Involvement of a proline-rich motif and RING-H2 finger of Deltex in the regulation of Notch signaling

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SUMMARY

The Notch pathway is an evolutionarily conserved signaling mechanism that is essential for cell-cell interactions. The Drosophila deltex gene regulates Notch signaling in a positive manner, and its gene product physically interacts with the intracellular domain of Notch through its N-terminal domain. Deltex has two other domains that are presumably involved in protein-protein interactions: a proline-rich motif that binds to SH3-domains, and a RING-H2 finger motif. Using an overexpression assay, we have analyzed the functional involvement of these Deltex domains in Notch signaling. The N-terminal domain of Deltex that binds to the CDC10/Ankyrin repeats of the Notch intracellular domain was indispensable for the function of Deltex. A mutant form of Deltex that lacked the proline-rich motif behaved as a dominant-negative form. This dominant-negative Deltex inhibited Notch signaling upstream of an activated, nuclear form of Notch and downstream of full-length Notch, suggesting the dominant-negative Deltex might prevent the activation of the Notch receptor. We found that Deltex formed a homo-multimer, and mutations in the RING-H2 finger domain abolished this oligomerization. The same mutations in the RING-H2 finger motif of Deltex disrupted the function of Deltex in vivo. However, when the same mutant was fused to a heterologous dimerization domain (Glutathione-S-Transferase), the chimeric protein had normal Deltex activity. Therefore, oligomerization mediated by the RING-H2 finger motif is an integral step in the signaling function of Deltex.

Key words: Notch, Deltex, Cell-cell interaction, Wing formation, SH3-domain, RING-H2 finger, Drosophila

INTRODUCTION

Local cell-cell interactions are essential for the development of multicellular organisms. Notch signaling is involved in cell-cell communications that regulate a broad spectrum of cell-fate determinations in organisms ranging from the fly to mammals (reviewed by Artavanis-Tsakonas et al., 1995; Blaumueller and Artavanis-Tsakonas, 1997; Gridley, 1997; Kimble and Simpson, 1997; Weinmaster, 1998; Greenwald, 1998; Artavanis-Tsakonas et al., 1999; Kadesch, 2000).

In Drosophila, Notch encodes a 300 kDa single-pass transmembrane receptor (Artavanis-Tsakonas et al., 1983). The extracellular domain of Notch contains 36 epidermal growth factor (EGF)-like repeats and three Notch/Lin-12 repeats. In the intracellular domain of Notch, there are six CDC10/Ankyrin repeats and a PEST-like sequence. Delta and Serrate have been identified as transmembrane ligands for Notch (Vässin et al., 1987; Nye and Kopan, 1995). There is strong evidence supporting the idea that the ligand-dependent activation of Notch induces the proteolytic cleavage of Notch itself, so that the intracellular domain of Notch is released from the cell membrane and moves to the nucleus (Lecourtois and Schweisguth, 1998; Schroeter et al., 1998; Struhl and Adachi, 1998). This cleavage has been shown to depend on the function of Presenilin and a γ-secretase-like proteinase (De Strooper et al., 1999; Struhl and Greenwald, 1999; Ye et al., 1999; Brou et al., 2000; Mumm et al., 2000). In the nucleus, the intracellular domain of Notch physically interacts with a transcription factor, Suppressor of Hairless [Su(H)], which functions as a suppressor of transcription when it is not complexed with the intracellular...
domain of Notch (Fortini and Artavanis-Tsakonas, 1994; Honjo, 1996; Klein et al., 2000). The complex involving the Notch intracellular domain and Su(H) is an activator of transcription and binds to promoter elements that regulate the expression of the target genes of Notch signaling, such as Enhancer of split and vestigial (Bailey and Posakony, 1995; Lecortois and Schweiguth, 1995; Kim et al., 1996).

