Synergistic signaling by two BMP ligands through the SAX and TKV receptors controls wing growth and patterning in Drosophila

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Accepted 3 August; published on WWW 14 September 1998

INTRODUCTION

Morphogens are diffusible molecules that influence cell fate in a concentration-dependent manner (reviewed by Neumann and Cohen, 1997). Although the general characteristics of morphogens have been well established, the ways in which their activities are modulated to influence the final output in a receiving cell are still poorly understood. For secreted morphogens, numerous mechanisms that involve both extracellular and intracellular control points are likely to influence ligand potency. In the source cells, control of ligand production levels will clearly influence morphogen activity. Control at this point can be exerted by positive, negative and autoregulatory effects on ligand transcription as well as mechanisms that influence translation and secretion of the ligand. Once outside the cell, the ligand can interact with a variety of binding factors that control access to signaling receptors (Fainsod et al., 1997; Piccolo et al., 1996). Other binding components in the extracellular matrix, such as proteoglycans, are also likely to impact diffusion and ligand potency (Haerry et al., 1997; Jackson et al., 1997). In addition, for several ligands, binding to their respective receptor complexes both promotes signal transduction and helps limit their spatial range of activity by impeding movement of the ligand (Casanova and Struhl, 1993; Chen and Struhl, 1996). A final level of complexity is imposed at the receiving cell, where multiple signals are integrated in both synergistic and inhibitory ways to control the output response (Cornell and Kimelman, 1994).

One of the better characterized families of extracellular morphogens is the TGF-β group of growth and differentiation factors (reviewed by Kingsley, 1994). Members of this family can form both homo- and heterodimers, which may have very different activities. Within the superfamily, members of the BMP subgroup play particularly prominent roles in several developmental processes such as organogenesis, spermatogenesis and apoptosis (reviewed by Hogan, 1996). Signaling results after binding of a family member to a combination of type I and II transmembrane serine/threonine kinase receptors. These receptors likely form a heterotetrameric complex in the presence of ligand (reviewed by Heldin et al., 1997). In the paradigm developed for TGF-β and Activin signaling, the type II receptor is the primary determinant of ligand binding specificity. Once the ligand binds to the type II receptor, a type I partner is recruited to the complex and is activated by phosphorylation from the type II receptor. In contrast, for BMP-type ligands, depending upon the particular set of receptors involved, binding to either the type I or type II receptor can take place in the absence of the other, but a higher affinity complex is formed when both are present (Letsou et al., 1995; Liu et al., 1995). The type I receptor is the primary downstream signaling component and acts by modifying SMAD transcription factors, which relocate from the cytoplasm to the nucleus upon phosphorylation (reviewed by Heldin et al., 1997; Massagué et al., 1997).

In Drosophila, Decapentaplegic (DPP), a BMP2/4 ortholog, controls a number of developmental events including embryonic dorsal ventral patterning and growth and patterning...
of imaginal discs (reviewed in Gelbart, 1989). DPP has also been shown to have morphogenetic properties. During early embryogenesis, a post-transcriptional activity gradient of DPP is involved in the establishment of cell identities and spatial boundaries within and between the amnioserosa and dorsal ectoderm (Ferguson and Anderson, 1992; Wharton et al., 1993). In the wing disc, DPP appears to differentially activate three downstream response genes spalt (sal), optomotor blind (omb) and vestigial (vg) in a concentration-dependent fashion (Kim et al., 1997; Letou et al., 1995; Nellen et al., 1996; Singer et al., 1997).

In each case, the downstream response to DPP is mediated by the type II receptor Punt (PUT) and the two type I receptors, Thick veins (TKV) and Saxophone (SAX) (Brummel et al., 1994; Letsou et al., 1995; Nellen et al., 1994; Penton et al., 1994; Ruberte et al., 1995; Xie et al., 1994). At present, it is uncertain whether SAX and TKV differentially activate distinct downstream signaling pathways and whether they respond only to DPP or to additional related ligands. Two other BMP family members have been identified in Drosophila that might also signal through these receptors. The first is the product of the screw (scw) gene, which encodes a novel BMP family member (Arora et al., 1994). The second is encoded by the glass bottom boat-60A (gbb-60A or gbb) locus and is related to the BMP5/6/7 subgroup (Doctor et al., 1992; Wharton et al., 1993).

Fly strains and constructs

Mutants
gbb alleles 1 and 4 are described in Khalsa et al. (1998).

