SNARE-Dependent Signaling at the Drosophila Wing Margin

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The wing of Drosophila melanogaster has long been used as a model system to characterize intermolecular interactions important in development. Implicit in our understanding of developmental processes is the proper trafficking and sorting of signaling molecules, although the precise mechanisms that regulate membrane trafficking in a developmental context are not well studied. We have therefore chosen the Drosophila wing to assess the importance of SNARE-dependent membrane trafficking during development. N-Ethylmaleimide-sensitive fusion protein (NSF) is a key component of the membrane-trafficking machinery and we constructed a mutant form of NSF whose expression we directed to the developing wing margin. This resulted in a notched-wing phenotype, the severity of which was enhanced when combined with mutants of VAMP/Synaptobrevin or Syntaxin, indicating that it results from impaired membrane trafficking. Importantly, we find that the phenotype is also enhanced by mutations in genes for wingless and components of the Notch signaling pathway, suggesting that these signaling pathways were disrupted. Finally, we used this phenotype to conduct a screen for interacting genes, uncovering two Notch pathway components that had not previously been linked to wing development. We conclude that SNARE-mediated membrane trafficking is an important component of wing margin development and that dosage-sensitive developmental pathways will act as a sensitive reporter of partial membrane-trafficking disruption.© 2001 Academic Press

Key Words: NSF; VAMP; Synaptobrevin; Syntaxin; Notch; Wingless.

INTRODUCTION

SNARE (soluble NSF attachment protein receptors) protein-dependent membrane trafficking has been the subject of intense study in recent years. The Syntaxin, VAMP, and SNAP-25 families of proteins are proposed to target and fuse transport vesicles with specific membrane compartments (McNew et al., 2000; Sollier et al., 1993; Weber et al., 1998). One of the identifying characteristics of SNARE proteins is their ability to form tight, SDS-resistant, ternary complexes. The SNARE complex is a parallel four-helix bundle with one helix contributed by each of Syntaxin and VAMP and two contributed by SNAP-25 (Sutton et al., 1998). The formation of a trans-membrane complex, with VAMP on the target membrane, is thought to lead to the fusion of the two membranes, resulting in a cis-membrane complex.

It follows that the cis-residing protein complexes need to be broken apart to make those proteins available for further trans-complex formation. This complex breakdown occurs under the action of N-ethylmaleimide-sensitive fusion protein (NSF) (Malhotra et al., 1988), an ATPase. NSF contains two nucleotide binding domains and demonstrable ATPase activity. Structural analyses have shown that NSF forms a hexamer in vivo (Hanson et al., 1997). NSF is a homolog of the yeast gene SEC18 (Wilson et al., 1989) and analysis of SEC18 function also revealed its requirement for intracellular membrane transport (Eakle et al., 1988). Banerjee et al. (1996) showed that NSF-dependent ATP hydrolysis is required to disassemble SNARE complexes, although it is not required for the fusion step. Thus the role of NSF in vesicular transport appears to be primarily one of priming vesicles for fusion and dissociation of SNARE complexes to permit their recycling. In Drosophila there are two homologs of NSF: dNSF1 and dNSF2 (Boulianne and Trimble, 1996).
1995; Ordway et al., 1994). dNSF1 is the gene product of comatose (Pallanck et al., 1995) and is primarily found in neurons, whereas dNSF2, in addition to being neuronally expressed, is broadly expressed within imaginal discs, salivary glands, and the ring gland (Boulianne and Trimble, 1995). Thus, dNSF2 is the most likely isoform to contribute to intracellular trafficking in nonneuronal tissue.

Despite their proposed role in most intracellular trafficking events, in vivo studies of SNARE proteins have concentrated on two main systems: the budding yeast and calcium-triggered exocytosis in neurons. Relatively little attention has been given to other in vivo contexts in which the SNARE proteins are likely to have important roles. For example, in signaling pathways it is self-evident that transmembrane receptors and ligands need to be delivered to the plasma membrane, although few studies have been devoted to specifically studying the role of SNARE proteins in this process and their potential influence on the strength of intracellular signaling.