Although an increasing number of genes have been identified as components of the Notch pathway, the biochemical function of the deltex gene product remains elusive. Drosophila deltex encodes a cytoplasmic regulator of Notch, although its function may not be essential for signaling (Xu and Artavanis-Tsakonas, 1990; Gorman and Girton, 1992; Busseau et al., 1994). The N-terminal region of Deltex physically interacts with the CDC10/Ankyrin repeats of the Notch intracellular domain (Busseau et al., 1994; Matsuno et al., 1995). This interaction appears to be crucial for the function of Deltex (Matsuno et al., 1995). Two other Deltex domains, a proline-rich motif and a RING-H2 finger motif have been identified previously (Matsuno et al., 1995). In general, proline-rich motifs are known as binding sites for various SH3-domains (Cohen et al., 1995; Di Fiore et al., 1997; Pawson and Scott, 1997; Kay et al., 2000). Indeed, it has been shown that human GRB2, a SH3-domain-containing protein, binds to the human Deltex homolog and to Drosophila Deltex (Matsuno et al., 1998). RING-H2 finger motifs have also been shown to function in protein-protein interactions in various systems (Freemont, 1993; Freemont, 2000; Joazeiro and Weissman, 2000). These three motifs in Deltex, which are all presumably involved in protein-protein interactions, are conserved among the mammalian homologs of Deltex, suggesting that they have functional importance (Pampeno and Meruelo, 1996; Matsuno et al., 1998; Frolova and Beebe, 2000; Kishi et al., 2001).

Genetic analysis in Drosophila and biochemical studies involving mammalian Deltex and tissue culture cells support the idea that Deltex is a positive regulator of Notch signaling (Xu and Artavanis-Tsakonas, 1990; Diederich et al., 1994; Fortini and Artavanis-Tsakonas, 1994). It has been shown that human GRB2, a SH3-domain-containing protein, binds to the human Deltex homolog and to Drosophila Deltex (Matsuno et al., 1998). RING-H2 finger motifs have also been shown to function in protein-protein interactions in various systems (Freemont, 1993; Freemont, 2000; Joazeiro and Weissman, 2000). These three motifs in Deltex, which are all presumably involved in protein-protein interactions, are conserved among the mammalian homologs of Deltex, suggesting that they have functional importance (Pampeno and Meruelo, 1996; Matsuno et al., 1998; Frolova and Beebe, 2000; Kishi et al., 2001).

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Creation of Deltex and Deltex mutant constructs
The amino acids of Deltex were numbered according to Busseau et al. (Busseau et al., 1994). A Chameleon double-stranded site-directed mutagenesis kit (Stratagene) was used to create the deletion and point mutation constructs of deltex. Dx
$^{\text{NBS}}$ and Dx
$^{\text{APRM}}$ lack a domain for binding to Notch (amino acids 46 to 204) (Matsuno et al., 1995) and the proline-rich motif (amino acids 475 to 483), respectively. Dx
$^{\text{NBS}}$ has mutations in which two histidine residues (amino acids 570 and 573) have been replaced by alanine residues. The cDNA encoding Dx
$^{\text{NBS}}$ was generated using the primer 5´CTGAATCTGCCTGC-AGGCTCTCATGGCTTGGGCAGTGCCTAA3´. All junctions and point mutations were confirmed by sequencing. The Dx
$^{\text{NBS-APRM}}$ construct was made by replacing by the XhoI fragment of the Dx
$^{\text{APRM}}$ cDNA with that of the Dx
$^{\text{NBS}}$ cDNA. The Dx
$^{\text{APRM}}$ cDNA encoding Dx
$^{\text{APRM}}$ was constructed by replacing the XhoI fragment of the Dx
$^{\text{APRM}}$ cDNA with that of the Dx
$^{\text{APRM}}$ cDNA. The Nol-Kpnl fragments from all the constructs were subcloned into a P-element transformation vector, pUAST (Brand and Perrimon, 1993).

The constructs producing fusion proteins of GST with various Deltex derivatives were generated as follows. A cDNA of S. japonicum glutathione-S-transferase (GST) with an extra Cdi site was generated by a PCR with two primers, 5´TGACGG-ATATGTCCTCATACTGAG3´ and 5´AATCGATATTGGAGGATGGTCT3´, using a pGEX vector (Amersham Pharmacia Biotech) as the template. The deltex cDNA fragment was amplified using two primers, 5´TCAGGTCGCGCTTCTTGCGC3´ and 5´GGGG-ACAATACCGTCACGGCCAG3´. The resulting two PCR fragments were used as the templates in a recombinant PCR and amplified with the following primers: 5´AATCGATATTGGAGGATGGTCT3´ and 5´TCAGGTCGCGCTTCTTGCGC3´. The 3´-noncoding region of deltex cDNA with an extra Cdi site was amplified using the following primers: 5´AATCGATATTGGAGGATGGTCT3´ and M13 reverse primer. The junctions of the resulting constructs and point mutations were sequenced for confirmation. These deltex and GST cDNA fragments were ligated into the pUAST-Deltex constructs to replace the corresponding cDNA fragments, as described above.