Gal4-driver lines
A9-Gal4 is an X chromosomal insertion line, identified in C. Goodman’s laboratory, which is strongly and ubiquitously expressed in wing and halter discs. Additional staining is present in the eye discs (low levels) and in the third leg discs (high levels). While the expression appears uniform using lacZ (perdurance) in late third instar wing discs, it is localized to the dorsal compartment of the wing pouch at late third instar larval stage using a dpp RNA probe. ptc-Gal4 was obtained from J. P. Conso and is expressed like the endogenous ptc gene, and dpp-diss-Gal4 line 39B2 was obtained from M. Hoffman and en-Gal4 from N. Perrimon.

pUAST constructs
A complete description of the subcloning steps of the constructs used in this study can be obtained on request. UAS-dpp: a second chromosome insertion line of UAS-dpp was obtained from the M. Hoffman. By jump start, an insertion line on the third chromosome was obtained that exhibits much weaker expression than the original line. The construction of UAS-gbb is described in Khalsa et al. (1998); UAS-putΔ: partial deletion of kinase domain, stop after 1353; UAS-sax-wt (HA); UAS-saxA (HA): Q263D mutation; UAS-sax-wtAE (HA); UAS-saxAΔE (HA): both constructs encode signal peptides and only nine amino acids of extracellular domain (ELDTSSISK); tldp>saxA (HA): 0.5 kb tid enhancer cloned upstream of saxA; UAS-saxΔE: deletion of kinase domain, stop after A261; UAS-tkv-wt (HA); UAS-tkvA (HA): Q199D mutation; UAS-tkv-wtΔE (HA), UAS-tkvAΔE (HA): both constructs encode signal peptides and five amino acids of extracellular domain (EFLHT); tldp>tkvAΔE (HA), saxA was replaced tkvAΔE in tldp>saxA; UAS-tkv1AAGS (HA): deletion of GS-box, leaves kinase domain intact; UAS-tkv1ΔGSK, UAS-tkv2ΔGSK: deletion of GS-boxes and kinase domains.

RNA in situ hybridizations

dpp, tkv and gbb expression were monitored by whole-mount in situ hybridization using digoxigenin-labeled antisense RNA probes. dppE55, a 4 kb dpp cDNA, tkv full-length or a fragment containing 1 kb of 3’ untranslated region (both in BS) and a 1.7 kb 60A cDNA (in NB40) were used as templates for probes. The probes were prepared according to the manufacturer’s directions (Boehringer-Mannheim 1277 073). Discs were hybridized and handled as previously described (Haerry et al., 1997).

Immunohistochemistry
Antibody stainings were carried out following the protocol published in Haerry et al. (1997), using AP-anti-rabbit and AP-anti-mouse antibodies from Promega and Sigma. Affinity-purified rabbit antisera against SAL (a kind gift from R. Schuh) was used at a dilution of 1:30. Mouse antisera against OMB was a kind gift from G. Pfugfelder and used at dilution of 1:200. Mouse antisera against the HA epitope were used at a concentration of 1:1000. Discs and wings were mounted in Canada balsam.

RESULTS

TKV and SAX can synergize to activate the downstream target gene OMB
The type I receptors TKV and SAX have been previously implicated in DPP signaling on the basis of genetic interaction data as well as ligand binding studies (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Xie et al., 1994). Whether these two receptors send quantitatively or qualitatively different signals during development has remained unclear. As a first step in determining how these two receptors contribute to pattern formation, we investigated the
phenotypic consequences of overexpressing constitutively active forms of these receptors in the developing wing using the GAL4-UAS system (Brand and Perrimon, 1993). For these studies we used the A9-Gal4 line, which drives high-level expression of Gal4 in the entire wing disc before it is restricted to the dorsal pouch at late third instar stage. In wild-type discs, the SAL and OMB products are symmetrically expressed along the anterior/posterior (A/P) boundary in response to DPP (de Celis et al., 1996; Grimm and Pfülgfelder, 1996; Fig. 1A). Normally, the SAL domain is restricted to cells in the wing pouch that are in close proximity to the DPP-expressing cells, while OMB responds to lower levels of DPP and is expressed in cells further away from the A/P boundary. The anterior boundary of SAL has been shown to specify the location where the longitudinal vein 2 (L2) is formed (Sturtevant et al., 1997), while the formation of L5 coincides approximately with the posterior boundary of the OMB domain, but a causal relationship has not yet been established.

