Animals expressing ectopic FNG alone at 4 hours AEL exhibit a reduction in viability of greater than 35% relative to control animals (Table 1). Examination of embryos ectopically expressing FNG alone or simultaneously expressing SER and FNG, failed to reveal significant morphological differences that might account for the reduction in viability (data not shown). Interestingly, the viability of animals expressing both SER and FNG during neurogenesis is comparable to that of animals expressing ectopic FNG alone. This may indicate that the reduced viability associated with FNG or FNG and SER embryonic expression results primarily from the actions of FNG alone and that FNG can completely block the effects of ectopic SER expression during this time period. It remains unclear whether the reduced viability associated with ectopic embryonic expression of FNG results from the actions of FNG on neurogenesis, the ability of FNG product to perdure and interfere with SER functions later in embryogenesis or disruptions of other processes in the developing embryo.



The N-terminal region of SER is required for FNG regulation

The available data suggest that FNG acts to restrict SER from interacting with and activating NOTCH within the dorsal wing compartment. This raised the possibility that FNG might affect *Ser* function by interfering with the N-terminal NOTCH-binding region of SER. To test this hypothesis, we generated a chimeric molecule that replaced the regions of the *Ser* cDNA that encode the signal peptide through the DSL domain with the corresponding region of the *Dl* cDNA (Fig. 4). This chimeric molecule was placed under the control of the UAS promoter from the Gal4 system (Brand and Perrimon, 1993), termed UAS-NT-DL/SER (NT stands for N-terminal), and

Fig. 2. Reduction in *fng* expression allows SER signaling in the dorsal wing disc. For all panels, a Gal4^{Ser2} promoter was used to express the gene products indicated in the *Ser* expression pattern (dorsal compartment). Orientation of discs is the same as in Fig. 1. (A) UAS-*nuc*^{lacZ} construct showing late third instar expression pattern of the Gal4^{Ser2} promoter in a wild-type disc. Most expression is limited to the dorsal compartment with the exception of the region corresponding to the future distal wing blade (arrow). (B) UAS-*Ser* driven by Gal4^{Ser2} shows nearly normal wing disc development (stained for *ap*-lacZ expression; compare to Fig. 1B). (C) In contrast to 2B, expression of UAS-*Dl* driven by Gal4^{Ser2} produces an overproliferation of cells in the dorsal compartment (stained for *ap*-lacZ). (D) The adult wing blade resulting from UAS-*Ser* driven by Gal4^{Ser2} is largely normal with the exception of the distal tip region, which shows some double margin formation and ventral overgrowth. (E) Adult wing overgrowth corresponds to that seen in the imaginal disc for UAS-*Dl* driven by Gal4^{Ser2}. (F) When UAS-*Ser* is driven by Gal4^{Ser2} in a genetic background with reduced *fng* expression, dorsal cell proliferation is observed (stained for *ap*-lacZ expression; note displacement of D/V margin similar to 2C). (G) Wild-type wing disc depicting the expression pattern associated with the *vg*-lacZ reporter construct. (H) When UAS-*Dl* is driven by Gal4^{Ser2}, expression of the *vg*-lacZ reporter construct is greatly reduced at the presumptive D/V wing margin (arrows) but not along the anterior-posterior boundary (arrowheads). (I) UAS-*Ser* driven by Gal4^{Ser2} in a reduced *fng* background (see text) induces expression of the *vg*-lacZ reporter construct along the D/V marginal zone. (J) Wild-type wing disc showing expression pattern of *cut* product. (K) UAS-*Dl* driven by Gal4^{Ser2} does not induce *cut* expression at the margin. (L) UAS-*Ser* driven by Gal4^{Ser2} in a reduced *fng* background consistently induces expression of *cut* along the presumptive D/V margin (arrows) and occasionally induces a second zone (arrowheads) along the inner edge of expression for UAS-*Ser* (compare to expression pattern, Fig. 2A).

