Signaling through both type I DPP receptors is required for anterior-posterior patterning of the entire Drosophila wing

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SUMMARY

The imaginal disk expression of the TGF-β superfamily member DPP in a narrow stripe of cells along the anterior-posterior compartment boundary is essential for proper growth and patterning of the Drosophila appendages. We examine DPP receptor function to understand how this localized DPP expression produces its global effects upon appendage development. Clones of saxophone (sax) or thick veins (tkv) mutant cells, defective in one of the two type I receptors for DPP, show shifts in cell fate along the anterior-posterior axis. In the adult wing, clones that are homozygous for a null allele of sax or a hypomorphic allele of tkv show shifts to more anterior fates when the clone is in the anterior compartment and to more posterior fates when the clone is in the posterior compartment. The effect of these clones upon the expression pattern of the downstream gene spalt-major also correlates with these specific shifts in cell fate. The similar effects of sax null and tkv hypomorphic clones indicate that the primary difference in the function of these two receptors during wing patterning is that TKV transmits more of the DPP signal than does SAX. Our results are consistent with a model in which a gradient of DPP reaches all cells in the developing wing blade to direct anterior-posterior pattern.

Key words: TGF-β, dpp, Drosophila, wing, signaling, gradient, morphogen, cell fate, anterior, posterior

INTRODUCTION

The adult Drosophila wing derives from the wing imaginal disk, a tissue that is specified during embryogenesis to form the adult structure (see Bate and Martinez-Arias, 1991). Originally made up of a small number of cells, the wing imaginal disk increases in size through cell division during postembryonic stages so that the adult wing blade contains over 30,000 cells (Garcia-Bellido and Merriam, 1971). The adult wing is a highly ordered structure, consisting of stereotypic arrangements of the wing margin, margin elements and veins. How the individual cells of the developing wing disk become determined to produce specific adult structures is currently an intensively studied question of developmental biology.

Recent work has elucidated some of the initial steps in the anterior-posterior patterning of the wing disk. The posterior compartment is established at the onset of disk development (Lawrence and Morata, 1977), by virtue of the positioning of the disk founder cells across the embryonic parasegment boundary (Cohen, 1990; Couso et al., 1993). Expression of engrailed in all cells of the posterior compartment leads to the production of Hedgehog protein (HH) in these cells. The secreted HH subsequently diffuses across the anterior-posterior (A-P) compartment boundary, where it interacts with anterior compartment cells to result in expression of the TGF-β superfamily member decapentaplegic (dpp) in a stripe of cells that abuts the boundary (Basler and Struhl, 1994; Tabata and Kornberg, 1994). dpp expression is prevented in the posterior compartment, despite the presence of HH, by activity of engrailed (Sancicola et al., 1995). Several other genes that are involved with hh in the proper spatial activation of dpp have been identified, including patched, protein kinase A (for review, see Blair, 1995; Perrimon, 1995) and cubitus interruptus (Schwartz et al., 1995; Domínguez et al., 1996).

The expression of dpp in its narrow stripe of cells is essential for proper A-P patterning of the entire wing (Posakony et al., 1991; Capdevila and Guerrero, 1994; Zecca et al., 1995). However, the events that occur after expression of dpp to further refine A-P pattern in the imaginal wing disk are not well understood. It has been proposed that the dpp protein (DPP), as a secreted ligand, could act only upon cells that are close to the stripe, or that it could act at a distance (Gelbart, 1989). Recently, three genes that act downstream of dpp in wing patterning, optomotor-blind (omb; Grimm and Pflugfelder, 1996), spalt-major (salm; de Celis et al., 1996; Sturtevant et al., 1997) and spalt-related (salr; de Celis et al., 1996; Reuter et al., 1996) have been identified. The expression patterns of these three genes have been used to show that ectopically expressed DPP can act at a distance and, in fact, that the domains of salm, salr and, possibly, omb are responsive to the local concentration of DPP (de Celis et al., 1996; LeCuit et al., 1996; Nellen et al., 1996; Sturtevant et al., 1997). Morphogenic activity of DPP has also been
observed in the specification of cell fates within the dorsal ectoderm during embryogenesis (Ferguson and Anderson, 1992; Wharton et al., 1993).

The DPP protein signals through members of the TGF-β receptor family of transmembrane serine-threonine (ser-thr) kinases (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Xie et al., 1994; Letsou et al., 1995; Ruberte et al., 1995). Both type I and type II receptors are required for signaling by the archetypal TGF-β1 ligand (for review, see Derynck, 1994). Different type I receptors appear to be associated with the same type II receptor, leading to the hypothesis that specificity of the signaling response may be mediated through the type II receptor. The stoichiometry of the actual TGF-β receptor complex is not known, although there is evidence that it may be a heterotrimer consisting of two type I and two type II receptor subunits (Yamashita et al., 1994). In Drosophila, two type I TGF-β receptors, encoded by the saxophone (sax) and thick veins (tkv) genes (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Xie et al., 1994), and one type II TGF-β receptor, encoded by the punt gene (Letsou et al., 1995; Ruberte et al., 1995), have been shown to be receptors for DPP.