When DPP is ubiquitously expressed in wing discs, they become overgrown and the expression of both SAL and OMB is expanded (Fig. 1B). Like DPP, overexpression of constitutively active TKV (TKVA) also leads to disc overgrowth and ectopic induction of SAL and OMB (Fig. 1C). All cells in wings derived from animals expressing either DPP or activated TKV appear to differentiate into vein tissue (Fig. 1B,C), as exemplified by production of vein-specific morphological markers such as dark pigment and longer bristles (de Celis, 1997). We also observe that the thick anterior margin triple-row bristles are transformed into long thin bristles (arrows in Fig. 1B,C), resembling the oversized double-row bristles found at more distal positions along the wing margin (higher DPP concentration; Singer et al., 1997). In addition, we find that the dorsal and ventral wing surfaces do not properly adhere to one another, resulting in a darkly pigmented and highly blistered wing.

We next examined the consequences of overexpressing an activated SAX receptor using the same driver. In contrast to TKV, expression of either one or two copies of SAXA or development at 30°C, which results in an approximately twofold increase of Gal4 activity, is not sufficient to expand either SAL or OMB and produces only weak adult phenotypes consisting primarily of ectopic and thickened veins with a small amount of wing blistering in the region of the posterior cross vein (Fig. 1D). This phenotype is similar to that seen in animals raised at 18°C that express low levels of TKVA. Although these findings suggest that SAX function may be qualitatively similar to that of TKV but simply weaker, higher levels of activated SAX activity (missing the GS box and the kinase domain), results in a strongly blistered wing overgrowth and the expansion of OMB (but not SAL), and results in a weak wing phenotype. The interaction of SAX and TKV is synergistic, since neither two copies of A9-Gal4>saxA nor two copies of tldp>tkvAAE show disc overgrowth, an expansion of OMB, or a strong adult wing phenotype (Fig. 1D,E). No enhancement is observed when A9-Gal4 is coexpressed with tldp>tkvAAE (data not shown), indicating that Gal4 is not interacting with the tld upstream sequence or other sequences present in the construct to produce this effect.

Taken together, these data suggest that SAX and TKV synergistically interact and control the expression of a common target gene, omb. Activation of omb expression requires a level of signaling that can be activated by either high levels of TKV activity alone or by a synergistic interaction between low levels of TKV and high levels of SAX activity. In contrast, SAL activation requires a higher level of signaling which, with our current constructs, can only be achieved by high levels of TKV activity (A9-Gal4>tkvA).

Reduced TKV and SAX activities affect different regions of the wing

Since both TKV and SAX, as well as the type II receptor PUT, have been implicated in mediating DPP signaling, we investigated whether the loss in signal activity of these receptors would cause similar patterning defects in the wing. If these three receptors all bind the same ligand and signal to the same sets of downstream genes, it was expected that a reduction in the activity of any individual receptor should result in qualitatively similar phenotypes that differ in severity only. Previous experiments inducing mutant clones in the wing imaginal disc have shown that TKV, SAX and PUT are required in cells throughout the wing disc for proper patterning and growth (Burke and Basler, 1996; Lecuit et al., 1996; Singer et al., 1997). Since clones that are null for tkv and put do not compete well with wild-type cells, their relative contributions to the growth and patterning process are difficult to assess with this type of analysis. Using a different approach, we overexpressed increasing levels of dominant negative receptors in different regions of the developing wing disc. Similar to using an allelic series of hypomorphic mutations, we expected that expression of increasing copy numbers of dominant negative receptors should result in progressively more severe phenotypes. We found that ubiquitous expression (using A9-Gal4) of 3-4 copies of either form of two dominant negative TKV1 constructs, tkv1ΔAGS (deleting just the GS box) and tkv1ΔGSK (missing the GS box and the kinase domain), results in additive effects.
in small wings with partial loss of L4 and both cross veins (data not shown). In addition, L2 and L3 are closer together and the triple-row margin bristles are shifted more distally/posteriorly, as expected if the level of DPP signal is reduced by titration of DPP into nonproductive complexes. At higher levels (6-8 copies) of dominant negative TKV1, very small adult wings are produced that show fusion of L2 and L3 as well as L4 and L5 (Fig. 2A). At third instar larval stage, wing discs expressing dominant negative TKV1 are strongly reduced in size and exhibit only residual SAL expression (when overstained), whereas by cell counts, the OMB domain is reduced approximately to the size of normal SAL expression domain (Fig. 2A). Similar phenotypes were produced by expressing dominant negative versions of the alternative isoform, TKV2, which has an N-terminal extended extracellular domain (data not shown), and also by expression of dominant negative PUT (putΔ: Fig. 2B). Both the SAL and the OMB domains are strongly reduced and the adult wings show fusion of L2 with L3 and L4 with L5. The phenotypes obtained with increasing levels of dominant negative TKV and PUT wing resemble those of certain combinations of dpp loss-of-function alleles such as dpp<sup>hr4</sup>/dpp<sup>d6</sup> (Brummel et al., 1994), which is consistent with the notion that DPP is primarily signaling through the combination of the TKV and PUT receptors.