In the fruit fly Drosophila melanogaster there is a long history in developmental biology and many of the signaling pathways that control development of the organism are well known. In parallel, Drosophila SNARE proteins have also been intensively studied, mostly in the context of synaptic transmission at the neuromuscular junction. The complete sequencing of the Drosophila genome has revealed all the members of the SNARE protein families and in general there is about one-third the number of fly genes compared to that estimated for mammalian species. Correspondingly, it appears that some Drosophila SNARE proteins are used in very different contexts. For example, Syntaxin 1A appears to be necessary both for cellularization of the early embryo and for neural synaptic transmission (Burgess et al., 1997; Schulze et al., 1995).

One of the most thoroughly studied developmental pathways in Drosophila is the development of the wing margin. These studies have revealed that the specification and establishment of the wing margin involves complex interaction between the Notch and Wingless signaling pathways (reviewed in Bray, 1998; Panin and Irvine, 1998). The early expression of the homeodomain protein Apterous establishes dorsal/ventral (D/V) polarity of the wing disc and initiates expression of Serrate, a Notch ligand. Serrate acts as a dorsal-to-ventral signal, to initiate the spatially restricted activation of the transmembrane receptor Notch (de Celis et al., 1996). The second Notch ligand, Delta, is expressed on both sides of the D/V border but is required only on the ventral side of the border (de Celis et al., 1996). Together these three molecules are believed to form a positive-feedback loop that enhances Notch activity at the D/V border and downregulates it outside this zone (de Celis et al., 1995; Ordway et al., 1994). dNSF1 is the gene product of comatose (Pallanck et al., 1995) and is primarily found in neurons, whereas dNSF2, in addition to being neuronally expressed, is broadly expressed within imaginal discs, salivary glands, and the ring gland (Boulianne and Trimble, 1995). Thus, dNSF2 is the most likely isoform to contribute to intracellular trafficking in nonneuronal tissue.

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and Bray, 1997). Notch is further required for expression of wingless, cut, vestigial, and achaete, genes that are important for wing development and sense organ formation along the wing margin (Kim et al., 1995, 1996; Neumann and Cohen, 1996; Rulifson and Blair, 1995). Given that Notch and its ligands are transmembrane proteins and that Wingless is a secreted protein, it is clear that membrane trafficking will be important to this signaling cascade.

To investigate the role of SNARE proteins within a defined developmental process, we took advantage of the key role of NSF in membrane-transport processes. Specifically, we expressed a dominant negative form of dNSF2 in wing imaginal discs and show that this disrupts proper wing margin formation. This phenotype is enhanced in transheterozygous combinations of mutant alleles of the SNARE proteins syntaxin or synaptobrevin, further supporting a role for SNAREs in this process. Using genetic and immunocytochemical analysis we show that this phenotype can be attributed to a failure in the signaling pathways that normally govern wing margin development. Thus, SNARE-dependent transport mechanisms are critical to wing formation and their manipulation may provide new insights into the mechanisms controlling developmentally important signaling pathways.

**MATERIALS AND METHODS**

**Fly Stocks**

Drosophila were raised on standard cornmeal medium at 25°C. UAS-dNSF2E/Q is a UAS transgenic line containing the dNSF2 open reading frame with a glutamate-to-glutamine amino acid substitution at position 326 (amino acid position according to Boulianne and Trimble, 1995). We generated several independent insertion lines; here we report data from one representative line, UAS-dNSF2E/Q. UAS-dNSF2WT is a transgenic line carrying the dNSF2 wild-type gene. Other UAS lines used were UAS-NICD, UAS-dNSF2EA2, UAS-dNSF2WT insertion lines; here we report data from one representative line, Boulouanne and Trimble, 1995). We generated several independent substitution at position 326 (amino acid position according to open reading frame with a glutamate-to-glutamine amino acid