Production of transgenic flies
The germline transformations and subsequent crosses were described previously (Sawamoto et al., 1994). In all experiments, several independent lines (~10) of each construct were established and examined. All crosses of UAS lines to patched-GAL4 (ptc-GAL4) were performed at 18°C (Johnson et al., 1995).

Western blot analysis
Transformant lines were crossed to an hs-GAL4 line (Brand and Perrimon, 1993). The resulting third-instar larvae were collected and heat shocked at 37°C twice for 1 hour, with a 1 hour 25°C interval between heat shocks. Larvae were homogenized in phosphate-buffered saline (PBS) (130 mM NaCl, 7 mM Na2HPO4, 3 mM NaH2PO4, pH 7.0) containing 1% SDS. The samples were boiled, and the protein concentration was determined using a bovine serum albumin (BSA) protein assay kit (Pierce). Protein samples were fractionated by SDS-PAGE on 7.5% or 10% acrylamide gels, transferred to Immobilon-P membranes (Millipore), and blocked. The protein blots were probed with rat anti-Deltex antibody (C645-17A) (Busseau et al., 1994) or rabbit anti-GST antibody (Santa Cruz biotechnology). The signal was detected using an HRP-conjugated secondary antibody (Cappel) and the ECL western blotting analysis system (Amersham Pharmacia Biotech).

Immunohistochemistry
Wing imaginal discs of the third-instar larvae were dissected in PBS and fixed in PLP (2% paraformaldehyde, 0.01 M NaOH, 0.075 M lysine, 0.037 M sodium phosphate, pH 7.2) (Tomlinson and Ready, 2000).
 Discs were washed in PBS-DT (0.3% sodium deoxycholate, 0.3% Triton X-100 in PBS) and incubated with the following primary antibodies: mouse anti-Wg (1:5) (van den Heuvel et al., 1989); rat anti-Deltex (1:25) (Busseau et al., 1994); mouse anti-Notch (1:5000) (Fehon et al., 1990); mouse anti-Delta (1:500) (Fehon et al., 1990); and rabbit anti-β-Galactosidase (1:500) (Cappel). After several washes in PBS-DT, the discs were incubated with fluorescently labeled secondary antibodies, rhodamine-conjugated goat anti-rat (Chemicon) and goat anti-mouse (Jackson Laboratories) antibodies, and FITC-conjugated goat anti-rabbit antibodies (Invitrogen), for 1-2 hours at room temperature, followed by washing in PBS-DT. The samples were mounted in 80% glycerol/PBS containing 1% N-propyl gallate.

**Cell culture and in vitro binding assay**

_Drosophila_ S2 cells were cultured and transfected as described previously (Fehon et al., 1990; Diederich et al., 1994). To produce Deltex derivatives or GST fusion to Deltex derivatives, UAS constructs encoding Deltex derivatives and pWA-GAL4 were co-transfected. pWA-GAL4 expressed GAL4 protein under the control of an actin gene promoter. A total of 2 µg of DNA and 8 µl of Cellectin reagent (Invitrogen) were mixed and added to cells in serum-free SFM medium (Invitrogen) and incubated for 4 hours, followed by incubation in a serum-containing medium for another 48 hours at 25°C. The cells were harvested and lysed in 200 µl TNE buffer (10 mM Tris-HCl pH 7.8, 1% NP-40, 0.15 M NaCl, 1 mM EDTA, 1 mM PMSF). After centrifugation at 18,000 g for 10 minutes at 4°C, the supernatant was incubated at 4°C for 1 hour with Glutathione-Sepharose 4B resin (Amersham Pharmacia Biotech), which was equilibrated with binding buffer (50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 100 mM NaCl, 10% glycerol, 0.5 mg/ml BSA, 5 mM β-mercaptoethanol). The resin was washed five times in binding buffer, then incubated in elution buffer (10 mM glutathione, 50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 100 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol) at room temperature for 20 minutes. Aliquots of the total lysates and the eluants from the Glutathione-Sepharose 4B resin were fractionated by 7.5% SDS-PAGE, and Deltex derivatives and the GST-Deltex derivative fusion proteins were detected on a western blot as described above, using an anti-Deltex antibody.