With the identification of these DPP receptors, it has become possible to use genetic tools to investigate the range of action of DPP during imaginal wing development. Clones of cells that lack the normal function of a DPP receptor can be generated in the wing imaginal disk and the effects of these clones can be observed in the adult wing. As the effects of defective DPP-receptor function presumably reflect the lack of transmission of the DPP signal, the areas of the wing in which clones show phenotypic effects indicate the range of DPP action. An analysis of clones lacking normal function of either of the two type I DPP receptors, SAX or TKV, is presented here. It is observed that these clones have consistent effects upon A-P wing patterning. Moreover, these clones show such effects throughout the wing. Thus, it is shown here that signaling by the type I DPP receptors is required in all cells of the wing for proper A-P patterning. These results indicate that DPP acts to specify the A-P identity of all cells in the Drosophila wing, most likely through a gradient of the DPP ligand emanating from the stripe abutting the compartment boundary.

**MATERIALS AND METHODS**

**Drosophila strains**

The mutants used in this work are referenced in FlyBase (1996) except for the null alleles sax4 and sax5, which are described in Twombly et al. (1996). No secondary lethals exist on the sax and tkv mutant chromosomes used for clonal analysis. The recessive lethality of the individual sax4, sax5 and tkv5 chromosomes were each rescuable with the appropriate sax+ or tkv+ transgene (V. T., unpublished results; Y. Chen, unpublished results). The tkv5 chromosome was used homozygous viable (A. P., unpublished results). Strains were cultured on standard cornmeal, yeast extract, dextrose medium at 25°C, unless otherwise stated.

**Adult clonal analyses**

Clones generated in this work made use of the FLP/FRT system (Golic and Lindquist, 1989). For the generation of sax null clones to be scored in adult wings, the adult external marker was an allele of shavenoid (sha), sha+. Wing blade cells that are homozygous for sha+ have trichomes that are stunted or altogether missing, allowing precise determination of clone boundaries on each surface of the wing (Lawrence et al., 1986). w1118 P{hsFLP} / Y males that were also either P{>w> }G13 sax3 sha+ / CyO or P{>w> }G13 sax5 sha+/CyO were crossed to y1 w1118, P{>w> }G13 M2R5/3/CyO females. Adults were transferred every 12 hours. To induce clones, progeny were heat-shocked for 90 minutes at 37°C at either 36±6 hours, 42±6 hours, or 56±6 hours. These time points correspond approximately to early first, mid-first and mid-second instars, respectively, taking into account the growth delay caused by the presence of the Minute (M(2R)53) mutation (Ferrus, 1975).

For the generation of adult-marked tkv6 clones, y w1118 P{hsFLP}; tkv6 P{neoFRT}40A / CyO males were crossed to y w1118 P{hsFLP}; P{Car20yJ25F} P{neoFRT}40A females. Adults were transferred every 12 hours. To induce clones, progeny were heat-shocked for 60 minutes at 37°C at either 36±6 hours (mid-first instar) or 56±6 hours (mid-second instar).

Upon eclosion, straight-winged females (for sax) or straight-winged flies of either sex (for tkv) were collected and their wings mounted in Euparal (ASCO Laboratories). Microscopy and photography were performed with bright-field optics using a Leica DMR. Wings were also drawn for detailed analysis of clone boundaries and phenotypes using a Zeiss microscope equipped with camera lucida.

**Generation of clones in imaginal wing disks**

To generate sax null clones to be scored in the imaginal disks, w1118 P{hsFLP} / Y; P{>w> }G13 sax3 sha+/Bc Elp males were crossed to y w1118 P{hsFLP}; P{>w> }G13 P{M}47F P{M}47F females. To generate tkv clones to be scored in imaginal disks, males that were y w1118 P{hsFLP} / Y and either tkv5 P{neoFRT}40A / Bc Elp or tkv5 P{neoFRT}40A / Bc Elp were crossed to y w1118 P{hsFLP}; P{M}21C P{M}36F P{neoFRT}40A females. The M construct is a fusion of the human c-myc protein and the N-terminal sequence of the Drosophila P element transposase, which is sufficient to target the chimeric protein to the nucleus (Xu and Rubin, 1993). Clones of sax+ or tkv+ cells were identified by an absence of labeling with an anti-MYC monoclonal antibody, while twin spots labeled more brightly than surrounding cells. All subsequent steps to generate clones were done as for adult wings above, except that, for each cross, wandering third instar Bc+ larvae were selected for subsequent antibody labeling.