**Production of a dNSF2 Point Mutant**

To generate a glutamate-to-glutamine amino acid substitution in dNSF2 we PCR-amplified a fragment of the cloned dNSF2 (Boulianne and Trimble, 1995) from pBluescript SK+ using T3 as the 5’ primer and AATGGCCTGAGATCTGCAGAATGATTG as the 3’ primer. This fragment was then used as the 5’ primer in a second PCR reaction with T7 as the 3’ primer, to amplify the entire dNSF2 cDNA containing the point mutation using XbaI-cut dNSF2 in pBluescript SK+. The DNA fragment was then used as the template DNA in a third reaction, in which T3 and the PCR product from the first reaction were 5’ primer and T7 was the 3’ primer. The DNA from this reaction was cut with XbaI and KpnI, subcloned into pBluescript SK+ and DNA-sequenced, to confirm that the E-to-Q substitution was the sole mutation. pUAS constructs were made by subcloning an 800-bp NotI/KpnI fragment from dNSF2WT and a 1.7-kb KpnI/XbaI fragment from dNSF2E/Q into the NotI/XbaI sites of the pUAS vector (Brand and Perrimon, 1993). Transgenic flies carrying dNSF2WT or dNSF2E/Q UAS constructs were created using standard methods (Rubin and Spradling, 1982).

**ATase Assay**

dNSF2WT and dNSF2E/Q constructs were subcloned into the pGEX-3X expression vectors and transformed into BSB72 cells. Protein expression was induced with 0.1 mM IPTG for 3 h. The cells were disrupted with a French press and the proteins were extracted with 1% Triton X-100 in PBS plus 5 mM EDTA and 0.3 M Na2SO4, and mixed with glutathione beads. The beads were then
packed into a column and washed with PBS with 1% Triton X-100. The protein was then eluted with a buffer containing 10 mM glutathione, 50 mM Tris (pH 7), 100 mM NaCl, and 0.5 mM ATP. The eluate was passed over a G-25 column into ATPase buffer (containing 25 mM Tris, pH 9; 0.2 mM ATP; 0.1 mM KCl; 0.5 mM DTT; 0.65 mM β-mercaptoethanol; 1 mM MgCl₂; 10% glycerol) on ice. For controls with N-ethylmaleimide (NEM), the proteins were incubated with 2.5 mM NEM for 30 min on ice prior to the determination of ATPase activity. Following the addition of 5 μl of [γ-32P]ATP to 1 μg/20 μL of NSF, the samples were transferred to 37°C and incubated for 10 min. Samples from the reaction were then taken and the reaction was stopped with a final concentration of 5 mM EDTA. The nucleotides were separated by thin-plate chromatography in developing buffer containing 0.7 M LiCl and 1 M acetic acid. The radioactive signals were obtained with a phosphorimager.

RESULTS

To investigate the function of SNARE-dependent transport mechanisms in Drosophila, we constructed point mutants in the ATP-binding region of the D1 domain of dNSF2. Each nucleotide-binding subdomain of NSF contains consensus ATP-binding domains known as the Walker A and Walker B motifs (Wilson et al., 1989). The DEAD box of the Walker B motif is conserved in a large number of ATP-dependent enzymes and was first identified in RNA helicases that use ATP hydrolysis to unwind RNA prior to translation. This motif binds the Mg²⁺ ion that coordinates the phosphates of ATP for hydrolysis. In RNA helicases, replacement of the glutamate residue within the modified DEAD box (DEID) eliminates ATP hydrolysis without affecting ATP binding (Pause and Sonenberg, 1992). In their study of mammalian NSF, Whiteheart et al. (1994) demonstrated in CHO cell-free extracts that a similar substitution within that protein’s DEID box, E329Q, reduces ATPase activity and NSF-dependent Golgi transport activity. NSF has been shown to form hexamers and, when mixed with wild-type protein NSF, forms hexamers that also lack ATPase activity, leading to a dominant negative effect. Drosophila NSF2 shows 59% overall amino acid identity with CHO NSF and nearly 100% conservation within the ATP-binding p-loop and DEID box of the D1 domain (Boulanne and Trimble, 1995). Thus the structural and functional properties of the dNSF2 ATPase domains are very likely to be identical to those previously defined in RNA helicases and mammalian NSF, and mutation of the glutamate residue with the Drosophila DEID box motif should also impair the ATPase activity of the protein.