**Rescue of deltex mutant by overexpression of Deltex derivatives**

_deltex^{2A}:hs-GAL4/TM5_ was crossed to either _UAS-Dx^{full}_ or _UAS-Dx^{mouse}+GST_. Progeny were raised at 25°C, heat shocked at 37°C for 1 hour at the early pupae stage and then cultured at 25°C.

**RESULTS**

Notch signaling is required for the proper formation of the wing margin in _Drosophila_ (for reviews, see Brook et al., 1996; Cohen, 1996; Irvine and Vogt, 1997). For example, partial loss of Notch activity results in the wing nicking phenotype for which Notch was named (Fig. 1C). Most _deltex_ mutant alleles also yield a recessive wing-notch phenotype similar to that of _Notch_ mutants, indicating the involvement of _deltex_ function in wing margin development (Fig. 1B). Our previous study showed that the ectopic expression of Deltex results in the activation of Notch signaling (Matsuno et al., 1995). Because ectopic activation of Notch signaling in the wing pouch of the third-instar larval wing disc induces an ectopic wing margin-like structure that includes ectopic wing outgrowth and bristle formation (Diaz-Benjumea and Cohen, 1995; de Celis and Bray, 1997), we examined whether the overexpression of Deltex might induce a similar ectopic wing margin structure (Matsuno et al., 1995). Using the UAS/GAL4 system, we overexpressed the full-length Deltex protein under the control of patched-GAL4 (_ptc-GAL4_) (Fig. 1D) (Brand and Perrimon, 1993; Johnson et al., 1995). As shown in Fig. 1D, in the wing discs of third-instar larvae from the _ptc-GAL4_ line, the region expressing the GAL4 protein was located between two veins (arrowheads in Fig. 1D), which corresponded to the longitudinal veins III and IV in the adult wing (arrowheads in Fig. 1A).

As a result of full-length Deltex (_Dx^{full}_) overexpression, an ectopic secondary wing margin-like structure was induced along the region expressing Deltex (see Fig. 3H). This secondary wing margin-like structure included ectopic outgrowth and ectopic bristle formation (see Fig. 3B,H). Taking advantage of this Deltex activity, we decided to analyze the function of three conserved motifs found in Deltex: (1) a domain that binds to the CDC10/Ankyrin repeats of the Notch intracellular domain, (2) a proline-rich motif, and (3) a RING-H2 finger motif (Fig. 2A). Constructs to produce mutant Deltex proteins were generated and introduced into flies by _P_-element-mediated transformation (Brand and Perrimon, 1993). The Deltex mutant proteins used in this study are shown (D). Progeny were raised at 25°C, heat shocked at 37°C for another 48 hours at 25°C. The cells were harvested and lysed in 200 µl TNE buffer (10 mM Tris-HCl pH 7.8, 1% NP-40, 0.15 M NaCl, 1 mM EDTA, 1 mM PMSF). After centrifugation at 18,000 g for 10 minutes at 4°C, the supernatant was incubated at 4°C for 1 hour with Glutathione-Sepharose 4B resin (Amersham Pharmacia Biotech), which was equilibrated with binding buffer (50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 100 mM NaCl, 10% glycerol, 0.5 mg/ml BSA, 5 mM β-mercaptoethanol). The resin was washed five times in binding buffer, then incubated in elution buffer (10 mM glutathione, 50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 100 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol) at room temperature for 20 minutes. Aliquots of the total lysates and the eluants from the Glutathione-Sepharose 4B resin were fractionated by 7.5% SDS-PAGE, and Deltex derivatives and the GST-Deltex derivative fusion proteins were detected on a western blot as described above, using an anti-Deltex antibody.

**Fig. 1. deltex and Notch are**

**required for the normal development of the wing margin.** (A) Wild-type adult wing. The wing veins III and IV are indicated by arrowheads (see D). (B) _deltex/Y_ wing. (C) _Notch^{Dp2A}/+_ wing. (D) Protein expression from a UAS responder line under the control of the _ptc-GAL4_ driver. _UAS-GFP_ was crossed to _ptc-GAL4_, and wing discs of the third-instar larvae were stained with mouse anti-Delta antibody. GFP and Delta are shown in green and red, respectively. Presumptive cells of wing veins III and IV expressing Delta are indicated by arrowheads.
All three motifs of Deltex are required for normal Deltex function