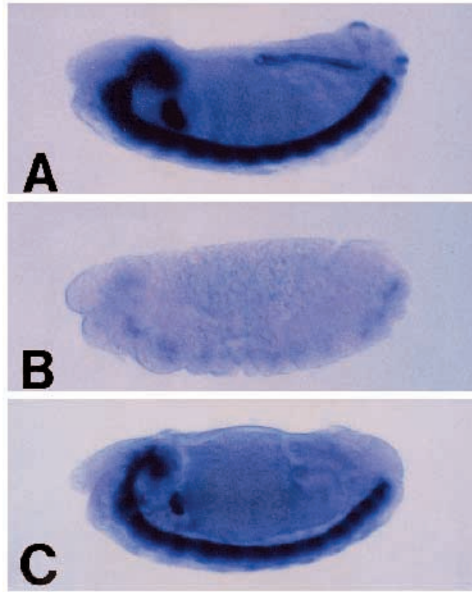


Fig. 3. Expression of FNG can block SER-mediated NOTCH activation. Embryos of the genotypes specified were subjected to a 37°C heat pulse at 4 hours AEL and analyzed for the expression of neural-specific antigens. Animals are at approximately stage 13 (Campos-Ortega and Hartenstein, 1985), shown with anterior to the left and dorsal up. (A) Control *UAS-Ser/+; Gal4*^{Hsp70}*/+* embryo without heat induction develop normally. The anti-HRP neural-specific marker (blue stain) demarcates the developing neuronal derivatives. (B) Following heat induction, *UAS-Ser/+; Gal4*^{Hsp70}*/+* animals display a drastic reduction in cells staining for the anti-HRP neural-specific marker. (C) *UAS-Ser/+; Gal4*^{Hsp70}*/UAS-fng*^{27} animal, following heat pulse, shows apparently wild-type staining for anti-HRP neural-specific marker.

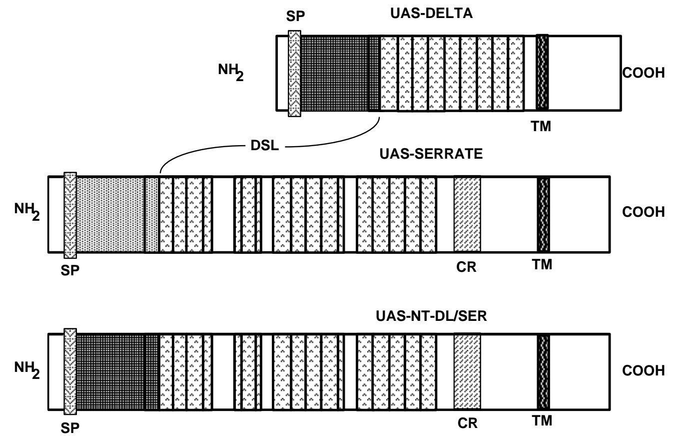


Fig. 4. Construction of the UAS-NT-DL/SER chimeric molecule. The expected proteins encoded by *Dl* (UAS-DELTA) and *Ser* (UAS-SERRATE) are diagrammed at the top and middle of the figure, respectively. We replaced the cDNA region encoding the N-terminal 283 amino acids of SER with the *Dl* cDNA region encoding the 229 amino acids of DL to produce the resulting UAS-NT-DL/SER construct (see Materials and Methods). NH_2, amino terminus; SP, signal peptide; CR, cysteine-rich region of SER; TM, transmembrane domain; COOH, carboxy terminus; chevroned boxes, EGF-like repeats. DSL refers to the C-terminal-most portion of the DSL domain (Tax et al., 1994) used as a common swap point between SER and DL.

used to generate transgenic lines via P-element-mediated germline transformation (Spradling, 1986).

We initially expressed UAS-NT-DL/SER under the Gal4^{ptc} promoter to test the effects of this molecule during wing development. Even though numerous independent UAS-NT-DL/SER inserts were tested, all resulted in lethality prior to the second instar stage. Similarly, expression of UAS-*Ser* under the Gal4^{ptc} promoter in a *fng*^{52}*/fng*^{80} results in lethality prior to the second instar period. In contrast, expression of UAS-NT-DL/SER under the Gal4^{Ser2} promoter in a wild-type genetic background produces animals that survive to the late pupal stage, allowing for a functional assessment of the chimeric molecule. As shown in Fig. 5A, the UAS-NT-DL/SER chimeric molecule is capable of inducing cell proliferation within the dorsal compartment in the presence of wild-type levels of *fng* expression. It appears, therefore, that the N-terminal region of SER is required for repression of *Ser* function by FNG in the dorsal wing compartment, although it is not known whether or not FNG interacts directly with SER in this region (see Discussion).