**Antibody labeling of imaginal wing disks**

Staining of wing disks with either mouse anti-MYC monoclonal antibody (kindly provided by S. Blair) or with rabbit anti-SALM antiserum (kindly provided by E. Bier from R. Schuh) was done as per Blair (1992), with the following specific procedures. Disks were fixed for 30 minutes in a Pipes buffer (Brower, 1986) containing 2% EM-grade formaldehyde (Polysciences). Disks were incubated overnight either in a 1:5 dilution of anti-MYC alone, or in a 1:5 dilution of anti-MYC and a 1:100 dilution of anti-SALM for double-labeling experiments. For labeling with anti-MYC alone, disks were subsequently incubated with a 1:200 dilution of FITC anti-mouse IgG. For double-labeling experiments, disks were first incubated with the FITC anti-mouse IgG and a 1:200 dilution of biotinylated anti-rabbit IgG, then washed and incubated in FITC anti-mouse and a 1:100 dilution of Cy5-streptavidin (all secondary and tertiary reagents from Jackson Immunolabs). Tissue was stored at 4°C in 30% glycerol mountant (in 50mM TRIS pH 8.8, 150 mM NaCl, 0.02% NaN3) and placed into 30% glycerol mountant with 0.5 mg/ml p-phenylenediamine (PDA, Sigma) before mounting. Tissue was initially observed with fluorescent optics using a Zeiss Axio phot and selected disks were viewed using a Biorad confocal microscope.
RESULTS

Effects of sax or tkv null clones upon wing development

The null alleles sax⁴ and sax⁵ used in this study were isolated in an EMS-mutagenesis screen for recessive lethals uncovered by deficiencies spanning the 43E18-43F2 region (V. T. and H. Kashevsky, unpublished results). These two sax alleles are indistinguishable from deficiencies of sax, and from each other, with respect to their recessive lethality and lack of enhancement of dpp phenotypes (V. T., unpublished results). Hence, a clone of cells homozygous for either allele will be referred to as a sax null clone. The tkv⁵ and tkv⁶ alleles have been classified as a null and a hypomorph, respectively (Terracol and Lengyel, 1994; Penton et al., 1994). Clones of cells that were homozygous for a null allele of either sax or tkv were generated using the FLP/FRT system (Golic and Lindquist, 1989).

We analyzed the effects of sax null clones on the development of the wing blade, using the trichome marker shavenoid (sha¹; see Materials and Methods). When somatic recombination is induced during the larval first instar, large clones of cells that lack sax⁺ function are recovered in the adult wing (Fig. 1A-C; a Minute mutation was used to provide the non-Minute sax⁻ clone with a growth advantage over the sax⁺ Minute tissue; Lawrence et al., 1986). These clones are recovered in all regions of the wing blade (Fig. 1B,C) including the region just anterior to the A-P compartment boundary, where dpp⁺ expression is known to be required (Posakony et al., 1991). dpp null clones induced at a similar stage are never observed in this region. In contrast, clones that lack sax⁺ function are recovered quite readily in this region and have relatively mild effects upon gross wing morphology (Fig. 1B). These effects include reduced wing size and a blunting of the wing tip, as well as ectopic venation.

In contrast, clones null for tkv function that are induced during the first or second larval instars are only very rarely recovered even in the late third instar wing pouch. Labeling

