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A dominant-negative form of *Serrate* acts as a general antagonist of *Notch* activation

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SUMMARY

Specification of the dorsal-ventral compartment boundary in the developing *Drosophila* wing disc requires activation of NOTCH from its dorsal ligand SERRATE and its ventral ligand DELTA. Both NOTCH ligands are required in this process and one cannot be substituted for the other. In the wing disc, expression of a dominant-negative, truncated form of SERRATE called BD^G, is capable of inhibiting NOTCH activation in the ventral but not the dorsal compartments. We demonstrate that BD^G can act as a general antagonist of both SERRATE and DELTA mediated NOTCH interactions, however, BD^G retains the SERRATE protein domain targeted by FRINGE, hence its antagonistic effects are restricted in the dorsal wing disc. Our findings suggest a model in which ligand binding to NOTCH is a necessary but insufficient step toward NOTCH activation.

Key words: *Drosophila*, *Notch*, *Serrate*, dominant-negative, wing development

INTRODUCTION

Growth and development of appendages in *Drosophila* depend upon the establishment of anterior-posterior (A/P) and dorsal-ventral (D/V) identities in groups of cells known as imaginal discs. Cells of an imaginal disc are initially specified during embryogenesis and proliferate during larval development where they acquire specific spatial and developmental cues required for establishment of the adult appendage. Using cell lineage analysis, it has been shown that A/P identities are established early in embryogenesis (Lawrence and Morata, 1977) and divide the wing disc into anterior and posterior compartments. A/P identity can be visualized by the expression of the posterior compartment specific transcription factor genes *engrailed* and *invected* (Kornberg et al., 1985; Sanicola et al., 1995; Tabata et al., 1995; Simmonds et al., 1995). Signaling from the posterior compartment, mediated by the secreted *hedgehog* gene product (Lee et al., 1992; Tabata et al., 1992), culminates in the localized anterior expression of *decapentaplegic* along the A/P boundary whose signaling activity is believed necessary to form an organizing center to regulate growth and patterning along the A/P axis (Capdevilla and Guerrero, 1994; Zecca et al., 1995; reviewed in Lawrence and Struhl, 1996).

Establishment of D/V compartment identity in the wing imaginal disc occurs considerably later than A/P establishment, arising near the end of the first larval instar period (Garcia-Bellido et al., 1976). D/V identity can be distinguished by the dorsal-specific expression of the APTEROUS (AP) transcription factor during larval instar stages (Cohen et al., 1992). During the establishment of the D/V organizer region, *ap* is believed to initiate expression of the gene *Serrate (Ser)*, the product of which (SER) acts as a ligand for the NOTCH receptor, thereby initiating a signal from the dorsal to ventral compartments (Diaz-Benjumea and Cohen, 1995; Couso et al., 1995). In turn, the *Delta (Dl)* gene product (DL), which also encodes a NOTCH ligand, signals reciprocally from the ventral to dorsal compartments (de Celis et al., 1996; Doherty et al., 1996). The combined actions of these reciprocal D/V signals result in production of the *wingless (wg)* signaling molecule and margin-specific expression of *vestigial (vg)* and *cut* (Kim et al., 1995; Rulifson and Blair, 1995; Diaz-Benjumea and Cohen, 1995; Couso et al., 1995; de Celis et al., 1996; Doherty et al., 1996). The combination of these latter genes, particularly *vg* and *wg*, are believed to induce formation of the D/V organizer (Kim et al., 1996; Neumann and Cohen, 1996).

NOTCH activation is essential to the establishment of the *Drosophila* wing margin (Shellenbarger and Mohler, 1978; Diaz-Benjumea and Cohen, 1995). Expression of activated forms of NOTCH are capable of inducing expression of margin specific genes including *wg*, *vg* and *cut* (de Celis et al., 1996; Doherty et al., 1996). In contrast, recent investigations have revealed that the NOTCH ligands SER and DL, elicit compartment-specific responses and moreover, cells along the presumptive wing margin respond differentially to SER- and DL-mediated NOTCH signals (Jönsson and Knust, 1996; Fleming et al., 1997). One aspect of the compartment-specific responses associated with NOTCH ligands can be explained by the ability of the dorsally expressed *fringe (fng)* gene product (FNG) to specifically inhibit SER-mediated NOTCH activation (Fleming et al., 1997; Panin et al., 1997). These findings explain why SER does not initiate a NOTCH signal within the dorsal com-

partment where it is expressed, even though the NOTCH receptor is present and is capable of activation by DL. The reciprocal process that prevents or reduces the ability of DL to signal within the ventral compartment is yet to be elucidated.

We have been examining a dominant-negative mutant form of SER encoded by the *Beaded of Goldschmidt (Bd^G)* allele of *Ser* (Hukriede and Fleming, 1997). Adult animals heterozygous for *Bd^G* exhibit loss of wing margin tissue suggesting that the product of the mutation (BD^G) interferes with NOTCH activation and establishment of the D/V organizer. The BD^G product is predicted to encode a truncated form of SER that retains the N-terminal regions through the 14 EGF-like repeats but is deficient for the extracellular cysteine-rich, transmembrane, and intracellular domains (see Fig. 1). The antimorphic nature of the mutation suggests that BD^G can compete with wild-type SER, thereby reducing the strength of the dorsal to ventral signal along the wing margin. Loss of *Ser$^+$* activity at the dorsal edge of the presumptive wing margin or loss of *Dl$^+$* activity along the presumptive ventral wing margin results in the loss of adult wing margin tissue (Diaz-Benjumea and Cohen, 1995; Couso et al., 1995; de Celis et al., 1996; Doherty et al., 1996). Since heterozygous *Bd^G* animals exhibit margin loss phenotypes similar to loss of SER or DL function and since both SER and DL bind within the same region of the NOTCH molecule (Rebay et al., 1991), we sought to investigate the mechanism by which *Bd^G* exerts its dominant-negative effects. Specifically, is the BD^G product a SER-specific antagonist, or can it also antagonize DL function, suggesting that it encodes a more general antagonist to NOTCH-mediated signaling?

In this report, we show that expression of the predicted transcript from the *Bd^G* mutation, encoding an N-terminal truncated form of SER (Fig. 1), is capable of reproducing all of the phenotypes associated with the *Bd^G* mutation. The resulting BD^G product is capable of antagonizing SER function in all events assayed and, in addition, will antagonize DL function during embryonic neurogenesis and in ventral regions of the wing imaginal disc. The BD^G protein retains the SER N-terminal domain acted upon by FNG and its dominant-negative effects can be blocked by FNG expression. These findings suggest that the dominant wing-loss phenotype of heterozygous *Bd^G* animals is due primarily, if not exclusively, to loss of SER activity during wing formation.

MATERIALS AND METHODS

Genetic stocks and markers

All strains used were raised at 25°C on standard media. Most strains, balancers and mutations have been described previously by Lindsley and Zimm (1992); Fleming et al. (1990); Irvine and Wieschaus (1994); Gu et al. (1995); and Hukriede and Fleming (1997). Additional chromosomes and aberrations used in this work include a Gal4^{ptc} driver (Hinz et al., 1994); p[mini-*w*$^+$: UAS-*Dl*] and p[mini-*w*$^+$: UAS-*lac*Z^{nuc}] (nuclear-localized *lac*Z reporter) were generated by T. Jacobson; p[mini-*w*$^+$: UAS-*fng^{27}*] insert on the third chromosome (gift from 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). *Df(3R)Ser^{J94}* is a deficiency removing the 97E-98A interval generated by irradiating 3- to 4-day old males of the genotype *w^{1118}/Y; Y488[w$^+$]/ Y488[w$^+$]* (Shelton and Wasserman, 1993) with 4000 R of gamma rays at approximately 450 R/minute (J.L. Sheppard Cs source). Selection was based on the phenotypic loss of the white$^+$ marker.

One or two copies of the *Ser* promoter (termed *Gal4^{Ser1}* and *Gal4^{Ser2}* respectively; Gu and Fleming, unpublished data), each located on the second chromosome, were used to express individual UAS constructs.

Crosses

To test the effects of ectopic BD^G expression during embryonic neurogenesis, the following crosses were performed. Homozygous *UAS-Bd II/UAS-Bd II; Dl^{BX6}/ TM3, lacZ Sb or UAS-Ser II/UAS-Ser II; Dl^{BX6}/ TM3, lacZ Sb* animals (*II* indicates second chromosome) were crossed with *Dl^{BX6} Gal4^{Hsp70}/TM3, lacZ Sb* animals to produce *UAS-Bd II or UAS-Ser II/+; Dl^{BX6}/Dl^{BX6} Gal4^{Hsp70}* progeny lacking *Dl$^+$* activity. Examination of Bd^G activity in wild-type genetic backgrounds used *UAS-Bd III/UAS-Bd III or UAS-Ser II/UAS-Ser II* animals crossed to *Gal4^{Hsp70}/ TM3,lacZ Sb* animals to produce *UAS-BD III/Gal4^{Hsp70} or UAS-Ser II/+; Gal4^{Hsp70}/+* experimental progeny or *UAS-Bd III/ TM3,lacZ Sb or UAS-Ser II/+; TM3,lacZ Sb/+* control siblings. To determine the effects of FNG in this system, *UAS-Bd III* was recombined onto a *UAS-fng^{27}* (third chromosome) by meiotic recombination. *UAS-Bd III UAS-fng^{27} /UAS-Bd III UAS-fng^{27}* animals were then crossed with *Gal4^{Hsp70}/ TM3,lacZ Sb* as above.