As mentioned above, overexpression of Dx\textsuperscript{full} under the control of \textit{ptc-GAL4} induced a secondary wing margin-like structure (Fig. 3B,H). As shown in Fig. 3N, ectopic sensory organ precursor (SOP) cells (green and arrowhead) along the region expressing Dx\textsuperscript{full} protein (red) were formed in these flies. The induction of SOPs appeared to be non-cell-autonomous and occurred only in the ventral compartment (Fig. 3N). This observation provides further support to the interpretation that the overexpression of Dx\textsuperscript{full} leads to the development of a secondary wing margin-like structure. The endogenous Deltex protein could be detected as faint, ubiquitous staining throughout the entire wing discs of the late third instar larvae (Fig. 3M, red). Using this overexpression system, we examined the activity of four different mutant forms of Deltex (Fig. 3C-R). A mutant Deltex protein lacking the domain that binds to the Notch CDC10/Ankyrin repeats (Dx\textsuperscript{NBS}) or carrying two amino acid substitutions in the RING-H2 finger motif (Dx\textsuperscript{mRZF}) failed to induce the secondary wing margin-like structure in the adult wing, although very slight effects were still observed occasionally (Fig. 3C,I,E,K). These findings were confirmed by the observation that ectopic SOPs were not formed in the wing discs expressing these two mutant proteins (Fig. 3O,Q). By contrast, the overexpression of a mutant Deltex lacking the nine amino acids (amino acids 475-483) that encompass the proline-rich motif (Dx\textsuperscript{PRM}) resulted in wing nipping (Fig. 3D,J). We noted that this phenotype resembled those of \textit{deltex/Y} and \textit{Notch/+} flies (Fig. 1).

Dominant-negative behavior of a mutant Deltex lacking the proline-rich motif

The wing-notch phenotype induced by the overexpression of Dx\textsuperscript{PRM} suggested that Dx\textsuperscript{PRM} might be a dominant-negative
form of Deltex that inhibited Notch signaling during wing margin development. To test this hypothesis, we performed two different lines of experiments. First, $\text{Dx}^{\text{PRM}}$ and $\text{Dx full}$ were overexpressed simultaneously under the control of $\text{ptc-GAL4}$. We expected that $\text{Dx}^{\text{PRM}}$ and $\text{Dx full}$ would counteract each other’s activity, if the $\text{Dx}^{\text{PRM}}$ was a dominant-negative protein. As described above, overexpression of $\text{Dx full}$ induced an ectopic wing margin-like structure (black arrowheads) and ectopic SOPs (white arrowhead). (C,I,O) Overexpression of $\text{Dx}^{\text{NBS}}$. Occasionally, a missing crossvein (white arrow) and a few extra bristles (white arrowhead) are observed. (D,J,P) Overexpression of $\text{Dx}^{\text{APRM}}$. Note the wing-notch phenotype. (E,K,Q) Overexpression of $\text{Dx mRZF}$. (F,L,R) Overexpression of $\text{Dx}^{\text{NBS-mRZF}}$.

Fig. 3. Effects of overexpression of Deltex derivatives under the control of the $\text{ptc-GAL4}$ driver. Transgenic flies carrying the various UAS constructs expressing the Deltex derivatives were crossed to $\text{ptc-GAL4}$ or $\text{ptc-GAL4/CyO; A101/TM6B}$. (A-L) Adult wings and high magnifications of regions of wings. (M-R) The SOP cells in the third-instar wing discs are shown in green, and the Deltex derivatives are shown in red. An enhancer trap line, A101, was used to visualize the SOP cells. (A,G,M) Wild-type wing and disc. (B,H,N) Overexpression of $\text{Dx full}$. Note a secondary wing margin-like structure (black arrowheads) and ectopic SOPs (white arrowhead). (C,I,O) Overexpression of $\text{Dx}^{\text{NBS}}$. Occasionally, a missing crossvein (white arrow) and a few extra bristles (white arrowhead) are observed. (D,J,P) Overexpression of $\text{Dx}^{\text{APRM}}$. Note the wing-notch phenotype. (E,K,Q) Overexpression of $\text{Dx mRZF}$. (F,L,R) Overexpression of $\text{Dx}^{\text{NBS-mRZF}}$.
First, Dx^{APRM} was co-expressed with N^{act} under the control of the ptc-GAL4 driver. As shown in Fig. 5B, the expression of N^{act} alone resulted in the formation of ectopic SOPs (Rebay et al., 1993; Struhl et al., 1993; Lyman and Yedvobnick, 1995). Ectopic SOPs are indicated by an arrowhead (Fig. 5B). As shown in Fig. 5C, the co-expression of Dx^{APRM} did not substantially affect the ectopic SOP induction caused by the overexpression of N^{act} (compare with Fig. 5B). Similarly, the co-expression of Dx^{APRM} did not cause any marked effect on the ectopic induction of Wg that was caused by overexpressed N^{act} (compare Fig. 5D with 5E). Although Dx^{full} induced ectopic SOPs only in the ventral compartment of the wing pouch, N^{act} induced SOPs and Wg expression in both the dorsal and ventral compartments (Fig. 3N, Fig. 5B,D). As shown in Fig. 5F, the overexpression of N^{full} resulted in the ectopic and non-cell-autonomous induction of Wg expression, which contrasted with the cell-autonomous induction of Wg by the overexpression of N^{act}. We found that co-expression of Dx^{APRM} suppressed the induction of the ectopic Wg expression that was caused by the overexpressed N^{full} (compare Fig. 5F with 5G). Therefore, these results suggest that Dx^{APRM} acted downstream of N^{full} and upstream of N^{act}.