We therefore created a dNSF2E/Q construct with a glutamate-to-glutamine substitution at position 326 of the dNSF2 D1 domain as described under Materials and Methods. In two separate ATPase assays we found that the NEM-sensitive ATPase activity of dNSF2E/Q was 47.5 and 51.7% that of dNSF2WT. The mean ATPase activity was 15.2 nmol Pi/µg/h for the wild-type protein and 7.8 nmol Pi/µg/h for the mutant protein. The remaining ATPase activity in dNSF2E/Q may be attributable to the second ATPase site within the D2 domain of the protein.

To express the mutant dNSF2 in the wing margin, we created transgenic flies carrying UAS-dNSF2E/Q and UAS-dNSF2WT constructs for use in the Gal4-UAS expression system. C96-Gal4 is expressed in developing wing discs in a pattern that is similar to, though slightly broader than, wing margin proteins such as Wingless (Figs. 1A and 1B; see also Gustafson and Boulianne, 1994). When UAS-dNSF2E/Q is driven by C96-Gal4, we observed loss of wing margin (Figs. 1C–1E). The expression of dNSF2WT did not cause any visible phenotype (data not shown), indicating that simple overexpression of dNSF2 in the wing margin is not a cause of the phenotype.

Our observation that dNSF2E/Q causes loss of wing margin implies that SNARE-dependent transport is important for wing margin formation. To test this further, we used mutant alleles of synaptobrevin and syntaxin, two well-characterized SNARE proteins, to determine whether they would enhance the wing phenotype (Fig. 2). Indeed, all trans-heterozygous combinations of dNSF2E/Q and synaptobrevin or syntaxin loss-of-function alleles enhanced the wing margin phenotype, thus providing further evidence of the involvement of SNARE proteins in wing margin development.
The wing phenotype we observe is similar to that observed with mutant alleles of Notch and Wingless signaling pathway genes. To determine whether components of these pathways could be contributing to the dNSF2<sup>E/Q</sup>C96 wing phenotype we first examined the protein pattern of Wingless in third-instar imaginal wing discs and observed a striking effect on the distribution of Wingless. In control discs Wingless appears as a three- to four-cell-wide stripe across the wing disc, whereas in discs expressing the mutant dNSF2 Wingless appears very narrow and patchy (Figs. 3A and 3B). We then examined Wg expression using a Wg-lacZ reporter construct and found an incomplete pattern of Wingless expression (Figs. 3C and 3D), as was observed for the Wingless protein.

Because Wg is a secreted protein we next examined Wg at higher magnification using confocal microscopy to determine directly whether Wg secretion was impaired (Fig. 4). In control discs there is punctate Wg staining, indicative of Wg secretion, in the tissue surrounding the narrow stripe of wing margin cells. In the regions of the mutant discs that were immunoreactive for Wg we also saw punctate staining surrounding the positive cells. However, the Wg signal was much stronger in those cells and confocal sectioning of the cells revealed the accumulation of Wg at the apical region of the wing margin cells. These data indicate that mutant dNSF2<sup>E/Q</sup> impairs, but does not eliminate, wingless secretion.

Because Wingless expression is impaired and its activation is under the control of Notch signaling, we next examined the distribution patterns of other proteins involved in the Notch pathway (Fig. 5). We first examined Notch protein distribution directly using a monoclonal antibody that recognizes the extracellular domain of Notch (gift of S. Artavanis-Tsakonas). At low magnification there is no major difference between mutant and control samples, with the antibody labeling the cell membranes in the wing pouch. However, at higher magnification, in addition to the membrane staining we also observed immunoreactive puncta within the cells of the mutant wing disc that were not readily observed in the control discs. These puncta likely represent improperly sorted Notch proteins.