To test the effects of ectopic BD^G expression during wing formation, *UAS-Bd II/UAS-Bd II* animals were crossed with animals homozygous for *Gal4$^{Ser1(or\ 2)}$ /Gal4$^{Ser1(or\ 2)}$* or *Gal4^{ptc}/Gal4^{ptc}* (which may have also carried homozygous *p[mini-w$^+$:vg^{en}]/ p[mini-w$^+$:vg^{en}]* on the second chromosome). Co-expression of *UAS-Bd* with *UAS-Dl* or *UAS-fng^{27}* was accomplished by crossing *UAS-Dl II (pUG1.2.6)/UAS-Dl II (pUG1.2.6); UAS-Bd III/ UAS-Bd III* or *UAS-Bd III UAS-fng^{27}/ UAS-Bd III UAS-fng^{27}* animals to *Gal4$^{Ser1(or\ 2)}$* or *Gal4^{ptc}* homozygous animals. By *crossing UAS-Bd II/CyO; Df(3R)Ser^{J94}/TM6B* animals with *GAl4^{Ser1}/Gal4^{Ser1}; Ser^{+r83k}/Ser^{+r83k}*, progeny of the genotype *UAS-Bd II/Gal4^{Ser1}; Df(3R)Ser^{J94}/Ser^{+r83k}* were obtained to determine the effects of the *Bd* transgene in a *Ser^{+r83k}* genetic background.

Immunohistochemistry

Labeling of embryos with anti-β-galactosidase (Promega) and anti-HRP antibodies (directly conjugated to alkaline phosphatase; Jackson Labs) was performed as described by Gu et al. (1995); dissection and staining of wing imaginal discs was performed as described by Fleming et al. (1997). 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), or mouse anti-αPS2 integrin (1:500; Wilcox et al., 1984). Detection was accomplished using horseradish peroxidase (HRP)-conjugated, goat anti-mouse secondary antibody (Promega) at 1/250 dilution. HRP detection was performed by standard methodologies and wing discs were mounted in glycerol for observation.

Expression constructs and germline transformation

Construction of the UAS-*Bd* transgene was initiated using the 5′ portion of the *Ser* cDNA (Fleming et al., 1990) up to base pair 3357 cut at the *Acc*I site found in the cDNA. This 5′ end of the *Ser* cDNA was spliced to the 3′ end of the partial *Bd^G* cDNA (Hukriede and Fleming, 1997) at the same *Acc*I site. A c-*myc* tag (9E10; Kolodziej and Young, 1991) was generated by PCR using the primers 5′ TGCAAGCTACCGGTATACAGCTCTGGAGCAGAAGCTGATTC-TCCGAG 3′ and 5′ GGGAGCTCGCCCGGTATACAGGT-TCAGGTCCTCCTCGGACATCAGC 3′ from a modified pSp64A c-*myc* tagged vector (gift from R. and L. Angerer). The c-*myc* PCR product was digested with *Acc* I and ligated into the *Acc* I site of the *Bd* cDNA above. The DNA sequence of the tag and the construct junctions were verified using the dideoxy chain-termination technique (Sanger et al., 1977) on double stranded DNA using Sequenase reaction protocols (U. S. Biochemical). The resulting *Bd* cDNA is a

single *Eco*RI fragment and was ligated into the *Eco*RI cloning site of the pUAST P element transformation vector (Brand and Perrimon, 1993) and selected for proper orientation. Transgenic animals were generated using standard germline transformation procedures (Spradling, 1986).

Embryo collection and heat shock induction

Embryos for the UAS-*Bd* transgene induction during neurogenesis 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.

From a cross of homozygous UAS-*construct* animals (where *construct* is either UAS-*Bd* or UAS-*Bd*; UAS-*fng*) to Gal4^{Hsp70}/TM3,*lacZ* animals, experimental (UAS-*construct*/ Gal4^{Hsp70}) and control animals UAS-*construct* / TM3*lacZ*, were obtained. Survival ratios are given as the number of experimental/control animals recovered as adults.

RESULTS

The *Bd^G* phenotype

The functional coding region of *Bd^G* has been previously determined to encode a truncated form of SER lacking the transmembrane and intracellular regions of the wild-type protein (Fig. 1; Hukriede and Fleming, 1997). The regions of the SER protein remaining in the BD^G isoform include the N-terminal regions of the protein that are sufficient for NOTCH binding (Fleming, Sun and Artavanis-Tsakonas, unpublished) and the 14 EGF-like repeats of the wild-type SER protein. Given that the BD^G isoform retained regions capable of binding with NOTCH, we sought to determine if the potential to bind with NOTCH is sufficient to activate NOTCH.

Animals homozygous for loss-of-function alleles of *Ser* die at the late embryo/first larval instar period and exhibit malformations in larval head structures, particularly the development of the mouth hooks (compare Fig. 2B with wild type in 2A; see also Speicher et al., 1994). The *Bd^G* mutation produces a dominant wing phenotype in heterozygous animals and encodes an antimorphic form of SER capable of interfering with normal SER function (Hukriede and Fleming, 1997). Homozygous or hemizygous *Bd^G* animals die at the late embryonic/first larval instar transition and appear phenotypically indistinguishable from animals homozygous for *Ser* null alleles (compare Fig. 2C with 2B). This implies that the BD^G product is incapable of supplying normal SER function. Moreover, in the presence of wild-type SER, BD^G will interfere with normal SER activity.

During embryonic neurogenesis, the interaction of *Dl* gene product (DL) with the NOTCH receptor is required for proper specification of neuroblasts and epidermal cells from pluripotent ectodermal precursors (reviewed by Artavanis-Tsakonas et al., 1995). Since ectopic expression of SER during embryonic neurogenesis can substitute, at least in part, for loss of DL activity in this process (Gu et al., 1995) and since the homozygous *Bd^G* phenotype implied that there is no wild-type activity associated with BD^G, we determined if the BD^G form simply lacked the ability to activate NOTCH or if it was specifically non-functional in roles requiring SER function.

Based on the *Bd^G* transcriptional product, we constructed a *Bd^G* cDNA and placed it under the control of the upstream activating sequence (termed UAS-*Bd*) of the two component Gal4 system (Brand and Perrimon, 1993; see Materials and Methods). Several independent transgenic lines were recovered via P element mediated transformation (Spradling, 1986). We tested the ability of UAS-*Bd* to activate NOTCH by examining its effects during embryonic neurogenesis. If BD^G retained the ability to activate NOTCH, then it should be able to substitute for the loss of DL expression during embryonic neurogenesis.

Animals lacking zygotic *Dl* activity exhibit neuronal hypertrophy at the expense of epidermal structures (Lehmann et al., 1983), a condition referred to as the neurogenic phenotype. Expression of wild-type SER under the control of a heat shock promoter can partially rescue *Dl* neurogenic phenotypes when expressed 4- to 5-hours after egg laying (AEL; Gu et al., 1995). We expressed UAS-*Bd* under Gal4^{Hsp70} control at 4 hours AEL in a *Dl^{BX6}* homozygous (null) background and examined the effects on development of the nervous system. Animals deficient for *Dl* activity exhibit severe neuronal hypertrophy (compare Fig. 3B with wild type in 3A). *Dl* deficient animals supplied with wild-type SER function via a single 1-hour 37°C heat pulse 4 hours AEL exhibit reduced neuronal hyperplasia (Fig. 3C). In contrast, when *Dl* deficient animals express UAS-*Bd* under identical conditions, no reduction in the severity of the *Dl* phenotype is observed (Fig. 3D). It appears, therefore,

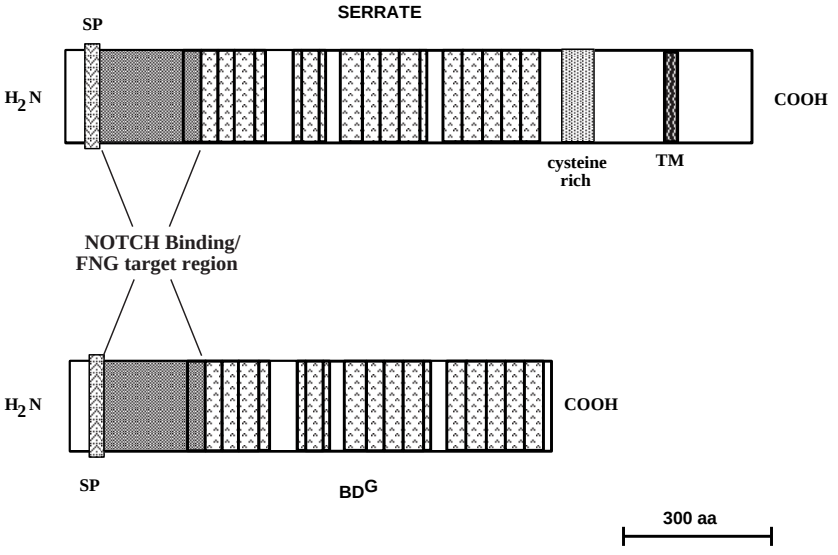

Fig. 1. The SER and BD^G protein products. The illustration depicts the predicted protein product for the wild-type SER product (upper panel) and the truncated BD^G product (lower panel). The N-terminal NOTCH binding region that also serves as the region upon which FNG regulation is imposed is shaded (Fleming et al., 1997). Other regions common to both molecules include the signal peptide (SP) and EGF-like repeats (chevroned boxes). The wild-type SER form also contains a cysteine-rich region (stippled box), transmembrane domain (TM) and intracellular regions. H_2N, amino terminus; COOH, carboxy terminus. Scale bar = 300 amino acids.