Fig. 1. Effects of clones generated early in larval development with null alleles of the two type I DPP receptors. (A) A normal wing. The standard pattern of the longitudinal veins L1 through L5 is indicated. (B) A large anterior clone of the null allele sax⁴ induced at late first instar (clone boundaries on the dorsal versus ventral surface of the wing are represented with dashed lines or dots, respectively). Mutant cells are marked with shavenoid (sha¹), which severely shortens or removes the trichomes of wing blade cells. This clone, which extends into the region where dpp null clones are never recovered (asterisks; Posakony et al., 1991), has mild effects upon wing morphology. Note the reduction in the size of the wing, blunting of the wing tip (arrow) and ectopic venation (arrowhead). (C) A large posterior clone of sax⁴ cells induced at late first instar. This clone also has mild effects upon wing morphology, including variable disruptions of venation (arrowhead). (D) Clones of the null allele tkv⁵, induced at the mid-second instar, are not present in the larval wing pouch at the late-third instar. The wing pouch has been labeled with an antisera to the ßM cell marker (Xu and Rubin, 1993) shows that tkv null clones induced even during the second instar usually are not recovered (Fig. 1D). The rare tkv null clones that are observed consist of two to four cells and are much smaller than their accompany-
the anterior wing margin. Arrows demarcate the boundaries of each of the morphologically distinct stretches of the three different bristle types (dco, distal costa; tr, triple row of bristles; dr, double row of bristles; pr, posterior row of hairs). (B-G) Comparison of the same areas in normal wings (B,D,F) to those in wings bearing sax clones (C,E,G; clone boundaries on the dorsal wing surface (dashed lines) and ventral wing surface (dots) are indicated). (B,C) Effect of sax clones upon cell fate in the anterior wing margin. (B) A normal wing showing the standard disposition of dr and pr. The site of the dr to pr demarcation (arrow) occurs very close to where L3 intersects the wing margin. (C) Within a large sax clone that encompasses this transition point, cells posterior to L3 now produce dr. The transition from dr to pr occurs further posterior (arrow), within the clone. An ectopic vein is also found just posterior to the sax clone boundary (arrowhead). (D,E) Effect of sax clones upon cell fate at more anterior areas of the wing margin. (D) A normal wing with the stereotypic pattern of tr and dr. (E) Within a sax clone, margin cells, which would normally produce dr, now produce tr (bracketed arrows) at the anterior edge of the clone. Within the clone, a transition from tr to dr (posterior to the ectopic tr) is observed. Again, ectopic venation occurs just posterior to the sax clone boundary (arrowhead) (F,G) Effect of sax clones upon cell fate within extreme anterior areas of the wing margin. (F) A normal wing, showing the standard positions of the dco and the tr. An area of naked margin just anterior to the tr is indicated (arrow). (G) Within a sax clone, cells in this area (arrow) now produce a structure that strongly resembles the dco; the structure rises above the normal curvature of the margin and the stout mechanosensory bristles that it produces are much larger than those produced posteriorly in the tr. Within the clone, the transition from this ectopic dco to tr still occurs, although there may be a loss of some or all of the naked margin.

Within sax null clones, there are typical anterior wing margin fate transformations to bristle types that are normally found further anterior along the margin (Fig. 2). While all margin cells within a clone appear to adopt more anterior fates, this is most evident at the anterior clone boundary, where cells entirely lacking sax+ function are apposed to cells that have normal sax+ function. For example, in a normal wing, the transition from the double row of bristles to the posterior row of hairs occurs essentially at the intersection of the longitudinal vein 3 (L3) with the margin (Fig. 2B). Within a sax null clone, however, several margin cells posterior to L3 now produce double row bristles (Fig. 2C), indicating that they are adopting fates normally found more anterior along the margin.

At other points along the anterior wing margin, shifts to more anterior cell fates within sax null clones are also observed. Within the area of the margin that normally produces the double row bristles (Fig. 2D), a subset of the margin cells within sax+ clones produce bristles of the triple row type (Fig. 2E). This ectopic triple row includes all three rows of bristles found in the normal triple row (Bryant, 1975), with essentially normal shape and spacing. It is also observed that this ectopic triple row is always produced at the anterior edge of sax null clones whose boundaries intersect the margin in this region (Fig. 2E). Posterior to the ectopic triple row but still within the sax null clone, double row is produced, so that within the clone, the elements of the anterior margin are iterated in their normal A-P sequence. Production of ectopic triple row bristles in areas that normally produce the double row type also typify clones of salm (see the accompanying paper by Sturtevant et al., 1997).
Changes of margin cell fates to those normally found more anterior occurs within \textit{sax} null clones even at the extreme edge of the anterior wing blade (Fig. 2F,G). Whereas in a normal wing the area just anterior to the triple row produces a naked stretch of margin (Fig. 2F), margin cells just within the boundary of a \textit{sax} null clone produce an ectopic stretch of the distal costa in this same area (Fig. 2G). As with the ectopic triple row (Fig. 2E), this ectopic distal costa is produced at the anterior edge of the clone, while posterior to this structure, triple row is produced within the clone (Fig. 2G).

These results provide evidence that SAX participates in the determination of A-P margin fate in the developing pouch, even at the extreme anterior edge (Fig. 2G). Margin cells within anterior compartment \textit{sax} null clones adopt fates normally found further anterior than the actual position of the clone. It is also apparent that within a \textit{sax} null clone, the normal A-P polarity is maintained, as multiple margin types are elaborated in their normal order.

\textbf{\textit{saxophone} null clones produce ectopic veins in both compartments in a position-dependent manner}

The effect of \textit{sax} null clones upon the production of extra veins in the adult wing further substantiates a role for SAX in A-P patterning in the wing (Fig. 3). Such additional vein material is produced adjacent to longitudinal vein 2 (L2) or longitudinal vein 5 (L5) only when the clone lies in a particular position relative to the endogenous vein.