We next examined the distribution of Cut, Delta, and Achaete, genes that are downstream of Notch activation in the wing margin signaling pathway, and we found all of these markers were disrupted in dNSF2<sup>E/Q</sup>C96 larval wing discs. Cut is normally found in a pattern that overlaps with Wg along the presumptive wing margin (Fig. 6A; see also Blochlinger et al., 1993), whereas in the mutant discs it appeared in a broken pattern (Fig. 6B) similar to that of Wg. Delta is normally expressed in two parallel bands along the D/V boundary (Fig. 6C; see also Kooh et al., 1993) and this pattern is thought to be the result of the downregulation of Delta in boundary cells by Cut and the upregulation of Delta in flanking cells by Wingless (de Celis and Bray, 1997). In dNSF2<sup>E/Q</sup>C96 wing discs the expression of Delta is reduced and the two parallel bands appear to be collapsed into a single band along the boundary (Fig. 6D). Achaete is normally expressed in two broad bands parallel to the D/V boundary in the anterior compartment of the wing disc defining a proneural cluster (Fig. 6E; see also Romani et al., 1989). In the dNSF2<sup>E/Q</sup>C96 discs this pattern is severely disrupted: the number of Achaete-expressing cells is reduced and there is complete absence of Achaete in some areas (Fig. 6F).

We found a similar pattern of disruption when we used lacZ reporter constructs to examine the expression of neuralized and vestigial, two other genes in the Notch pathway. neu<sup>101E/Q</sup>-lacZ is normally detected in sensory organ precursors (SOPs) located in two rows of single cells parallel to the D/V boundary in the anterior compartment of late third-instar wing discs (Fig. 6G). In the mutant discs this pattern is disrupted and lacking in some areas along the wing margin, while SOPs elsewhere in the disc are unaffected (Fig. 6H). Similarly, vg<sup>E/Q</sup>-lacZ expression was disrupted. In wild-type discs it is seen in the D/V and anterior/posterior (A/P) boundaries, whereas in the mutant discs the expression in the D/V boundary is disrupted (Figs. 6I and 6J). Interestingly, expression in the A/P boundary remains, although the C96-Gal4 expression pattern overlaps this region. Taken together, these results demonstrate that dNSF2<sup>E/Q</sup> affects the distribution and expression of several downstream components of the Notch signaling pathway.

To confirm the effect of dNSF2<sup>E/Q</sup> on Notch signaling we examined loss-of-function alleles of several genes in the Notch and Wingless pathways for their ability to enhance the adult wing phenotype caused by dNSF2<sup>E/Q</sup> expression. In that Notch signaling is known to be highly sensitive to haploinsufficiency of interacting gene products, we reasoned that these loss-of-function alleles should show genetic interaction. We tested two alleles of Notch and one each of Delta, Serrate, wingless, and fringe and found that they all enhanced the wing phenotype in transheterozygous combination with dNSF2<sup>E/Q</sup>C96 (Fig. 7). The severity of the phenotype produced by each allele was similar, although Df(1)N8, a null allele of Notch, did produce a more severe phenotype than did N<sup>0</sup> (not shown), a hypomorphic allele. With the exception of Df(1)N8 (Fig. 7B, inset), none of these mutants produces a wing-nicking phenotype when examined alone as heterozygotes. Thus, the enhancement of the adult wing phenotype by mutants in the Notch pathway supports the conclusion that dNSF2<sup>E/Q</sup> expression causes a defect in wing margin signaling pathways.

Finally, we tested the ability of UAS-constructs of Notch, Delta, and Serrate to rescue the wing phenotype generated by dNSF2<sup>E/Q</sup>C96 (Figs. 8A–8D). Complete rescue could be obtained with both Notch and Delta constructs. Serrate generally appeared to rescue less well than did the other constructs because minor nicks in the distal wing persisted. Furthermore, no rescue effect was seen when crosses were made to UAS-lacZ lines (not shown), indicating that competition for Gal4 protein was not responsible for rescue of the phenotype. The observation that UAS-Notch and UAS-Delta could completely rescue the dNSF2<sup>E/Q</sup> wing phenotype further indicates that the mutation affects intracellular...
Having established that dNSF2^{E/Q} disrupts signaling at the wing margin in a SNARE-dependent manner, and that we could easily detect enhancement of the phenotype.
attributed to haploinsufficiency of known genes, we sought to determine whether the wings of the dNSF2E/Q flies could be used as a sensitized background to find novel genes involved in wing margin formation. To this end we conducted a small-scale screen for enhancers and suppressors of the phenotype.