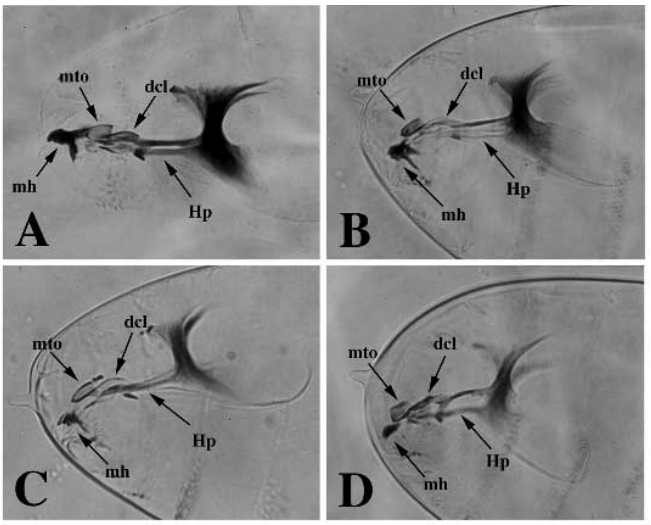

Fig. 2. Head skeleton defects of *Ser* mutations. First instar larvae of each of the genotypes below were collected, cleared and mounted. In all panels, anterior is to the left and dorsal is up. Hp = H-piece; dcl = dorsal neck clasp; mto = median tooth; mh = mouth hooks. (A) Wild type larva showing normal development of mouth hooks. (B) A homozygous *Ser* null embryo ($Ser^{rev6\text{-}1}$) displays malformations, primarily of mouth hook structures. (C) Homozygous Bd^{G} larvae appear phenotypically similar to *Ser* null larvae with mouth hook defects (compare to 2B). (D) Expression of the UAS-*Bd* transgene under a $Gal4^{e22c}$ promoter produces larval mouth hooks with defects comparable to loss of function *Ser* alleles (compare to 2B and C).

that even though the BD^{G} form retains the NOTCH binding region, this molecule is incapable of activating NOTCH under these conditions.

In order to demonstrate that the UAS-*Bd* product can function, we expressed it under the control of the $GAl4^{e22c}$ promoter, which is expressed in most tissues beginning around the time of blastoderm formation. If the $GAl4^{e22c}$ promoter is used to express wild-type UAS-*Ser*, animals die at approximately the first instar larval stage but fail to exhibit any gross morphological abnormalities. This finding indicates that expression of the $GAl4^{e22c}$ promoter must be at relatively low levels in the embryonic neuroectoderm since ectopic SER production by this promoter does not appear to interfere with neurogenesis (data not shown). Expression of UAS-*Bd* by $GAl4^{e22c}$ also results in late embryonic/early larval lethality. Again, neuronal development appears fairly normal in these animals, however, head skeleton defects remarkably similar to the mouth-hook defects seen for *Ser* nulls are consistently observed (Fig. 2D). It is likely that this phenotype results from an ability of BD^{G} to antagonize endogenous SER activity in this region.

To further substantiate that UAS-*Bd* can be expressed and is capable of mimicking phenotypes associated with the original Bd^{G} mutation, we expressed the transgene under the control of partial *Ser* promoters (Gu and Fleming, unpublished). These promoters are expressed in a pattern similar to wild-type expression in the developing third instar larval disc (a wing disc drawing is provided in Fig. 4A for reference). As shown in Fig. 4B, wild-type SER protein is detected primarily along the presumptive dorsal wing margin during the third larval instar stage. We used a chromosome carrying the *Ser* promoter in one copy ($Gal4^{Ser1}$; Fig. 4C) or two copies ($Gal4^{Ser2}$; Fig. 4D) to express UAS-*Bd* in a dose-dependent fashion because the Bd^{G} mutation is sensitive to wild-type Ser^{+} dosage (Hukriede and Fleming, 1997). The $Gal4^{Ser2}$ promoter, when used to express the UAS-*Ser* cDNA, consistently produces higher concentrations of SER along the dorsal boundary than does the $Gal4^{Ser1}$ promoter (compare Fig. 4D with 4C). Animals with two Ser^{+} doses and one Bd^{G} allele have phenotypically normal wings (Fig. 4E) but animals with a single Ser^{+} dose and the Bd^{G} allele exhibit loss of adult wing margin (Fig. 4F). In the presence of wild-type levels of endogenous SER, expression of UAS-*Bd* by $Gal4^{Ser1}$ or $Gal4^{Ser2}$ results in animals exhibiting wing margin loss phenotypes (Figs 4G and 4H, respectively). As expected, the severity of margin loss increases as the number of *Ser* promoters is increased. It is interesting to note that the severity of the wing margin loss phenotype caused by expressing the transgene under a single *Ser* promoter ($Gal4^{Ser1}$; Fig. 4G) is more severe in the presence of two wild-type *Ser* alleles than is the wing phenotype associated with the Bd^{G} mutation heterozygous for a single wild-type *Ser* allele (Fig. 4F). This result is likely to be a consequence of the Gal4 expression system used to drive the transgene since it appears that the levels of SER produced under the control of $Gal4^{Ser2}$ promoters is significantly higher than those produced by endogenous *Ser* promoters (compare Fig. 4B with 4C and 4D).

Animals heterozygous for the Bd^{G}/Ser^{+r83k} combination of *Ser* mutations die as pharate adults and exhibit striking phenotypic abnormalities (Gu et al., 1995). These include abnormalities of the adult leg such as fusion of the femur and tibial segments (Fig. 5B; compare with wild type, 5A) and the near absence of wing blade material (Fig. 5E; compare with wild

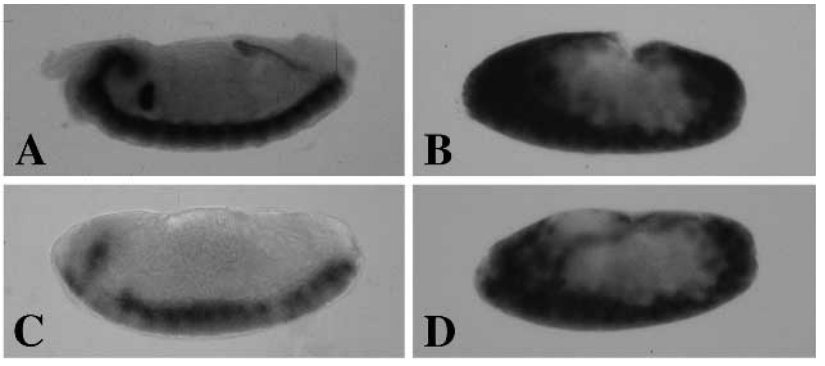

Fig. 3. Functions of SER and BD^{G} during neurogenesis. The effects of ectopic SER and BD^{G} expression during embryonic neurogenesis were examined using the anti-HRP nervous system specific marker (Jan and Jan, 1982). Orientation is dorsal up, anterior left; all animals are at approximately stages 12-13. (Campos-Ortega and Hartenstein, 1985). (A) Wild-type embryo with normal neuronal architecture. (B) Homozygous Dl^{BX6} embryo, lacking zygotic *Dl* function, displays characteristic neuronal hyperplasia. (C) Embryo lacking zygotic *Dl* expression as in B but was provided with ectopic SER, by a 1-hour 37°C heat shock at 4 hours AEL, shows greatly reduced neuronal hyperplasia (compare to 3B). (D) Embryo lacking zygotic *Dl* provided with ectopic BD^{G} expression under the same conditions as in C shows neuronal hyperplasia comparable to 3B.

type, 5D). The *Ser^{+r83k}* mutation itself is homozygous and hemizygous viable suggesting that the antimorphic nature of the *Bd^G* mutation is responsible for the mutant phenotypes observed in *Bd^G/Ser^{+r83k}* animals. To assess how comparable the *Bd* transgene is to the *Bd^G* mutation, we expressed UAS-*Bd* by Gal4^{Ser2} in a *Ser^{+r83k}* hemizygous background (see Materials and Methods). These animals exhibit phenotypes that are indistinguishable from the *Bd^G/Ser^{+r83k}* mutant combination (compare Fig. 5C with 5B and 5F with 5E). Together with the previous transgene expression results, we are confident that the *Bd* transgene faithfully reproduces the effects of the *Bd^G* mutation.

Developmental effects of ectopic BD^G expression

Our results support the contention that the BD^G product is not capable of activating the NOTCH receptor under all conditions tested. However, BD^G is capable of antagonizing SER functions. The simplest model to account for these observations is that BD^G may be able to bind with the NOTCH receptor in a non-productive manner and, in so doing, block wild-type SER from accessing and transducing a signal through the NOTCH receptor. Such a model would predict that, since SER and DL bind within the same two EGF-like repeats of NOTCH (Rebay et al., 1991), BD^G should function as an antagonist to DL as well as to SER.