\textit{sax} null clones that lie anterior to L2 (in the anterior compartment) do not cause ectopic venation (Fig. 3A). However, if the clone lies posterior and adjacent to L2, an ectopic vein is produced within the \textit{sax} null tissue (Fig. 3B). This ectopic vein is produced as a ridge on the ventral wing surface, as is the normal L2 but not the normal L3 (Garcia-Bellido and Santamaria, 1972). The point where the ectopic vein separates from the normal L2 vein is located precisely where the \textit{sax}\textsuperscript{−} clone boundary diverges from the normal L2. Distal to the branch point, the ectopic vein often does not lie right at the boundary of \textit{sax}\textsuperscript{+} and \textit{sax}\textsuperscript{−} cells (Fig. 3B). In the posterior compartment, the relationship between the position of \textit{sax} null clones and ectopic venation exists with mirror-like symmetry across the A-P compartment boundary. A \textit{sax} null clone that lies posterior to L5 does not cause ectopic venation (Fig. 3C), but a clone that lies anterior to L5 does produce an ectopic vein (Fig. 3D). The ectopic vein in this case is produced as a ridge on the dorsal surface, as is the normal L5 but not the normal L4 (Garcia-Bellido and Santamaria, 1972). As with effects at L2, the ectopic vein branches from the normal L5 at the point where the \textit{sax}\textsuperscript{−} clone boundary leaves the normal vein and, distal to the branch point, the ectopic vein often does not lie right at the clone boundary. These venation phenotypes appear very similar to those observed with clones of \textit{salm} in the

\textbf{Fig. 3.} Ectopic venation associated with specific positions of \textit{sax} null clones. (A-D) Adult wings bearing \textit{sax}\textsuperscript{4} clones, clone boundaries on the dorsal wing surface (dashed lines) and ventral wing surface (dots) are indicated. (A) A clone of \textit{sax}\textsuperscript{4} cells positioned anterior to L2 has no effect upon the normal pattern of venation. The small spur of ectopic vein near L5 is a background effect of \textit{M(2R)53} and is unrelated to the presence of a \textit{sax} clone. (B) When a clone of \textit{sax}\textsuperscript{4} cells lies posterior to L2, an ectopic vein (arrowhead) is produced. (C) A clone of \textit{sax}\textsuperscript{4} cells positioned posterior to L5 has no effect upon normal venation. (D) When a clone of \textit{sax}\textsuperscript{4} cells lies anterior to L5, an ectopic vein (arrowheads) is produced. As with the ectopic vein in B, this vein is found entirely within \textit{sax} null tissue.
accompanying paper (Sturtevant et al., 1997), as well as with clones of a small deficiency that removes both salm and salr (de Celis et al., 1996). We have not observed effects of sax null clones upon ectopic venation near longitudinal veins 3 or 4.

The positional specificity with which these sax null clones affect ectopic venation is striking. When a group of cells on the posterior side of L2 lacks sax+ activity, a subset of this group is apparently directed to produce a structure that normally appears more anterior (i.e., the L2 vein). When a group of cells on the anterior side of L5 lacks sax+ activity, a subset of them produce a structure that is normally found more posterior (the L5 vein). In each case, cells that lack sax+ activity appear to be taking on the fate of cells that are normally found farther from the A-P compartment boundary than the actual position of the clone. Thus, these results not only support a role for SAX in A-P patterning in the wing, but also show that SAX functions similarly within the two compartments. Furthermore, as the effect of a sax null clone in one compartment is a mirror-image of the effect in the other compartment, these results suggest that the SAX receptor responds to a signal that emanates from the vicinity of the A-P compartment boundary.

saxophone regulates SALM through its role in A-P patterning of the wing pouch

Some of the effects of sax null clones are comparable to those observed with clones of salm (Sturtevant et al., 1997), as well as with clones doubly mutant for salm and the adjacent gene salr (de Celis et al., 1996); these effects include ectopic stretches of triple row produced in areas that would normally make double row, and ectopic venation. To determine if these effects of sax null clones might be mediated through salm, wing pouches bearing sax null clones upon ectopic venation near longitudinal veins 3 or 4.

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in wing patterning is mediated through regulation of SALM expression. They are also consistent with the previously observed effects of sax null clones upon A-P patterning (Figs 2, 3), but these results show that these effects extend to all cells of the wing pouch, not simply those cells fated to become margin or vein. In short, the effect of a loss of sax+ activity in the wing pouch is to cause cell fate shifts to more anterior positions in the anterior compartment and to more posterior positions in the posterior compartment. Cells in clones positioned at the edges of the SALM domain are shifted in fate to cells that normally lie outside of the SALM domain and, hence, these cells lose all SALM expression. Alternatively, cells in a clone in the middle of the SALM domain are shifted to cell fates that, while equivalent to more lateral fates than found normally at the position of the clone, still lie within the SALM domain. Thus SALM is still expressed in these clones, albeit at a reduced level (see Discussion).