In the first set of experiments we used specific alleles of two genes, big brain and porcupine, that have been shown to be important in Notch and Wingless signaling in other developmental contexts but were not previously known to be important for wing margin development. In the dNSF2E/QC96 background we found that both mutant alleles of these genes enhanced the dNSF2E/QC96 wing margin phenotype (Figs. 4G and 4H). This result is the first report of the involvement of these two genes in wing margin development and suggests that dNSF2E/QC96 wings provide an ideal sensitized background for conducting forward genetic screens to identify novel genes involved in wing margin development.

In the second set of experiments we tested for genetic interactions with deficiencies that uncover most of the Drosophila genome. Of the deficiencies we tested, we identified 33 interacting lines that enhanced or suppressed the wing margin phenotype (Table 1). At present we have not determined which of the genes uncovered by these deficiencies led to the interacting phenotype. Indeed, because components of both the Notch and Wg signaling pathway and membrane trafficking genes may be involved, more than one gene within each deficiency may be affected. The further characterization of these loci may reveal novel components of the SNARE or Notch and Wg signaling pathways.

**DISCUSSION**

Despite extensive study of the function of SNARE proteins, their role in mediating developmentally important processes is poorly understood. In Drosophila the presence of dNSF2 in imaginal discs and the maternal contribution of Syntaxin and Synaptobrevin indicates that those proteins have an important role in development. Burgess et al. (1997) used germ-line mosaics of hypomorphic Syntaxin alleles to show that Syntaxin is necessary for cellularization of the early blastoderm. Schulze et al. (1995) found that certain combinations of syntaxin alleles gave rough eye- and wing-nicking phenotypes, indicating a potential role in eye and wing development. However, given the possible cell lethality caused by homozygous mutations of syntaxin (Burgess et al., 1997; Schulze and Bellen, 1996), it is unclear whether the main effect in that study was on a developmental pathway or cell viability.
In this investigation we examined the role of SNARE-dependent transport mechanisms during development by expressing a dominant negative form of dNSF2 at the developing Drosophila wing margin. In view of current membrane-trafficking models, we expect that expressing dNSF2\textsuperscript{E/Q} will impair the ability of NSF to dissociate cis-SNARE complexes, making fewer SNARE proteins available for functional trans-membrane complex formation and thus reducing intracellular transport. When we expressed dNSF2\textsuperscript{E/Q} within a narrow band of cells in the developing wing disc, we observed loss of wing margin and disruption of protein patterns in the wing disc. We further showed that this is not a cell-lethal phenotype because it can be rescued by UAS-Notch and UAS-Delta constructs, indicating that the cells are viable. We also showed that single-copy mutations of both syntaxin and synaptobrevin can enhance the wing phenotype, providing solid evidence that these SNARE proteins are important in wing margin formation. This implies that the mutant NSF must suppress but not block all membrane traffic. Indeed, we observed mislocalization of some, but not all, Wingless and Notch immunoreactive signals. Because wing margin development is particularly sensitive to gene dosage we were able to detect sublethal disruptions in membrane trafficking.