In contrast to these observations, dominant negative SAX (sax<sup>ΔI</sup>) constructs produce different results. Binding studies have previously shown that the affinity of SAX for BMP-2 (the vertebrate ortholog of DPP), is lower than that of TKV (Brummel et al., 1994), and we expected that overexpression of dominant negative SAX should result in phenotypes that were similar but weaker than dominant negative TKV or PUT. We found however, that when increasing copy numbers (1-8 copies) of sax<sup>ΔI</sup> were expressed using A9-Gal4, the discs become smaller and the OMB domain is reduced to the size of the normal SAL domain (Fig. 2C). But unlike expressing dominant negative TKV, the SAL domain is not affected. In the adult wing, L5 and the posterior cross vein are lost compared to losing L3 and L4 after expression of dominant negative TKV or PUT (Fig. 2A,B). In addition, L2 is shifted more proximally and proximal triple-row bristles that expand more distally/posteriorly in dominant negative TKV wings are replaced by more proximal costa bristles. While the distance between L3 and L4 is normal, the overall shape of the wing becomes more ‘strap-like’, suggesting loss of peripheral tissue rather than the central tissue that is deleted in animals expressing TKV or PUT dominant negative receptors. These results suggest that dominant negative SAX acts in a qualitatively different manner from dominant negative TKV.

**gbb mutations phenocopy dominant negative SAX effects in the wing**

Our results indicate that while the reduction of TKV and PUT...
activity affects the whole disc (SAL, OMB and growth), the expression of dominant negative SAX only affects the peripheral region of the disc (OMB and peripheral growth). If the dominant negative receptors function primarily by titrating DPP, then it is curious why the overexpression phenotypes of dominant negative SAX are different. One possibility is that these receptors do not simply signal in response to DPP but also in response to the binding of other ligands as well. Of the other two BMP-type ligands that have been described in Drosophila, scw shows no detectable expression at this stage (Arora et al., 1994). However, glass bottom boat (gbb) is expressed broadly in wing discs, and mutant analyses indicate that gbb is required for normal wing development (Chen et al., 1998; Khalsa et al., 1998). Given its role in wing patterning, we examined the effects of heteroallelic gbb mutations on SAL and OMB expression. Similar to discs expressing dominant negative SAX (Fig. 2C), we found that SAL expression in gbb mutant discs is normal while the OMB domain is reduced, particularly in the dorsal compartment (Fig. 2D). At 25°C, less than 5% of the expected heteroallelic mutant combination survives to adulthood. The wings of these animals show a reduction of L5 and L4 and lack the posterior cross vein (Fig. 2D), a phenotype also reminiscent of flies expressing low copy numbers of saxΔI.

These observations are consistent with the notion that a second BMP-type ligand, GBB, is required in addition to DPP for proper OMB expression. Furthermore, the similarity of the gbb loss-of-function and the dominant negative SAX phenotypes is consistent with recently described genetic interactions between gbb and sax mutations (Khalsa et al., 1998) and suggests that GBB could signal in part through SAX.

**Differential suppression of ligand overexpression phenotypes by dominant negative receptors**

To further investigate how GBB influences wing development, we overexpressed GBB and examined SAL and OMB expression. Unlike DPP (Fig. 1B), we found that ubiquitous overexpression of moderate levels of GBB using A9-Gal4 at 25°C did not result in excessive disc overgrowth and did not alter the distribution of SAL and OMB (Fig. 3A). The resulting wings are slightly larger and exhibit minor venation defects along L2 and L5. However, similar to DPP or TKVA (Fig. 1B,C), higher levels of GBB overexpression (development at 30°C) expands both SAL and OMB and results in blistered and pigmented adult wings (Fig. 3B). Since only activated TKV but not SAX is able to expand SAL and OMB expression (Fig. 1), these findings are consistent with the notion that expression of moderate levels of GBB leads to signaling preferentially through SAX, producing relative mild phenotypes, while higher concentrations of GBB may also result in signaling through TKV, producing phenotypes similar to activated TKV.