**Partial reductions in thick veins function to lead to phenotypes similar to the saxophone null alleles**

While clones that lack all tkv+ function do not survive when they are induced early in imaginal development (Fig. 1D), clones that have partial loss of tkv+ function can be recovered in the adult wing (Fig. 5A) and the late third instar wing pouch (Fig. 5B-D). The effects of clones of the hypomorphic allele tkv6 in these tissues are very similar to those of null alleles of sax. These include anterior shifts in cell fate along the adult anterior wing margin, as evidenced by the production of ectopic triple row bristles in areas that would normally produce double row bristles (compare Fig. 5A to Fig. 2E). In the imaginal wing pouch, cells within tkv hypomorphic clones also lose expression of SALM (Fig. 5B-D). These results show that both type I DPP receptors, SAX and TKV, interact with the same developmental pathways required for proper anterior-posterior patterning of the wing. It is interesting to note from these results that the consequences of homozygosity for this particular hypomorphic allele of tkv appear to be somewhat more severe than homozygosity for a null allele of sax (compare Fig. 5B-D to Fig. 4B-D). tkv6 clones lose all SALM expression even in clones located near the middle of the SALM domain (Fig. 5C,D).

**DISCUSSION**

Clonal analysis of sax and tkv reveal a role in A-P patterning of the wing

The results presented here demonstrate that both of the known type I DPP receptors, SAX and TKV, participate in A-P patterning of the entire Drosophila wing blade. The DPP signal is non-autonomously required for A-P patterning of the whole
wing (Posakony et al., 1991; Capdevila and Guerrero, 1994; Zecca et al., 1995). Here, we show that all cells of the wing blade depend upon proper activation of both the SAX and TKV receptors in order to be properly specified as to their A-P fate. This conclusion is supported by several observations. In the specification of the anterior wing margin, as well as veins L2 and L5, a loss of sax+ function results in cells differentiating as cell types normally found at other positions along the A-P axis. This occurs even for structures quite distant from the DPP signal emanating from the A-P compartment boundary, such as the juncture of the anterior blade and wing hinge where cells are transformed into distal costa. In the wing pouch itself, the expression of the wing patterning gene salm is regulated by sax in a manner consistent with a role for sax in A-P patterning. Finally, mutant clones that have reduced, but not complete loss of, tkv+ activity show similar effects to those of sax null clones, demonstrating that TKV is involved in the same set of patterning events during wing pouch development as SAX.

While we believe that much of the A-P patterning of the wing depends upon proper activation of SAX and TKV, we have encountered exceptions in the ontogeny of two wing veins: L3 and L4. Unlike the case for veins L2 and L5, sax null clones that include portions of L3 or L4 have no phenotypic effects on these veins; that is, L3 and L4 are positioned and structured normally, and no ectopic L3 or L4 veins are observed. The most likely explanation for this is that the positions of L3 and L4 are determined independently of DPP signaling. Consistent with this, Sturtevant et al. (1997) suggest that the position of L3 may be determined directly by a threshold response to Hedgehog secreted across the A-P compartment boundary and that the position of L4 may be initiated in alignment with the A-P compartment boundary. We think that the ontogeny of L3 and L4 are not representative of the patterning of other elements of the wing blade. Even within the domains that will give rise to L3 and L4, sax null and tkv hypomorphic clones show alterations in SALM expression consistent with global effects on A-P patterning. In the ensuing discussion of how these receptors mediate A-P patterning, we will generalize from the fate shift observations that we consider more typical, namely anterior wing margin development, L2 and L5 vein formation, and SALM expression.

A gradient of DPP may directly provide A-P positional information to all cells in the developing wing

The phenotypes of sax null and tkv hypomorphic clones indicate that these two TGF-β receptors are responding to a gradient of positional information that emanates from near the A-P compartment boundary and which patterns both the anterior and posterior compartments of the wing. The reduced receptor levels in these clones have the consistent effect of causing cell fate to shift to positions farther from the A-P compartment boundary than the actual position of the clone (Fig. 6A). Thus SAX and TKV are not responsible for elaborating a specific subregion or cell type in the wing, but instead pattern each cell according to its position relative to the A-P compartment boundary.