The disruption of molecular markers, such as Wg, Delta, Achaete, Cut, Vestigial, and Neuralized, indicates that the dNSF2\textsuperscript{E/Q} wing phenotype we observed is the result of impaired signaling at the developing wing margin. This is consistent with data presented in other studies that manipulated the signaling pathway directly. For example, reduction of Notch activity with \textit{N}\textsuperscript{ts} alleles can lead to reduced and patchy Wingless expression (Diaz-Benjumea and Cohen, 1995). Wingless and Cut expression is also reduced and patchy in Notch mutant wing discs (de Celis et al., 1996; Micchelli et al., 1997; Rulifson and Blair, 1995). Stripes of Delta and Serrate that normally flank the D/V boundary collapse into a single stripe along the margin in \textit{N}\textsuperscript{ts} alleles exposed to restrictive temperature (de Celis and Bray, 1997). In our dNSF2\textsuperscript{E/Q}C96 wing discs we observe changes in Wingless, Cut, and Delta patterns that are similar to those that occur when Notch activity is directly manipulated; therefore, it seems that dNSF2\textsuperscript{E/Q} expression phenocopies genetic mutants of Notch.

Because the Notch and Wingless signaling pathways are so intertwined in controlling wing margin development it is difficult to determine whether the dNSF2 mutants cause a primary defect in one or the other of these proteins, although it seems likely that there are parallel effects on both. Our experiments show not only a direct impairment of Wingless trafficking but also that Wg-IacZ expression is disrupted. The latter suggests that an upstream activator of Wingless expression is impaired (although this could be Wingless itself) (Rulifson et al., 1996). We find that Notch subcellular localization is disrupted and that a Wg-independent target of Notch signaling, the vestigial boundary enhancer, is also disrupted. Because this vestigial enhancer element is thought to be under the sole control of

**FIG. 6.** Notch pathway markers disrupted in mutant wing discs. Control (A, C, E, G, I) and dNSF2\textsuperscript{E/Q}C96/+ (B, D, F, H, J) wing discs stained with anti-Cut (A, B), anti-Delta (C, D), anti-Achaete (E, F), anti-IacZ neuralized\textsuperscript{A101}-IacZ (G, H), vestigial\textsuperscript{A101}-IacZ (I, J). All markers are disrupted in the mutant discs.
Notch (Neumann and Cohen, 1996) this supports the idea that dNSF2E/Q has a direct effect on Notch signaling. Thus our data point to direct effects on both Wg and Notch. Moreover, because these molecules are at the top of the hierarchy controlling signaling at the wing margin this provides the likely explanation for the disruption of downstream targets of these genes.

A targeted screen using the dNSF2E/Q wing as a sensitized background allowed us to easily identify enhancement of the wing phenotype with several known members of the Notch signaling pathway; large-scale screens using this genotype could reveal new members of the pathway. In this study we identified two genes, porc and bib, not previously known to be involved in wing margin formation. Porcupine is a molecule thought to be important for Wingless secretion, in that Wingless is observed to accumulate in Wingless-secreting cells in a porcupine mutant background (van den Heuvel et al., 1993). Our finding that the adult wing phenotype is enhanced by porc mutants supports this role and indicates that porc is important in multiple tissue types. Our immunocytochemistry revealed the accumulation of Wg in wing margin cells and our finding that porc mutants enhance the wing margin phenotype supports the idea that Wg secretion is compromised by dNSF2E/Q expression.

It was previously proposed that bib modulates Notch signaling (Doherty et al., 1997), although the mechanistic role this protein plays remains unclear. Doherty et al. (1997) did not identify a wing margin defect when bib clones were generated in wing imaginal discs, even though they do report bib to be expressed specifically in the wing margin. In the present study we found that dNSF2E/Q expression leads to a reduction in Notch signaling and we did find an enhancement by bib1 alleles. Clonal analysis of bib in a Notch hypomorphic background may yield a similar result. Thus it seems the relative levels of Notch...
TABLE 1

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<th>Bloomington stock number</th>
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X chromosome

Df(1)G4eLH24iR, f1 5E3-8;6B Suppressed
Df(1)J4e, In(1)dl-49, f1 7A2-3;7C1 Suppressed
Df(1)4t1v, y1 7B2-4t;7C3-4 Slight suppression
Df(1)JA26 11A-1;11D-E Enhanced
Df(1)N12, rass1, v1 11D1-2;11F1-2 Lethal