We further examined wing discs expressing UAS-NT-DL/SER under the Gal4^{Ser2} promoter for the expression of the margin-specific genes *vg* and *cut.* As shown in Fig. 5B and C, expression of the UAS-NT-DL/SER chimeric construct induces expression of *vg* and *cut* along the marginal zone. This result is the same whether the UAS-NT-DL/SER chimera is expressed in a wild-type genetic background or the *fng*^{52}*/fng*^{80} background. These results contrast with those seen for the expression of DL under the same conditions (Fig. 2G,H), suggesting that the

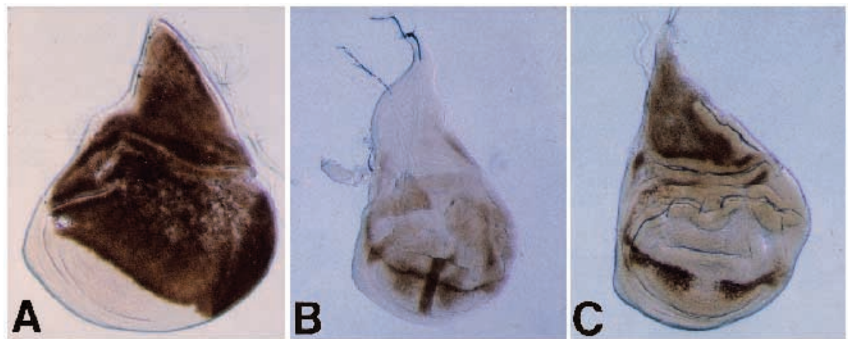


Fig. 5. FNG requires the N-terminal region of SER to restrict signaling. The Gal4^{Ser2} promoter was used to express the UAS-NT-DL/SER chimeric molecule under the *Ser* expression pattern (Fig. 2A) in a genetic background with wild-type *fng* expression. Orientation of discs is the same as in Fig. 1. (A) Wing disc stained for *ap*-lacZ expression showing dorsal outgrowth when UAS-NT-DL/SER is driven by Gal4^{Ser2}. (B) In contrast to the expression of UAS-DL (see Fig. 2H), expression of UAS-NT-DL/SER by Gal4^{Ser2} results in *vg*-lacZ expression along the presumptive D/V wing margin. (C) Similarly, expression of UAS-NT-DL/SER by Gal4^{Ser2} results in *cut* expression along the presumptive D/V wing margin (contrast with UAS-DL under similar conditions; Fig. 2K).

quality of the signal generated by the chimeric molecule more closely resembles that of SER than of DL and that the replacement of the N-terminal region of SER with that of DL serves only to alleviate the suppression imposed by FNG.

DISCUSSION

During the specification of the D/V boundary in the developing wing imaginal disc, activation of NOTCH is required for subsequent expression of margin-specific genes such as *wg*, *cut* and *vg* (Rulifson and Blair, 1995; Diaz-Benjumea and Cohen, 1995; De Celis et al., 1996; Neumann and Cohen, 1996). The correct spatial activation of NOTCH in the wing appears to require both of its identified ligands, SER and DL (Diaz-Benjumea and Cohen, 1995; Couso et al., 1995; De Celis et al., 1996; Doherty et al., 1996). In this paper, we have initiated an investigation to determine how the SER and DL ligands elicit compartment-specific responses through NOTCH. Our findings clearly demonstrate that *fng* expression within the dorsal compartment is largely responsible for the restriction of *Ser* activity to the ventral compartment.