Previously, it was demonstrated that ectopic SER expression during neurogenesis will suppress neuroblast formation (Gu et al., 1995). The effects of ectopic expression of SER by Gal4^{Hsp70} at 4 hours AEL in otherwise wild-type animals can be seen in Fig. 6B (compare to wild type; Fig. 6A). It is believed that this mis-expression of SER during neurogenesis acts similarly to overexpression of DL, causing the inappropriate activation of the NOTCH receptor and suppression of neuroblast formation. Since BD^G does not appear to be capable of NOTCH activation during embryonic neurogenesis, we reasoned that if BD^G antagonizes SER by blocking access to the NOTCH receptor, it may interfere with DL signaling in a similar fashion. We therefore ectopically expressed UAS-*Bd* by Gal4^{Hsp70} at 4 hours AEL in wild-type embryos and examined the animals for developmental effects. Not surprisingly, UAS-*Bd*/Gal4^{Hsp70} promoter animals die as embryos while UAS-*Bd* /TM3, β-gal control siblings develop normally (11/175 animals for 6% survival; see Materials and Methods). More importantly, as shown in Fig. 6C, the UAS-*Bd* / Gal4^{Hsp70} heat pulsed animals exhibit neuronal hypertrophy with a concomitant reduction in hypodermal differentiation (compare Fig. 6E with wild type, Fig. 6D). These results are consistent with BD^G binding with NOTCH, thereby limiting the access of DL to the NOTCH receptor. Thus, BD^G can act as a general NOTCH antagonist that has the ability to block the access of both SER and DL to the NOTCH receptor.

Antagonist of the *Notch* signaling pathway

The BD^G product will compete with DL during neurogenesis and with SER during appendage formation. We have previously shown that two copies of the wild-type *Ser* locus with

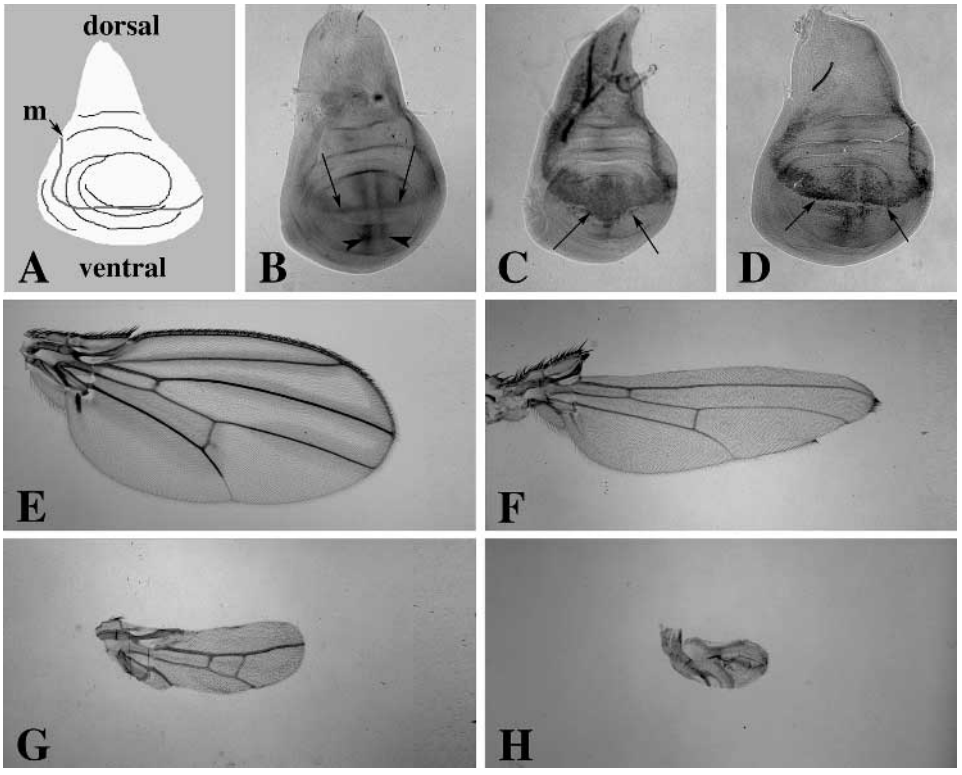

Fig. 4. Expression of UAS-*Bd* under *Ser* promoters reproduces *Bd^G* phenotypes. Expression of *Ser* promoters and the effects of UAS-*Bd* expression under the control of those promoters are shown by anti-SER staining of late third instar wing discs and adult wing whole mounts. For all discs, dorsal is up and anterior to the left. (A) Drawing depicting normal third instar wing imaginal disc. 'm' indicates the location of presumtive wing margin formation at the D/V boundary. (B) Wild-type wing disc depicting endogenous SER expression pattern along the dorsal edge of the D/V boundary (arrows) and A/P boundary (arrowheads). (C) Wild-type wing disc in which the Gal4^{Ser1} promoter has been used to express UAS-*Ser*. Greatly increased amounts of SER product are evident around the presumptive wing margin (compare to 4B). Gal4^{Ser1} has some ectopic expression near the A/P boundary in the ventral compartment (arrows). (D) When two copies of the *Ser* promoter (Gal4^{Ser2}) are used to express UAS-*Ser*, very high levels of SER product are observed, especially along the dorsal edge of the wing margin (arrows). (E) Wing blade from a *Bd^{Grev7}* animal, which has two wild-type *Ser* alleles and the *Bd^G* mutant allele (Hukriede and Fleming, 1997), shows normal patterning and margin formation. (F) *Bd^G/+* wings display the characteristic dominant loss of wing margin phenotype. Dose sensitivity of the *Bd^G* mutation with wild-type *Ser* is demonstrated by comparing this wing with 4E. (G) Expression of UAS-*Bd* by Gal4^{Ser1} in a wild-type *Ser* background (two *Ser$^+$* doses) results in loss of wing margin which is more severe than *Bd^G/+* animals (4F; see text). (H) Expression of UAS-*Bd* by Gal4^{Ser2} in a wild-type background produces a more severe phenotype than that seen in 4G, demonstrating dose sensitivity of UAS-*Bd*.

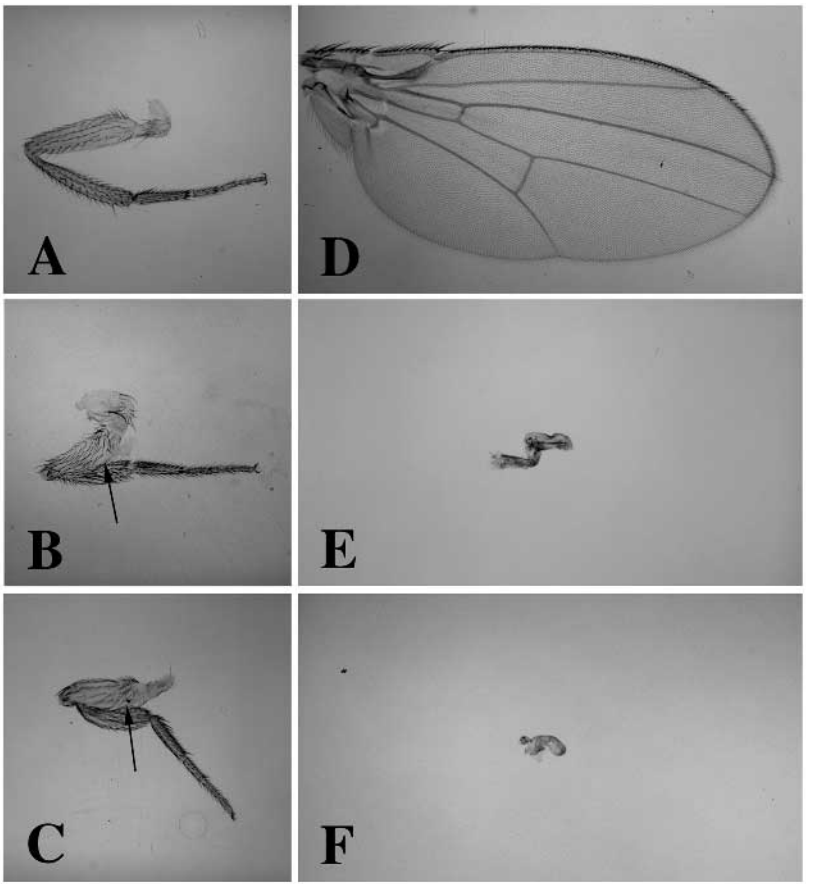

Fig. 5. UAS-*Bd* expression recapitulates the effects of the Bd^G mutation. Heterozygous Bd^G/Ser^{+r83k} animals are late pupal lethal and display characteristic leg and wing defects that can be mimicked by expressing UAS-*Bd* by $Gal4^{Ser1}$ in *a* Ser^{+r83k}*/deficiency (Df(3R)*Ser^{J94}*)* background. (A) Wild-type adult leg. (B) Adult leg from a Bd^G/Ser^{+r83k} animal with characteristic fusion of femur and tibial segments (arrow). (C) UAS-*Bd* expression in the Ser^{+r83k} background results in femur-tibial leg fusions similar to 5B (arrow). (D) Wild-type adult wing. (E) Wing recovered from a Bd^G/Ser^{+r83k} animal displays little wing blade tissue. (F) Wings removed from UAS-*Bd* expressing Ser^{+r83k} animals show reduction in wing blade development similar to 5E. All wing pictures at same magnification.