Second chromosome

Df(2)J1e-LMF 21A1-2;27B8-8 Suppressed
Df(2)L14, dpp1ho, ed1 23A1-2;23C3-3 Suppressed
Df(2)L11, cn1 11A1-1;11D-E Enhanced
Df(2)L139 31C-D;32D-E Slight suppression
Df(2)L1p1, b3, Adh1n2 pr1 cn1, sca1 338b-3;34A1-2 Enhanced
Df(2)LJW50, cn1 36E4-1;38A6-7 Suppressed
Df(2)LJW84, 1(2)74i1, Tt1 LarTW844 37F5-38A1;39D3-E1 Suppressed
Df(2)Jnap9 42A1-2;42E6-F1 Suppressed
Df(2)Jpe78 54E6-1;55B9-C1 Suppressed
Df(2)JpD11B, al1 dpp1v, b3, et1 54F6-55A1;55C1-3 Suppressed
Df(2)Jpu-D17, cn1, bw1 sp1 57B4-58B Slight suppression
Df(2)JX58-7, pr1 cn1 58A1-2;58E4-10 Suppressed
Df(2)JX58-12 58D1-2;59A Suppressed
Df(2)Jx2 60C5-6;60D9-10 Suppressed

Third chromosome

Df(3)Lh1-22, h1, R11 Ki1 roe1 p2 66D10-11;66E1-2 Enhanced
Df(3)L29A9, kni1, p1 66F5-67B1 Enhanced
Df(3)Lx66 67E1-67C2 Lethal
Df(3)Lwin2, ru1 h1 gl2, et1 ca1 67F2-68D6 Suppressed
Df(3)LLy, mwh1, Ly1 70A2-3;70A3-5 Enhanced
Df(3)Lb1k9 73A3-74F Enhanced
Df(3)LW10, ru1 h1 75A6-7;57C1-2 Suppressed
Df(3)L31A 78A-78E Enhanced
Df(3)R6-7 82D3-8;82F Enhanced
Df(3)Ry712, red1, et1 84D4-85B6 Enhanced
Df(3)Ry10, red1, et1 85D8-9;85F1 Enhanced
Df(3)Ryx1-1-Kx1 86C1-87B1-5 Suppressed
Df(3)Ry615 87B11-13;87E8-11 Suppressed
Df(3)JT1-1, et1 ca1 97A9A-2 Enhanced

Signaling may be critical to the role of bib and our data are consistent with the idea that bib serves as a positive modulator of Notch signaling in wing margin formation as it does in neuroectoderm determination.

Using available deficiencies that uncover most of the Drosophila genome we identified a number of lines that enhanced or suppressed the dNSF26O C96 wing phenotype. Among these lines some of the deficiencies uncovered clear candidate genes. For example Df(3)T1-1, et1 ca1, with breakpoints at cytological location 97A:98A1-2, enhanced our phenotype and likely disrupts Serrate. In other cases either there is no clear candidate or contradictory interactions were found. This result may arise as a result of the uncertainty surrounding the chromosomal breakpoints and the genes that lie within the deficiencies. Alternatively, it may occur because each of the deficiencies removes many individual genes, some of which may have counterbalancing effects on the phenotype. Nevertheless, it is clear that the dNSF26O C96 wing provides a highly sensitized background and further study of the interacting loci are likely to reveal novel components of the intracellular trafficking pathway and the signaling pathways that control wing development.

The molecular and genetic interactions that regulate developmentally important signaling pathways are important for defining the final outcome of the signaling cascade. For example, previous studies identified several molecules, including Fringe, Big Brain, and Numb, that are proposed to influence Notch signals (Doherty et al., 1997; Guo et al., 1996; Panin et al., 1997). Because the SNARE proteins interact with many protein partners, some of which are proposed to regulate their availability (e.g., Syntaxin's interaction with rop/nsec-1), our data indicate that regulation of SNARE-dependent transport steps may represent an additional mechanism by which signal transduction pathways can be modulated during development.

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REFERENCES