**Deltex signaling activity is regulated by oligomerization mediated by its RING-H2 finger**

The above results demonstrate that the Deltex RING-H2 finger...
motif is essential for Deltex function. RING-H2 finger motifs have been shown to mediate various protein-protein interactions (Freemont, 1993; Freemont, 2000; Joazeiro and Weissman, 2000). Ste5, a yeast protein, and Deltex have similar RING-H2 finger motifs (Inouye et al., 1997). Ste5 is an essential component of the mitogen-activated protein kinase (MAPK) cascade in a yeast pheromone response pathway (for a review, see Madhani and Fink, 1998; Schaeffer and Weber, 1999). In response to pheromone, Ste5 binds through its RING-H2 finger motif to the free Gβγ complex, which is composed of Ste4 and Ste18 (Whiteway et al., 1995; Inouye et al., 1997; Feng et al., 1998). This interaction leads to the activation of the MAPK cascade (Feng et al., 1998). The interaction between Ste5 and Ste4 is a prerequisite for Ste5 to self-associate and to function as part of the signal-transduction pathway (Whiteway et al., 1995). Moreover, it has been shown that the RING-H2 finger motif of Ste5 is also required for this self-association (Inouye et al., 1997).

The homology between the RING-H2 finger motifs of Ste5 and Deltex raised the possibility that the Deltex RING-H2 finger motif might have a similar function to the RING-H2 finger motif in Ste5. To test this hypothesis, we first performed an in vitro binding experiment. Two chimeric forms of Deltex, a wild-type Deltex (Dxfull+GST) and a Deltex carrying mutations in the RING-H2 finger motif, DxmrZF (DxmrZF+GST), in which GST was fused to the C terminus, were made in Drosophila tissue culture cells (the S2 cell line). We co-expressed each GST fusion protein with either wild-type Deltex or DxmrZF in S2 cells. The GST fusion form of the Deltex derivatives that bound to Glutathione-Sepharose 4B resin could be recovered and detected on a western blot using an anti-Deltex antibody. If Deltex formed homo-oligomers, non-GST fusion forms of Deltex should be co-purified with the fusion proteins and detected on the same western blot. We found that Deltex (non-GST fusion) bound to Dxfull+GST and was co-purified (Fig. 6, lane 11), but DxmrZF did not bind to the corresponding fusion protein (Fig. 6, lane 12). Neither wild-type Deltex nor DxmrZF bound to DxmrZF+GST under the same conditions (Fig. 6, lanes 13, 14). These results show that Deltex self-associates and that the RING-H2 finger motif is required for this oligomerization.