a single copy of the Bd^G allele results in a wild-type wing (Hukriede and Fleming, 1997). This suggests that the endogenous SER product will compete with the BD^G product. Since *Dl* also functions during wing formation (de Celis et al., 1996; Doherty et al., 1996), we questioned if it was possible for extra copies of the endogenous *Dl* locus to compete with the Bd^G mutation in the wing. We constructed flies that had three doses of Dl^+ (using $Dp(3;3)bxd^{110}$) and that were heterozygous for the Bd^G mutation. Adult wing blades from these animals are indistinguishable from those of heterozygous Bd^G animals with normal *Dl* doses (data not shown). This suggested that BD^G may not directly interfere with DL

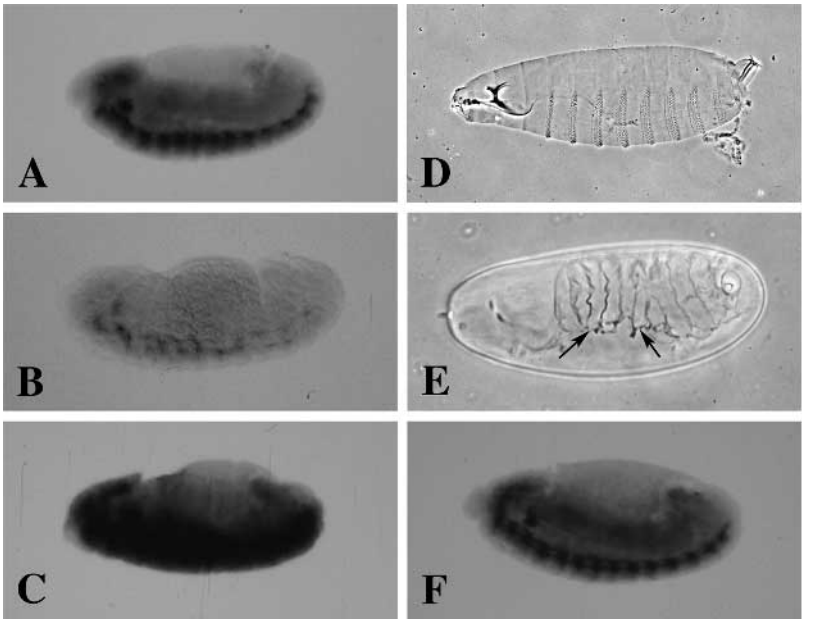

Fig. 6. Expression of UAS-*Bd* can antagonize DL during embryonic neurogenesis. Wild-type embryos were examined for neuronal development following ectopic, heat shock induced expression of UAS-*Ser*, UAS-*Bd* and UAS-*Bd*; UAS-fng^{27} combinations at 4 hours AEL. Orientation and staging of embryos is the same as in Fig. 3. (A) Wild-type embryo with normal neuronal architecture (stained for anti-HRP neuronal marker; Jan and Jan, 1982). (B) Wild-type embryos supplied with ectopic SER at 4 hours AEL have suppressed neuronal differentiation as a result of excessive NOTCH activity (see text). (C) In contrast to 6B, wild-type embryos expressing BD^G at 4 hours AEL, exhibit neuronal hypertrophy, suggesting that normal DL-NOTCH activation is being inhibited. (D) Wild-type first instar cuticle preparation showing normal hypodermal tissue differentiation. (E) Cuticle preparations from embryos expressing BD^G at 4 hours AEL show characteristic loss of ventral epidermal structures (arrows) characteristic of animals lacking normal *Dl* activity. (F) Animals simultaneously expressing UAS-*Bd* and UAS-fng^{27} develop phenotypically normal nervous systems demonstrating that FNG can regulate BD^G expression as it does SER expression (see text).

function in the wing even though it is capable of interfering with DL during neurogenesis.

One potential caveat to the genetic competition experiment is that BD^G and DL are not expressed in the same cells of the wing imaginal disc. In the *Bd^G* mutant, expression of BD^G is driven on the dorsal side of the wing margin by the *Ser* promoter and not in the ventral wing compartment where DL expression is required (de Celis et al., 1996; Doherty et al., 1996). Therefore, BD^G may not directly compete with DL during wing formation. Even though BD^G may be a secreted protein (Hukriede and Fleming, 1997; Sun and Artavanis-Tsakonas, personal communication), it may not be capable of diffusion, hence it might not block DL activity along the ventral wing margin. An alternative explanation for the inability of extra *Dl$^+$* doses to affect *Bd^G* expression may stem from the possibility that there exist differences in the effects of SER- and DL-mediated signals through NOTCH (Jönsonn and Knust, 1996; Fleming et al., 1997). If this is true, BD^G may not be able to antagonize DL-mediated NOTCH activation during wing formation simply because BD^G is still recognized as having SER identity.

We addressed these questions directly by ectopically expressing BD^G, DL, or BD^G and DL in the developing wing imaginal disc. When UAS-*Bd* is expressed under the control of Gal4^{Ser2}, the regions of the wing imaginal disc corresponding to the presumptive wing blade are consistently smaller and misshapen relative to wild type (Fig. 7B, compare with 7A). The corresponding adult wings produced by these animals are shown in Fig. 4H. In contrast, expression of a UAS-*Dl* transgene by Gal4^{Ser2} induces dorsal outgrowths of the wing imaginal disc (Fig. 7C) and adult wings with excess dorsal wing blade material (Fig. 7D).

If BD^G can bind and block access to NOTCH by DL in the wing disc similar to its effects during neurogenesis, then co-