The results presented here are consistent with the TGF-β ligand DPP being directly responsible for this gradient of anterior-posterior positional information. Expression of dpp lies just anterior to the A-P compartment boundary (Gelbart, 1989; Masucci et al., 1990; Posakony et al., 1991) and, further, dpp is known to be required for A-P patterning in the wing (Capdevila and Guerrero, 1994; Zecca et al., 1995). Both SAX and TKV are identified DPP receptors (Brammel et al., 1994; Nellen et al., 1994; Panton et al., 1994; Xie et al., 1994). Finally, recent work has shown that DPP is capable of acting at long range in the wing disk (Lecuit et al., 1996; Nellen et al., 1996). Taken together, these data support the proposal that cells throughout the presumptive Drosophila wing blade adopt A-P fates in direct response to the local concentrations of a graded DPP signal.

There is insufficient information available to determine if other TGF-β ligands contribute to the gradient of positional information that activates the SAX and TKV receptors during wing development. It is not known if the other identified Drosophila TGF-β ligands, SCW (Arora et al., 1994) and 60A (Wharton et al., 1991), are expressed in the wing imaginal disk or are involved in wing patterning. All known TGF-β ligands are dimeric; both homodimers and heterodimers have been observed (Massagué, 1990). It is possible that another TGF-β ligand that is co-expressed with DPP contributes to a heterodimeric ligand and that the true gradient is a complex mixture of homodimers and heterodimers. However, possibilities that invoke spatially separate sources of different TGF-β ligands seem to be ruled out by our observations.

Roles of the two type I DPP receptors in wing development

It is evident from the work presented here that the two identified type I DPP receptors promote the same A-P patterning functions in the cells of the developing wing. Because of this functional overlap, a variety of A-P fates can still be produced in sax null or tkv hypomorphic clones by activation of the residual receptor complex in response to the DPP gradient. Consistent with observations during other phases of dpp action (Nellen et al., 1994), the primary difference in function between SAX and TKV during wing A-P patterning seems to be that TKV transmits a higher level of the DPP signal to the receiving cell. The reduced viability or proliferative ability of tkv null clones that we observe (which has also recently been reported by Burke and Basler, 1996) presumably reflects this higher level of TKV signal reception. A cell that only has SAX does not receive sufficient DPP signal for viability or proliferation. In contrast, the complete elimination of SAX activity does not reduce the level of DPP signal reception below that necessary for cell survival or proliferation. However, our work demonstrates that SAX remains a critical component of the DPP signal reception system for proper A-P patterning processes in the wing.

Also consistent with the greater role of TKV in mediating the DPP signal is the difference between sax null and tkv hypomorphic clones in their effects on SALM expression. Both sax and tkv clones have similar effects on SALM expression when located in the lateral portion of the normal SALM domain: no detectable SALM protein remains in these clones. However, medially located sax null clones exhibit reduced SALM levels whereas medially located tkv hypomorphic clones completely lack residual SALM protein. The most likely explanation for this is that the fate change observed in medial tkv hypomorphic clones is more dramatic than for similarly located sax null clones. Cells in the medial tkv hypomorphic clones are shifted
to fates that are normally in a domain that does not express SALM, whereas cells in the medial sax null clones are shifted to fates that are of a more lateral SALM expression domain. Consistent with this, Nellen et al. (1996) note that there is graded expression of SALM at the anterior and posterior edges of the SALM domain. Hence, while the amount of residual TKV activity in tkvβ is unknown, nonetheless it seems that partial loss-of-function of TKV is sufficient to produce a more dramatic fate shift than complete loss-of-function of SAX. These results emphasize the more dramatic contribution of TKV than SAX to A-P patterning.

While this work has shown that SAX and TKV function in the same cells to promote A-P patterning, several questions remain concerning type I DPP receptor function. One is whether the two type I DPP receptors might be components of a single receptor complex. Work in vertebrate cell lines on the same cells to promote A-P patterning, several questions TKV than SAX to A-P patterning.

A related issue is the degree to which these two type I receptors are quantitatively or qualitatively distinct from one another. It is thought that the type I receptors, through the phosphorylation of a type I receptor-specific cytoplasmic domain, the GS-box, activate downstream signaling pathways (Wrama et al., 1994). We have shown that both SAX and TKV participate in A-P patterning of the wing, but we cannot resolve whether their roles are limited to a cumulative contribution to the activation of a common signal transduction pathway, or whether one or both of these receptors have additional, unique pathways of signaling. The rescue of sax loss-of-function phenotypes with ubiquitously expressed tkv+ transgenes (Brummel et al., 1994) indicates that TKV can activate all downstream signaling pathways essential to SAX. The converse observation (sax+ transgene rescue of tkv) has not been reported, leaving open the possibility that TKV may have unique signaling activities.