We then tested the possibility that the oligomerization of Deltex that was mediated by the RING-H2 finger motif had a crucial role for the activity of Deltex. The GST protein forms a stable dimer complex, both in solution and in protein crystals (Lim et al., 1994; McTigue et al., 1995), and this dimerization can functionally substitute for the dimerization domain of a heterologous protein (Maru et al., 1996; Riley et al., 1996). The Deltex derivative-GST fusion proteins were expressed in vivo using the UAS/GAL4 system (Fig. 2B). A western blot analysis using the anti-GST antibody revealed that all the GST fusion proteins were expressed with the expected molecular weight (Fig. 2D). Each fusion protein was expressed under the control of the ptc-GAL4 driver, and the transgenic flies were examined for formation of an ectopic wing margin-like structure and ectopic induction of SOPs (Fig. 7). Expression of DxmrZF+GST resulted in the formation of an ectopic wing margin-like structure and the ectopic induction of SOPs (Fig. 7D,J,P), while DxmrZF (the non-GST form) had no significant effect on either (Fig. 3E,K,Q). Therefore, the GST-mediated dimerization was sufficient to restore the function of DxmrZF. Furthermore, we found that overexpression of DxmrZF+GST under the control of a heat-shock promoter could rescue the mutant phenotype of the loss-of-function deltex in the wing veins (Fig. 8C). DxmrZF+GST was as competent as wild-type Deltex in rescuing the wing vein thickening phenotype (compare Fig. 8E with 8F). Occasionally, a wing vein was found to be missing in wings overexpressing DxmrZF+GST or wild-type Deltex (data not shown). By contrast, a control GST fusion form of wild-type Deltex did not show a substantial effect in the adult wing or on the formation of SOPs (Fig. 7A,G,M). These results suggest that the self-association of Deltex that is mediated by the RING-H2 finger motif in the wild-type protein is essential for the signaling activity of Deltex.

**DISCUSSION**

We have dissected the functions of three Deltex domains. These domains have been described previously, but their functions are not understood. Our results showed that the proline-rich and the RING-H2 finger motifs are required for distinct Deltex functions and are indispensable for Deltex activity. Although the identity of the proteins binding to these Deltex motifs and the nature of the protein-protein interactions
are still elusive, our results suggest that unidentified factor(s) are an integral component of Deltex function and the regulation of Notch signaling.

A dominant-negative form of Deltex

A proline-rich motif in the middle region of Deltex has been reported previously (Busseau et al., 1994; Matsuno et al., 1998). This motif shows homology to a consensus amino acid sequence of a binding site for SH3-domain proteins (Cohen et al., 1995; Di Fiore et al., 1997; Pawson and Scott, 1997; Kay et al., 2000). Indeed, we have previously demonstrated that human Grb-2, an SH3-domain protein, binds to Deltex (Lowenstein et al., 1992; Matsuno et al., 1998). In this paper, we show that Deltex lacking the proline-rich motif (DxPRM) behaves as a dominant-negative form. Based on these observations, we speculate that an as-yet-unidentified SH3-domain protein interacts with the proline-rich motif of Deltex and is an integral part of Deltex activity.

Nonetheless, the mechanism of the dominant-negative action of this mutant Deltex remains to be elucidated. Because proline-rich motifs are also found in the human, chicken and mouse Deltex homologs, the underlying mechanisms of this dominant-negative behavior may be evolutionarily conserved (Pampeno and Meruelo, 1996; Matsuno et al., 1998; Frolova and Beebe, 2000; Kishi et al., 2001). Previously, we showed that the expression of Deltex domain I fragment (amino acids 1-303), which lacks approximately two-thirds of the C-terminal region of the molecule, rescued a loss-of-function deltex phenotype and did not show dominant-negative function (Matsuno et al., 1995). Therefore, in addition to the absence of the proline-rich motif, the presence of some other part(s) of the Deltex domain II-III is required for the DxPRM mutant to act as a dominant-negative form of the Deltex protein (see Fig. 2A).

While DxPRM behaved as a dominant-negative protein during wing margin development, overexpression of DxPRM under the control of a heat-shock promoter during early embryogenesis did not result in a neurogenic phenotype, which is an indication that Notch signaling was not disrupted (data not shown). Therefore, the dominant-negative action of DxPRM may depend on the developmental context of cells, although the cellular component(s) responsible for this context-dependence remains to be identified. In this regard, it is noteworthy that none of the existing deltex alleles show the neurogenic phenotype (Xu and Artavanis-Tsakonas, 1990).
Oligomerization mediated by the RING-H2 finger motif is required for Deltex activity