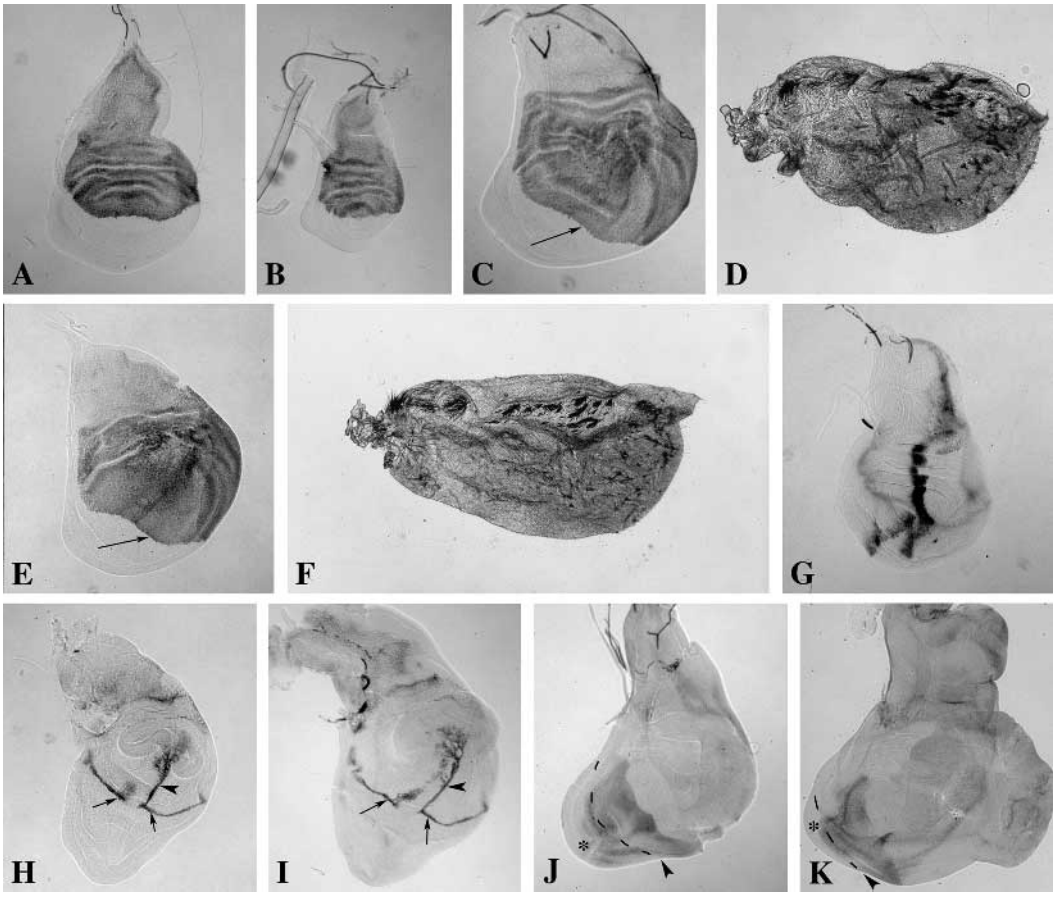

Fig. 7. Phenotypic effects of UAS-*Bd* expression during wing formation. UAS-*Bd* was expressed under the control of Gal4^{Ser2} (see Fig. 4C) and Gal4^{ptc} promoters to assay the effects of BD^G product on wing development. Orientation of wing discs is the same as in Fig. 4. (A) Wild-type wing disc stained for dorsal-specific expression of an *ap-lacZ* reporter. (B) When UAS-*Bd* is expressed under control of Gal4^{Ser2}, the region of the wing disc that produces the wing blade is greatly reduced in size (stained for *ap-lacZ* expression). (C) Expression of a UAS-*Dl* construct under Gal4^{Ser2} control results in over-proliferation of cells within the dorsal compartment (stained for *ap-lacZ* expression; note displacement of D/V region; arrow). (D) The adult wing corresponding to the wing disc shown is 7C is greatly distorted as a result of dorsal tissue overgrowth. (E) UAS-*Bd* and UAS-*Dl* were co-expressed under Gal4^{Ser2}. The resulting wing disc appears indistinguishable from that of UAS-*Dl* expression alone by Gal4^{Ser2} (note margin displacement (arrow); compare to C), suggesting the BD^G will not compete with DL in the dorsal wing compartment. (F) Adult wing corresponding to E appears indistinguishable from expression of UAS-*Dl* alone (D). (G) Wild type wing disc stained for expression of a UAS-lacZ reporter construct under the control of the Gal4^{ptc} promoter. Note promoter expresses along A/P boundary in both the dorsal and ventral compartments. (H) Expression of UAS-*Dl* under Gal4^{ptc} produces cell proliferation in both the dorsal and ventral wing compartments. Staining pattern for cut protein shows normal marginal expression (arrows) and ectopic expression along *ptc* expression pattern in the dorsal compartment only (arrowhead). (I) Co-expression of UAS-*Bd* and UAS-*Dl* by Gal4^{ptc} does not alter cut expression pattern (compare to H) consistent with no functional Bd^G effects in the dorsal compartment (see text). (J) The extent of ventral overgrowth induced by UAS-*Dl* by Gal4^{ptc} is variable but generally greater than the overgrowth observed in K when UAS-*Dl* and UAS-*Bd* are co-expressed under Gal4^{ptc}. Ventral overgrowth is greatest adjacent to the *ptc* expression stripe and can be detected as a ridge of outgrowth from the disc (arrowheads in J and K). By extrapolating the position of the ridge and the expected size of the normal ventral compartment (dotted line), the extent of ventral overgrowth can be discerned (asterisks). Discs in J and K are stained for ventral specific staining of the αPS2 integrin (Wilcox et al., 1984).

expression of BD^G and DL by $Gal4^{Ser2}$ would be expected to reduce the severity of overgrowth produced by ectopic expression of DL alone. Significantly, co-expression of UAS-*Bd* and UAS-*Dl* by $Gal4^{Ser2}$ did not alter the extent of cell proliferation in the dorsal wing compartment in discs or adult wing blades (Figs 7E and 7F respectively). We further tested for the ability of BD^G to compete DL by co-expressing these molecules under a *patched* promoter ($Gal4^{ptc}$) that expresses in a stripe through the dorsal and ventral compartments (Fig. 7G). Consistent with the above results, examination of wing discs produced by these animals revealed that BD^G does not effectively block the ability of DL to induce dorsal expression of the margin-specific *cut* gene product (compare Figs 7H and 7I). However, it does appear that co-expression of BD^G with DL leads to variable but consistent reduction in the extent of ventral compartment cell proliferation as compared with DL expression alone (compare Figs 7J and 7K). Expression of the BD^G protein could be detected with anti-SER antibody in both the dorsal and ventral wing compartments, though the staining was diffuse and not confined to the stripe of *ptc* expression (data not shown). These findings suggest that BD^G can antagonize DL, however, the dominant-negative effects of BD^G are compartmentally restricted in the wing disc.

BD^G interactions with *fringe*

We have recently shown that the presence of the *fringe (fng)* gene product (FNG) in the dorsal compartment of the wing disc blocks the ability of SER to activate NOTCH in that region (Fleming et al., 1997). Moreover, the N-terminal region of SER is required for FNG to regulate SER function. Since BD^G retains this N-terminal domain, and since our data suggest that the actions of BD^G are blocked within the dorsal wing compartment, it is reasonable to suspect that FNG also regulates the ability of BD^G to interact with NOTCH. The possibility of FNG regulating BD^G would serve to explain why BD^G can antagonize DL function during embryonic neurogenesis and in the ventral wing compartment where FNG is not expressed (K. Irvine, personal communication; Irvine and Weischaus, 1994), but not in the dorsal wing compartment where FNG is expressed.

We tested the ability of a *fng* transgene (UAS-fng^{27}) to suppress the dominant-negative effects of the *Bd* transgene during neurogenesis and pupal development by co-expressing FNG with BD^G. During embryogenesis, we ectopically expressed UAS-*Bd* and UAS-fng^{27} under the control of $GAL4^{Hsp70}$ with a 1-hour 37°C heat pulse 4 hours AEL. Instead of the neural hypertrophy seen when BD^G is expressed alone (Fig. 6C), co-expression of BD^G and FNG produce animals with apparently normal CNS development (Fig. 6F). Moreover, the viability of these animals is significantly improved when compared to ectopic expression of BD^G alone (57% survival for UAS-*Bd*; UAS-fng^{27} [84/147 animals] versus 6% survival for UAS-*Bd* [11/175 animals]; see Materials and Methods). This suggests that FNG protein will suppress the ability of BD^G to antagonize DL during neurogenesis.

A further test of FNG's ability to suppress the *Bd* transgene was performed during pupal development. When UAS-*Bd* is expressed under the control of a $Gal4^{ptc}$, animals die during early pupal formation prior to imaginal disc eversion (data not shown). Co-expression of UAS-*Bd* and UAS-fng^{27} by $Gal4^{ptc}$ produces animals that are late pupal lethal but have everted their imaginal discs and develop to pharate adults. In fact, even though the pharate adults will not eclose, when dissected from their pupal cases, the animals are alive and exhibit complete though shortened legs and no wing blades (data not shown). It is unclear why FNG expression at these times is unable to completely block the actions of BD^G, though it is possible that differential diffusion of the secreted BD^G form relative to that of FNG might account for these observations. Taken together, the data support the proposal that of FNG can block the ability of BD^G to interact with NOTCH similar to the effect FNG has on wild-type SER.

DISCUSSION

The Bd^G mutation is associated with the insertion of a transposable element, *roo*, into the coding region of the *Ser* locus (Hukriede and Fleming, 1997). As a result of this insertion, a novel transcript is produced under control of the *Ser* promoter that is predicted to encode a truncated version of the SER protein (Fig. 1). In this report, we confirm that expression of this altered *Ser* transcript is capable of generating the phenotypes associated with the original Bd^G mutation. In addition, upon examining the effects of this mutant SER form we conclude that BD^G can function as a general antagonist of the *Notch* signaling pathway. However, similar to wild-type SER, its ability to interact with NOTCH is regulated by the product of the gene *fringe*.

FNG affects expression of BD^G

It has been previously demonstrated that the FNG protein product selectively inhibits SER from transducing a signal through NOTCH (Fleming et al., 1997; Panin et al., 1997). More specifically, FNG appears to regulate SER by targeting the N-terminal NOTCH binding domain of the molecule. The co-localization of FNG and SER in the dorsal compartment of the wing imaginal disc (Irvine and Weischaus, 1994; Diaz-Benjumea and Cohen, 1995; Couso et al., 1995) is necessary, therefore, to prevent promiscuous activation of NOTCH in that region. Due to the presence of FNG and the N-terminal NOTCH binding domain within BD^G, the reason that ectopic BD^G expression is unable to compete with ectopic DL expression in the dorsal wing is readily apparent. Moreover, the selectivity of FNG targeting upon the SER N-terminal domain of BD^G is evident from our co-expression studies during embryonic neurogenesis. Animals expressing BD^G alone exhibit neuronal hyperplasia and die as embryos but animals co-expressing BD^G and FNG exhibit normal neuronal differentiation and can survive through adulthood. If FNG affected the NOTCH terminal binding domain of DL, then expression of FNG during neurogenesis should have also blocked DL-NOTCH interactions and produce neurogenic embryos.

Based on amino acid sequence comparisons, it has recently been suggested that FNG and FNG-like molecules may encode proteins related to galactosyltransferases (Yuan et al., 1997). Such a role for FNG is consistent with our data and suggests that the binding of SER to NOTCH may be inhibited by some type of modification event within the binding regions of these molecules. Since the region of the SER protein targeted by FNG is capable of binding with NOTCH, studies will be

required to determine if a protein modification is involved in regulation by FNG and if so, whether the modification is produced on SER or on NOTCH.

The dominant-negative nature of the BD^G product

Gene dosage studies supply the initial evidence that *Bd^G* encodes an antimorphic or dominant-negative form of *Ser*. The dominant wing margin defect associated with heterozygous *Bd^G* animals is effectively eliminated when an additional wild-type *Ser$^+$* gene copy is supplied (Hukriede and Fleming, 1997). This implies that a competition exists between wild-type SER and the BD^G form. Our expression studies, particularly during wing development, confirm these results by demonstrating that the margin-loss phenotype associated with expression of the UAS-*Bd* transgene under control of *Ser* promoters is similarly dosage sensitive. In addition, ectopic expression of UAS-*Bd* by Gal4^{e22c} produces phenotypes in embryos with wild-type levels of SER nearly identical to phenotypes seen with loss-of-function *Ser* alleles, suggesting that the transgene product competes with SER during embryonic periods as well.