As shown by our ectopic expression studies during embryonic neurogenesis, the presence of FNG protein product appears capable of blocking SER activity even in an environment where SER itself is not normally expressed. This suggests either that FNG alone is sufficient to disrupt SER-NOTCH signaling or that FNG can activate a pathway, present during both neurogenesis and wing formation, that is capable of blocking *Notch* signaling. The latter possibility seems unlikely since the presence of FNG specifically blocks SER-NOTCH signals but does not appear to block DL-NOTCH signals. If FNG acted to induce a general inhibitory pathway on NOTCH signaling, it would be expected to block both SER-mediated and DL-mediated signals. Therefore, if FNG does act to induce a secondary pathway impinging on *Notch* signaling, that pathway must have the ability to differentiate between SER-mediated and DL-mediated NOTCH signals. Given that our data support the existence of a qualitative difference between SER-mediated and DL-mediated signals, at least with respect to induction of a specific pattern of gene expression along the presumptive wing margin, the possibility of a FNG-induced secondary pathway cannot be discounted at this time. However, irrespective of the molecular mechanism, the specific inhibitory effects of FNG on SER are sufficient to explain the restriction of SER activity to cells of the ventral wing compartment.

Molecular interactions of FNG and SER

We find that replacing the N-terminal NOTCH-binding domain of SER with the similar region of DL allows for *Ser*-like signaling within the dorsal compartment even in the presence of wild-type levels of *fng* expression. Since the N-terminal regions of both SER and DL have been implicated in binding to the 11th and 12th EGF-like repeats of NOTCH (Fleming, Sun and Artavanis-Tsakonas, unpublished data; Shepard and Muskavitch, personal communication), it is possible that FNG may physically interfere with the ability of SER to interact with NOTCH. The chimeric molecule contains the N-terminal regions of DL yet retains a *Ser*-like signaling capability, suggesting that signal specificity of a given NOTCH ligand is not generated within its N-terminal NOTCH-binding region but resides elsewhere within each molecule. This also suggests that there are secondary sites of interaction between NOTCH and SER or DL that have yet to be determined.

Our findings indicate that FNG interferes with SER-NOTCH interactions but do not specifically address whether or not FNG physically interacts with SER, NOTCH or some other molecule(s). If FNG acts directly on the *Notch* pathway rather than via a secondary or parallel route, then it is possible that FNG binds directly to SER and thereby blocks its ability to interact with NOTCH. In this case, FNG would act in a fashion similar to that proposed for noggin or chordin, which interact with BMP4 to restrict TGF-β signaling during axis establishment in *Xenopus* (Zimmerman et al., 1996; Piccolo et al., 1996). An equally plausible model would allow FNG to bind to NOTCH and interfere with the ability of SER to access NOTCH. Such binding need not necessarily occur at the location of the 11th and 12th EGF-like repeats of the NOTCH molecule which are necessary for the binding of the SER and DL ligands in vitro (Rebay et al., 1991) but might reside outside this region to induce conformational changes in NOTCH, thereby regulating its ability to bind a particular ligand.

With respect to these proposed models, expression of UAS-SER under Gal4^{ptc} produces SER protein at levels far above those of endogenous SER production (see Fig. 1G). Nonetheless, when *fng* is expressed at wild-type levels, excess SER levels fail to generate a response within the dorsal compartment. Similarly, reduction of FNG levels in the fng^{52}/fng^{80} mutant background does not produce dorsal disc overgrowth in the presence of wild-type doses of *Ser*, yet clones deficient for *fng* in the dorsal wing do generate proliferative outgrowths (Irvine and Weischaus, 1994). The aforementioned hypothesis, where FNG may bind with NOTCH rather than to SER, would account for this observation since a FNG/NOTCH complex would be expected to be unreceptive to SER regardless of the SER concentration. The possibility that FNG binds with SER cannot be ruled out, however, since FNG may be produced in the dorsal compartment in significant excess over the amount normally necessary for restriction of SER activity. Excess FNG relative to SER could result from the simple production of excess quantities of FNG, the ability of one molecule of FNG to bind more than one molecule of SER or FNG acting in some undefined enzymatic role to block SER function. The lattermost alternative is made more plausible by the recent finding that FNG and FNG-like molecules contain motifs related to galactosyltransferases (Yuan et al., 1997). Clearly, further molecular and biochemical experimentation will be required to specifically address these possibilities.