In this paper, we have shown that Deltex forms homooligomers, and this oligomerization is integral for Deltex function. GST-mediated dimerization substituted for the function of the Deltex RING-H2 finger motif. The activity of DxmRZF +GST did not seem to be neomorphic, because the activity of wild-type Deltex to GST (Dxfull +GST) did not show substantial activity in our system. Therefore, the GST-mediated dimerization may abolish the activity of wild-type Deltex; it is also possible that Dxfull +GST is not functional because the fusion to GST leads to some nonspecific disruption of the protein structure. However, it has been reported that the Ste5 oligomerization mediated by its RING-H2 finger motif serves as both a positive and negative regulatory step (Inouye et al., 1997). An oligomerization of Ste5 that regulates it negatively is relieved by the Ste5-Ste4 interaction, and this interaction then permits Ste5 to form an oligomer mediated by its RING-H2 finger motif, in that order. Therefore, sequential oligomerization taking place in the proper order may be also important for Deltex function. However, in the case of Deltex, the factor that might relieve it from its inhibitory oligomeric state remains to be identified. It is not likely that Notch functions as such a relieving factor, because a GST-fusion with a double mutant Deltex, DXAnBS-mRZF +GST, lost the activity of DxmRZF +GST (Fig. 7D,J,P,E.K,Q), suggesting that the binding of Deltex to the Notch CDC10/Ankyrin repeats was apparently still required in DxmRZF +GST, despite the fact that this protein presumably bypassed the inhibitory oligomerization state and was competent to signal. This also suggests that the binding of Deltex to Notch is not a prerequisite for the self-association of Deltex, as the Notch-binding domain of Deltex is still indispensable for the activity of the artificially dimerized Deltex GST (Fig. 7D,J,P,E.K,Q).

Implications from the dominant-negative form of Deltex

Previously, we have shown that the loss-of-function deltex phenotype could be rescued by the expression of an activated form of Notch (Matsuno et al., 1995). This observation suggested that Deltex might act upstream of the activated form of Notch, although the nature of the deltex alleles used in that study had not been characterized very well. The present study shows that the dominant-negative form of Deltex acts upstream of an activated form of Notch and downstream of wild-type Notch. Although we need to be cautious in using a dominant-negative form of a protein to speculate about an epistatic relationship, the above two results are consistent. Therefore, we speculate that this dominant-negative form of Deltex may inhibit the activation or maturation of the Notch receptor. For example, possible target steps include the ligand-dependent cleavage of Notch, the processing of Notch to its mature form or the ligand susceptibility of Notch. Alternatively, it is possible that the dominant-negative Deltex specifically decreases the stability of full-length Notch.

Differences in the inductive properties of Dxfull and Nact

We have shown that overexpression of Dxfull induces an ectopic wing margin-like structure, which is similar to the consequence of the ectopic expression of Nact (Dias-Benjumea and Cohen, 1995; de Celis and Bray, 1997). However, these two proteins appear to have distinct inductive properties in the wing pouch. As shown in Fig. 3N, Dxfull induces SOPs only in the ventral compartment of the wing pouch, while Nact induces SOPs in both the dorsal and ventral compartments (Fig. 5B). Furthermore, Dxfull induces SOPs in cells other than and distant from those expressing Dxfull. From these results, we speculate that induction of Serrate may be a part of these events. Nact has been shown to induce Serrate within the wing pouch, and Serrate effectively activates Notch only in the ventral compartments (Panin et al., 1997). The activation of Notch results in the Wg induction that in turn induces SOPs in the neighboring cells (Rulifson and Blair, 1995). Furthermore, high-level expression of Serrate autonomously inhibits the induction of the genes within the wing pouch that are dependent upon Notch signaling (Jonsson and Knust, 1996; Klein et al., 1997; Micchelli et al., 1997). Thus, the induction of Serrate would explain, at least in part, the result that Dxfull induced SOPs only in the ventral compartment, and ectopic SOPs were formed slightly remove from the cells expressing Dxfull.

Putative factor(s) binding to the proline-rich motif of Deltex

The dominant-negative behavior of DXAprm suggests that
putative factor(s) that interact with the proline-rich motif might be essential for Deltex function. Suppressor of deltex [Su(dx)] is a good candidate. Su(dx) genetically suppresses deltex and Notch mutant phenotypes and encodes an E3-ubiquitin ligase (Fostier et al., 1998; Cornell et al., 1999). Su(dx) has many domains that bind to proline-rich motifs in general (Cornell et al., 1999). In a mammalian system, a mammalian homolog of Su(dx), Itch, binds to the intracellular domain of Notch and ubiquitinates it (Qiu et al., 2000). Therefore, Deltex may function to suppress Su(dx), a negative regulator of Notch signaling, through an interaction that may be mediated by the proline-rich motif of Deltex and the WW domain of Su(dx).

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