Two other significant findings concerning the BD^G product provide clues as to how the dominant-negative effects of this molecule are realized. Firstly, at least during the developmental periods tested, the BD^G protein does not retain the ability to activate NOTCH. This is true for homozygous *Bd^G* embryos, which appear phenotypically identical to homozygous *Ser* null embryos, and for ectopic expression during embryonic neurogenesis in animals lacking *Dl* activity. In both cases, the data are consistent with an absence of NOTCH activity associated with the BD^G protein. Second, and more importantly, BD^G has the ability to antagonize DL. Ectopic BD^G production during embryonic neurogenesis produces a neurogenic phenotype similar to that seen for *Dl* mutations (Lehmann et al., 1983). The results are therefore consistent with BD^G functioning to interfere with endogenous DL-NOTCH interactions during neurogenesis. During wing development, ectopic expression of BD^G has little if any detectable effect on ectopic DL expression in the dorsal wing compartment most likely due to the presence of FNG. Along the marginal zone, BD^G expression usually interferes with endogenous SER activity and disrupts the formation of the wing margin. Interestingly, there are no obvious margin defects associated with co-expression of DL and BD^G under the *Ser* expression pattern. Wings that express either DL alone or both DL and BD^G under the *Ser* pattern however exhibit only limited anterior margin formation (Fig. 7D,F). This result is likely due to loss of margin-specific gene expression brought about by ectopic expression of DL along the dorsal marginal zone (Fleming et al., 1997), leading to the absence of a defined margin. Hence, in the absence of a discernible margin, the effects of BD^G in the marginal zone are likely to go unnoticed.

Expression of DL in the ventral wing compartment leads to ventral cell overgrowth (Fig. 7J). Co-expression of BD^G and DL in the ventral wing also causes ventral cell overgrowth, however, that overgrowth is variably reduced relative to DL expression alone (Fig. 7K). Amongst the possible explanations that might explain why the antagonistic BD^G form does not fully compete with DL for signaling in the ventral wing compartment are differential expression of the two UAS constructs, differences in BD^G and DL binding affinity for NOTCH or the effective local concentration of each ligand during NOTCH activation. The biochemical nature of the differences in DL and BD^G action remain to be tested. However, the lattermost explanation seems most plausible given that the BD^G product is predicted to be secreted and our anti-SER antibody detection demonstrated that it does not remain localized to the *ptc* expression stripe in the wing disc (see also Sun and Artavanis-Tsakonas, 1997). Thus, within the *ptc* expression stripe, the secreted BD^G product would be expected to have lower relative concentrations than the membrane-bound DL product, thereby increasing the likelihood of positive NOTCH signaling interactions.

It is interesting to note that NOTCH ligands lacking transmembrane and intracellular domains in *Drosophila* have dominant-negative effects (Sun and Artavanis-Tsakonas, 1996; Hukriede and Fleming, 1997; Sun and Artavanis-Tsakonas, 1997). While these findings are consistent with those found for the *Xenopus* homolog of *Dl, X-Delta-1* (Chitnis et al., 1995), they stand in direct contrast to that seen for the *C. elegans* counterparts to the *Drosophila Notch* signaling pathway. In that system, expression of the extracellular domains of the ligands *lag-2* or *apx-1* are capable of functioning in a near wild-type capacity through the *lin-12/glp-1* receptors and can rescue *lag-2* loss-of-function mutations (Fitzgerald and Greenwald, 1995). While these fundamental differences appear to present a paradox in signaling mechanisms, it should be noted that in *C. elegans*, *lin-12/glp-1* ligands appear to be completely interchangeable in their functions (Fitzgerald and Greenwald, 1995) while *Ser* and *Dl* have limited abilities to substitute for one another (Gu et al., 1995; Fleming et al., 1997). This difference, coupled with the structural differences between *C. elegans* and *Drosophila lin-12/Notch* family ligands (Simpson, 1995), may reflect basic differences in the mechanism governing activation and regulation of signaling through the respective receptors in these two systems.

Implications for BD^G action and NOTCH signaling

When one examines the predicted BD^G protein, only the N-terminal domains, which include a region capable of binding NOTCH (Fleming, Sun and Artavanis-Tsakonas, unpublished) and the fourteen EGF-like repeats remain of the wild-type SER protein. An extracellular cysteine-rich region along with the transmembrane and intracellular domains have been eliminated in the mutant form (Hukriede and Fleming, 1997). How then does this mutant product exert its dominant-negative effects? Since it has been established that SER acts as a NOTCH ligand, the level of this interaction could be either in sequestering wild-type forms of NOTCH ligands or by sequestering NOTCH itself. Sequestering NOTCH ligands could be accomplished if those ligands function in a multimeric form. In this regard, homotypic interactions have been noted for cells expressing DL in cell culture (Fehon et al., 1990). If NOTCH ligands do dimerize, BD^G could potentially titrate out functional ligand dimers by generating non-functional heterodimers, thereby reducing effective NOTCH-LIGAND interactions. Although this possibility cannot be entirely discounted at this time, it seems unlikely. BD^G is a form of the SER protein and, in tissue culture experiments, neither homotypic interactions amongst SER expressing cells nor heterotypic SER-DL interactions have been observed (Rebay et al., 1991).

Since both SER and DL bind to within the 11th and 12th EGF-like repeats of NOTCH (Rebay et al., 1991) and the

NOTCH-binding region of the SER protein is retained by BD^G, it is more plausible that BD^G acts to sequester NOTCH receptor than NOTCH ligands. In this model, BD^G would bind with NOTCH and occupy the ligand binding site in the 11th and 12th EGF-like repeats. Endogenous SER (or DL) would then have to displace BD^G in order to activate NOTCH; thereby establishing the dominant-negative effect of the BD^G form.

This model accounts for all of the available data but, if correct, it raises a more basic question concerning NOTCH activation. Implicit in this model is the suggestion that the binding of a ligand to within the 11th and 12th EGF-like repeats of NOTCH is not sufficient for NOTCH activation. This model predicts that BD^G binds within that region yet our data show that BD^G is incapable of transducing a signal. At the same time however, the model supports the conclusion that binding within this region is necessary for NOTCH activation since in the presence of BD^G, wild-type ligands have impaired signaling ability. Taken together, these data suggest that binding within the 11th and 12th EGF-like repeats of NOTCH is necessary but not sufficient for receptor activation.

Additional data support the hypothesis that ligand binding is insufficient for NOTCH activation, or at least for the generation of ligand-specific responses by NOTCH. During wing development, both SER and DL are required for margin specification through NOTCH (Diaz-Benjumea and Cohen, 1995; Couso et al., 1995; de Celis et al., 1996; Doherty et al., 1996), and differential cellular responses of SER- and DL-mediated NOTCH activation have been demonstrated (Jönsson and Knust, 1996; Fleming et al., 1997). In particular, ectopic expression of DL along the dorsal wing margin under the control of a *Ser* promoter abolishes the expression of margin-specific genes. In contrast, expression of SER under conditions where dorsal signaling is permitted, will produce margin-specific gene activation (Fleming et al., 1997). This implies that the signal sent by DL-NOTCH interaction is somehow different than that mediated by SER-NOTCH interaction. Importantly, when the NOTCH binding region of DL is substituted for the corresponding region of SER and the chimeric molecule is similarly expressed along the dorsal wing margin, margin-specific gene expression is induced, indicating that the signal is comparable to a SER signal, not one from DL (Fleming et al., 1997). These data suggest strongly that the specificity of the NOTCH signal generated by interactions with SER and DL originate from regions residing outside of the NOTCH binding domains of these molecules and imply that other properties of NOTCH ligands are required for NOTCH activation. Of significant interest will be the determination of how signal specificity is generated by individual NOTCH ligands and the derivation of the molecular nature of the different signals transduced by NOTCH.

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REFERENCES

Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M.E. (1995). *Notch* signaling. *Science* **268**, 225-232.

Brand, A.H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Campos-Ortega, J.A., and Hartenstein, V. (1985). *The Embryonic Development of* Drosophila melanogaster. Berlin-Heidelberg-New York-Tokyo: Springer-Verlag.

Capdevila, J. and Guerrero, I. (1994). Targeted expression of the signalling molecule *decapentaplegic* induces pattern duplications and growth alterations in *Drosophila* wings. *EMBO J.* **13**, 4459-4468.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D. and Kinter, C. (1995). Primary neurogenesis in *Xenopus* embryos regulated by a homologue of the *Drosophila* neurogenic gene *Delta*. *Nature* **375**, 761-766.

Cohen, B., McGuffin, M.E., Pfeifle, C., Segal, D. and Cohen, S.M. (1992). *apterous*: a gene required for imaginal disc development in *Drosophila* encodes a member of the LIM family of developmental regulatory proteins. *Genes Dev.* **6**, 715-729.

Couso, J.P., Knust, E. and Martinez-Arias, A. (1995). *Serrate* and *wingless* cooperate to induce vestigial gene expression and wing formation. *Curr. Biol.* **5**, 1437-1448.

de Celis, J.F., Garcia-Bellido, A. and Bray, S.J. (1996). Activation and function of *Notch* at the dorsal-ventral boundary of the wing imaginal disc. *Development* **122**, 359-369.