Finally, there is the issue of whether these type I receptors are dedicated to receiving DPP signals, or whether they also participate in reception of signals from other TGF-β ligands. Very little information on this point is currently available, except that studies of the roles of DPP and SAX during oogenesis implicate a role for sax in patterning the posterior oocyte, whereas dpp appears not to be required in this domain or to beexpressed nearby (Twombly et al., 1996).

Some of the issues that we raise here can be addressed now that some components of the TGF-β signal transduction pathway have been identified. Recent observations demonstrate that the Drosophila MAD protein (Raftery et al., 1995; Sekelsky et al., 1995; Newfeld et al., 1996; Wiersdorff et al., 1996) is the prototype of a family of proteins that are components of DPP/BMP receptor signal transduction pathways (and probably all TGF-β receptor pathways) (Savage et al., 1996; Graff et al., 1996; Hoodless et al., 1996; Liu et al., 1996). Of particular interest is the identification of three different MAD-like proteins in C. elegans, which appear to be in a common developmental pathway downstream of a TGF-β receptor (Savage et al., 1996). That these three MAD-like proteins are not functionally redundant suggests that they may interact directly or indirectly with different type I receptors, or that they act combinatorially to mediate a receptor signal. Similarly, multiple MAD-like proteins have been identified in vertebrates (Graff et al., 1996; Hoodless et al., 1996; Liu et al., 1996). The study of this protein family, which we refer to as the DOT (Downstream Of TGF-β receptor) family, may allow resolution of the roles of the different type I receptors.

Anterior-posterior patterning by a gradient of DPP in the developing wing

As stated above, a model of A-P patterning of the wing by a DPP gradient correlates well with our results (summarized in Fig. 6A), as well as the results of others (Lecuit et al., 1996; Nellen et al., 1996). During normal imaginal wing pouch development, both SAX and TKV are present to interpret the level of DPP at any given position as this level falls off from its high point near the A-P compartment boundary (Fig. 6B). At different DPP concentrations, various thresholds are reached for the production of the different cell fates along the A-P axis, presumably through the activation of wing patterning genes such as salm and salr (de Celis et al., 1996; Lecuit et al., 1996; Nellen et al., 1996; Sturtevant et al., 1997; this work). Taken together, these findings consistently argue that the reception of a threshold level of a DPP signal is both necessary and sufficient for activation of SALM. Currently, contradictory reports on omb regulation by the DPP signal make it unclear if additional mechanisms exist for patterning of the A-P axis (Nellen et al., 1996; Lecuit et al., 1996). With the exception of the formation of some longitudinal veins (discussed above), our data are consistent with patterning of the entire wing blade in direct response to a long-range DPP signal. However, while our data do not require us to invoke additional patterning mechanisms, we cannot necessarily rule out their existence.

Regardless of these conflicts, our data clearly indicate that the different cell fates established by the DPP gradient result in the proper sequence of the elements of the adult wing, such as the longitudinal veins L2 and L5, and the bristle-types of the anterior wing margin. A DPP gradient model also explains some of the specific wing margin transformations observed in the sax null clones (Fig. 6C). Within such a clone, the extracellular DPP signal is received through receptor complexes whose type I subunits are all TKV, which produces an attenuated receptor activation response. Since the level of DPP signal is greater on the medial side of the clone than on its lateral side, more TKV is activated medially than laterally. Thus, we would predict that proper A-P polarity should be maintained in sax null (or by similar reasoning, tkv hypomorphic) clones. This prediction is fulfilled by the observed clones. For example, in a sax null clone that produces ectopic triple row bristles in the normal double row area, the ectopic triple row is always
produced at the anterior edge of the clone and posterior to that, double row is made within the clone.

The possibility that DPP might act at a distance to pattern the imaginal wing disk was recognized when its localized requirement and expression were first observed (Gelbart, 1989), and several studies have now indicated that this is indeed the case (Lecuit et al., 1996, Nellen et al., 1996; this work). In terms of the actual range of diffusion of DPP in this celluular environment, it is important to take into account that throughout most of imaginal development, the edges of the wing pouch are actually relatively close to the DPP stripe, while the DPP stripe itself appears to be more or less constant in width (Masucci et al., 1990; Sturtevant et al., 1997). As cell division in the wing disk results in the edges of the wing pouch being many cell diameters away from the DPP stripe by late stages of imaginal development, the position of these edge cells relative to the DPP stripe must change significantly during development of the wing disk. Thus, the A-P patterning influence that DPP has upon cells not only at the edge of the wing pouch of the wing imaginal disk, but also anywhere within the pouch, is likely a cumulative effect of the interpreted concentrations of DPP (through signaling by DPP receptors) over the entire period of larval development. Proper patterning of the wing thus appears to involve the response of individual cells to differing levels of a morphogenetic activity throughout appendage development.

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