Does FNG regulate *Ser* transcription?

It has been shown previously that ectopic expression of FNG results in the activation of SER in cells at the border between fng^+ and fng^- cells (Kim et al., 1995). However, areas ectopically expressing high levels of *fng* that are not adjacent to cells lacking *fng* expression do not appear capable of inducing SER expression. These observations suggest that *fng* alone is insufficient to induce SER expression and must rely upon other factors. Our experiments co-expressing FNG and SER are inconsistent with the primary role of FNG being to up-regulate SER expression at least during early embryogenesis. Ectopic

expression of FNG during neurogenesis produces a moderate reduction in viability but has no discernible effect on development of the nervous system. More importantly, FNG expression blocks the ability of SER to interfere with neuronal development. Since we ectopically expressed FNG under a heat-shock promoter and it should therefore be expressed ubiquitously, if FNG resulted in increased SER expression, the expected result should have been similar to, or more severe than, that seen for ectopic expression of SER alone and should have produced neuronal hypotrophy.

A possible explanation for SER induction at *fng*$^{+}$/*fng*$^{-}$ borders can be derived from a combination of our results showing that FNG inhibits SER activity with the previously proposed model of a feedback loop during NOTCH activation at the wing margin (De Celis et al., 1996; Doherty et al, 1996). The ability of FNG to up-regulate SER expression may result from FNG's ability to generate of an area that is incapable of receiving a SER-NOTCH signal (the cells expressing FNG). In a paper by V. M. Panin, V. Papayannopoulos, R. Wilson and K. D. Irvine (in press), it is proposed that in addition to our finding that FNG inhibits SER signaling, FNG may also act to facilitate DL signaling. Such facilitation may allow for the cells that are unreceptive to SER signaling to receive a DL-NOTCH signal. Thus, as in the model proposed by Doherty et al. (1996), reception of the DL-NOTCH signal would serve to up-regulate SER expression. Likewise, at the boundaries where FNG expression is low, SER-NOTCH interactions would, in turn, serve to reinforce DL expression. In this indirect manner, FNG could act to regulate the expression of SER and therefore serve to form a boundary and ultimately aid in the specification of the wing margin.

Ligand-specificity in *Notch* signaling

Alleviation of the restrictions on SER-mediated signaling in the dorsal compartment either by the reduction of *fng* levels (*fng*^{52}/*fng*^{80}) or the use of the UAS-NT-DL/SER chimera induces cell proliferation and margin-specific gene expression characteristic of a *Ser* signal. A question remaining to be resolved is what differences remain within the ventral compartment that allow SER to activate margin-specific genes there, while DL cannot. Given that FNG may facilitate DL signaling (Panin et al., personal communication), the presence of FNG in the dorsal compartment may allow DL to signal and specify margin-specific gene expression there. Similarly, the absence of FNG could prevent DL-induced margin-specific gene expression within the ventral compartment.

While these possibilities serve to explain why DL does not activate margin-specific genes ventrally, they do not explain why SER can induce ventral activation of those genes. Functional difference(s) between SER- and DL-mediated NOTCH signaling must remain, such that SER is capable of eliciting margin-specific gene expression when ectopically expressed within the ventral compartment. Differences in the quality of a SER- versus DL-mediated signal might arise from as yet unidentified molecule(s) within the ventral compartment that either facilitate a SER signal or inhibit a DL signal. It has been previously proposed that the WG protein, which is present in the ventral compartment, may act in the NOTCH signaling pathway (Couso and Martinez-Arias, 1994; Couso et al., 1995). However, it has been shown recently that WG does not appear to act as a SER facilitator in the ventral compartment (Jönsson and Knust, 1996). The differences between SER-NOTCH and DL-NOTCH activation at the presumptive wing margin and the determination of whether such differences result from intrinsic properties of the SER and DL ligands or the presence of yet-to-be-identified molecule(s) within the ventral wing disc are expected to provide fundamental insights into the nature of NOTCH activation.

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