Diaz-Benjumea. F.J. and Cohen, S.M. (1995). *Serrate* signals through *Notch* to establish a *Wingless*-dependent organizer at the dorsal/ventral compartment boundary of the *Drosophila* wing. *Development* **121**, 4215-4225.

Doherty, D., Feger, G., Younger-Shepard, S., Jan, L.Y. and Jan, Y,N. (1996). *Delta* is a ventral to dorsal signal complementary to *Serrate*, another *Notch* ligand, in *Drosophila* wing formation. *Genes Dev.* **10**, 421-434.

Fehon, R.G., Kooh, P.J., Rebay, I., Regan, C.L., Xu, T., Muskavitch, M.A.T., and Artavanis-Tsakonas, S. (1990). Molecular interactions between the protein products of the neurogenic loci *Notch* and *Delta*, two EGF-homologous genes in *Drosophila*. *Cell* **61**, 523-534.

Fitzgerald, K. and Greenwald, I. (1995). Interchangeability of *Caenorhabditis elegans* DSL proteins and intrinsic signalling activity of their extracellular domains in vivo. *Development* **121**, 4275-4282.

Fleming, R.J., Scottgale, T.N., Diederich, R.J. and Artavanis-Tsakonas, S. (1990). The gene *Serrate* encodes a putative EGF-like transmembrane protein essential for proper ectodermal development in *Drosophila melanogaster*. *Genes Dev.* **4**, 2188-2201.

Fleming, R.J., Gu, Y. and Hukriede, N.A. (1997). *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. *Development* **124**, 2973-2981.

Garcia-Bellido, A., Ripoll, P. and Morata, G. (1976). Developmental compartmentalization in the dorsal mesothoracic disc of *Drosophila*. *Dev. Biol.* **48**, 132-147.

Gu, Y., Hukriede, N.A. and Fleming, R.J. (1995). *Serrate* expression can functionally replace *Delta* activity during neuroblast segregation in the *Drosophila* embryo. *Development* **121**, 855-865.

Hinz, U., Giebel, B. and Campos-Ortega, J. (1994). The basic-helix-loop-helix domain of *Drosophila* lethal of scute protein is sufficient for proneural function and activates neurogenic genes. *Cell* **76**, 77-87.

Hukriede, N.A. and Fleming, R.J. (1997). *Beaded of Goldschmidt*, an antimorphic allele of *Serrate*, encodes a protein lacking transmembrane and intracellular domains. *Genetics* **145**, 359-374.

Irvine, K. and Weischaus, E. (1994). *fringe*, a boundary-specific signaling molecule, mediates interactions between dorsal and ventral cells during *Drosophila* wing development. *Cell* **79**, 595-606.

Jan, Y.L., and Jan, Y.N. (1982). Antibodies to horseradish peroxidase as specific neuronal markers in *Drosophila* and grasshopper embryos. *Proc. Natl. Acad. Sci. USA* **79**, 2700-2704.

Jönsson, F. and Knust, E. (1996). Distinct functions of the *Drosophila* genes *Serrate* and *Delta* revealed by ectopic expression during wing development. *Dev. Genes. Evol.* **206**, 91-101.

Kim, J., Irvine, K.D. and Carroll, S.B. (1995). Cell recognition, signal induction, and symmetrical gene activation at the dorsal-ventral boundary of the developing *Drosophila* wing. *Cell* **82**, 795-802.

Kim, J., Sebring, A., Esch, J., Kraus, M.E., Vorwerk, K., Magee, J. and

Carroll, S.B. (1996). Integration of positional signals and regulation of wing formation and identity by *Drosophila vestigial* gene. *Nature* **382**, 133-138.

Kolodziej, P.A. and Young, R.A. (1991). Epitope tagging and protein surveillance. *Meth. Enzymol.* **194**, 508-519.

Kornberg, T. B., Siden, I., O'Farrell, P. and Simon, M. (1985). The *engrailed* locus of *Drosophila*: in situ localization of transcripts reveals compartment-specific expression. *Cell* **40**, 45-53.

Lawrence, P.A. and Morata, G. (1977). The early development of mesothoracic compartments in *Drosophila*. *Dev. Biol.* **56**, 40-51.

Lawrence, P.A. and Struhl, G. (1996). Morphogens, compartments and pattern: Lessons from *Drosophila*? *Cell* **85**, 951-961.

Lee, J.J., von Kessler, D.P., Parks, S. and Beachy, P.A. (1992). Secretion and localized transcription suggests a role in positional signaling for products of the segmentation gene *hedgehog*. *Cell* **71**, 33-50.

Lehmann, R., Jiménez, F., Dietrich, U. and Campos-Ortega, J.A. (1983). On the phenotype of mutants of early neurogenesis in *Drosophila melanogaster*. *Roux's Arch Dev Biol*. **192**, 62-74.

Lindsley, D.L., and Zimm, G. G. (1992). The Genome of *Drosophila melanogaster*. San Diego, CA: Academic Press.

Neumann, C.J. and Cohen. S.M. (1996). A hierarchy of cross-regulation involving *Notch*, *wingless*, *vestigial* and *cut* organizes the dorsal/ventral axis of the *Drosophila* wing. *Development* **122**, 3477-3485.

Panin, V.M., Papayannopoulos, V., Wilson, R. and Irvine, K.D. (1997). Fringe modulates Notch-ligand interactions. *Nature* **387**, 908-912.

Rebay, I., Fleming, R.J., Fehon, R.G., Cherbas, L., Cherbas, P., and Artavanis-Tsakonas, S. (1991). Specific EGF Repeats of *Notch* mediate interactions with *Delta* and *Serrate*: Implications for *Notch* as a multifunctional receptor. *Cell* **67**, 687-699.

Rulifson, E.J. and Blair, S.S. (1995). *Notch* regulates *wingless* expression and is not required for reception of the paracrine *wingless* signal during wing margin neurogenesis in *Drosophila*. *Development* **121**, 2813-2824.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain termination inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.

Sanicola, M., Sekelsky, J., Elson, S. and Gelbart, W.M. (1995). Drawing a stripe in *Drosophila* imaginal disks: negative regulation of *decapentaplegic* and *patched* expression by *engrailed*. *Genetics* **139**, 745-756.

Shellenbarger, D. L. and J. D. Mohler. (1978). Temperature-sensitive periods and autonomy of pleiotropic effects of *l(1)N*^{ts1}, a conditional *Notch* lethal in *Drosophila*. *Dev. Biol.* **62**,432-446.

Shelton, C.A. and Wasserman, S.A. (1993). *pelle* encodes a protein kinase required to establish dorsoventral polarity in the *Drosophila* embryo. *Cell* **72**, 515-525.

Simmonds, A.J., Brook, W.J., Cohen, S.M. and Bell, J.B. (1995). Distinguishable functions for *engrailed* and *invected* in anterior-posterior patterning in the *Drosophila* wing. *Nature* **376**, 424-427. **Simpson, P.** (1995). The Notch connection. *Nature* **375**, 736-737.

Speicher, S.A., Thomas, U., Hinz, U. and Knust, E. (1994). The *Serrate* locus of *Drosophila* and its role in morphogenesis of the wing imaginal discs: control of cell proliferation. *Development* **120**, 535-544.

Spradling. A.C. (1986). P element-mediated transformation. In *Drosophila*: *A Practical Approach*. (ed. D. B. Roberts), pp. 175-197. Oxford, England: IRL Press.

Sun, X. and Artavanis-Tsakonas, S. (1996). The intracellular deletions of DELTA and SERRATE define dominant-negative forms of the *Drosophila* Notch ligands. *Development* **122**, 2465-2474.

Sun, X. and Artavanis-Tsakonas, S. (1997). Secreted forms of DELTA and SERRATE define antagonists of Notch signaling in *Drosophila*. *Development*. **124**, 3439-3448.

Tabata, T., Eaton, S. and Kornberg, T.B. (1992). The *Drosophila hedgehog* gene is expressed specifically in posterior compartment cells and is a target of *engrailed* regulation. *Genes Dev.* **6**, 2635-2645.

Tabata, T., Schwartz,C., Gustavson, E., Ali, Z. and Kornberg, T. B. (1995). Creating a *Drosophila* wing de novo, the role of *engrailed*, and the compartment border hypothesis. *Development* **121**, 3359-3369.

Wilcox, M., Brown, N., Piovant, M., Smith, R.J. and White, R.A.H. (1984). The position-specific antigens are a family of cell surface glycoprotein complexes. *EMBO J.* **3**, 2307-2313.

Williams, J.A., Paddock, S.W., Vorwerk, K. and Carroll, S.B. (1994). Organization of wing formation and induction of a wing-patterning gene at the dorsal/ventral compartment boundary. *Nature* **368**, 299-305.

Yuan, Y.P., Schultz, J., Mlodzik, M. and Bork, P. (1997). Secreted Fringe-like signaling molecules may be glycosyltransferases. *Cell* **88**, 9-11.

Zecca, M., Basler, K. and Struhl, G. (1995). Sequential organizing activities of *engrailed*, *hedgehog* and *decapentaplegic* in the *Drosophila* wing. *Development* **21**, 2265-2278.

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