Next, we investigated whether GBB contributes to wing development primarily in the form of homodimers or GBB/DPP heterodimers. First, we found that the level of gbb RNA appears to be significantly less than dpp, based on RNA in situ hybridization (data not shown; Khalsa et al., 1998), indicating that heterodimers are not likely to be very abundant assuming similar translational efficiencies. Second, localized overexpression of gbb in the dpp-expressing cells using the dppdiscΔ-Gal4 driver does not result in any mutant phenotypes (data not shown). Finally, expression of GBB in the posterior compartment using the en-Gal4 driver results in overgrowth, an expansion of the SAL and OMB domains and in adult wing defects all restricted to the posterior compartment only (Fig. 3C). Since en-Gal4 expression does not overlap with DPP-secreting cells, no DPP/GBB heterodimers should form, since heterodimer formation requires expression of both proteins in the same cell. Therefore, GBB functions most likely as a homodimer. This finding is consistent with recent genetic analysis showing that clones of GBB mutant cells that do not include dpp-expressing cells nevertheless produce patterning defects (Khalsa et al., 1998).

Overexpression of GBB, as well as genetic interaction between gbb, tkv and sax (Khalsa et al., 1998), suggest that...
both receptors may mediate the GBB signal. To further examine the relative contribution of these receptors to GBB signaling, we examined whether dominant negative TKV and SAX constructs exhibit different abilities to suppress the GBB overexpression phenotype (Fig. 3D). We found that coexpression of dominant negative SAX with GBB at 30°C (or at 25°C) almost completely suppresses the GBB overexpression phenotype, resulting in wings that are of normal size and only rarely exhibit minor L5 defects. Coexpression of TKV1AGSK with GBB at 30°C also results in suppression, but the resulting wings still have aspects of both GBB overexpression (larger size) and dominant negative TKV phenotypes (L4 defects). When the same experiment is done coexpressing DPP instead of GBB (Fig. 3E), we find that SAXAI is unable to suppress the DPP overexpression phenotype. On the other hand, TKV1AGSK partially suppresses blistering, restores all five longitudinal and both cross veins and reverts the ectopic double row margin bristles back to the normal triple row bristles. These results show that dominant negative TKV is more potent than SAX for inhibiting DPP signaling, while dominant negative SAX is a stronger suppressor than TKV of GBB signaling.

Control of DPP expression and diffusion by TKV concentration levels

In contrast to sax expression, endogenous tkv expression in third instar wing imaginal discs is not uniform, but notably absent in the center and elevated in the periphery (Brummel et al., 1994; de Celis, 1997). We noted that increased tkv expression is present adjacent to the OMB domain (Fig. 4A). Since receptor down-regulation is very common in many developmental processes, it seemed likely that DPP might down-regulate tkv expression within the OMB domain. However, when DPP or TKVA was ubiquitously expressed, we found that while OMB responds to the increase in DPP or TKVA signaling by expanding, tkv expression is not down-regulated (Fig. 4B). In addition, we found that other transgenes like GBB or the synergy combination of SAXA and TKV A regulate (Fig. 4B). Expression of OMB, therefore, we conclude that expression of tkv must be regulated independently of DPP and GBB in third instar wing discs.

Recent experiments have shown that overexpression of dpp within its normal pattern using a dppdisc-Gal4 driver can expand SAL but not OMB (Lecuit et al., 1996). To explain this result, the authors proposed a model in which OMB expression does not respond to different levels of the DPP gradient but is propagated by growth. However, our observation that OMB is surrounded by high levels of TKV, together with the finding that TKV expression is not controlled by the DPP gradient, suggested an alternative model in which high levels of TKV may function as a barrier that inhibits DPP diffusion and thereby restricts the size of the OMB domain. To examine this hypothesis in more detail, we first overexpressed DPP using dppdisc-Gal4 and examined the expression of OMB and tkv. To investigate any role of GBB/DPP heterodimers, we also coexpressed GBB with DPP using the same Gal4 line. We found that both DPP or DPP/GBB expression results in pouch overgrowth (Fig. 4C), but fails to expand OMB. The finding that tkv expression is still found adjacent to the OMB domain within these overgrown discs, is consistent with the model that the high levels of TKV receptor in the periphery might limit DPP diffusion and restrict OMB expression.

To test this model directly, we asked whether increased levels of wild-type TKV in the wing pouch would produce patterning defects. We expected that increased amounts of wild-type TKV (TKV-WT) receptor would reduce long-range DPP diffusion. Consistent with this view, we found that ubiquitous overexpression of one copy of full-length TKV-WT using A9-Gal4 results in less signaling not more (Fig. 5A). We found that the size of the discs as well as the SAL and OMB domains are reduced compared to wild type. The SAL domain was approximately four cells smaller (counting SAL-expressing cells in 20 discs of each). OMB expression was increased along the A/P boundary but substantially reduced in the periphery further away from the DPP source. Adult wings that emerge from these discs show the loss of L4, which is similar to the dominant negative TKV phenotype (Fig. 2A). This similarity indicates that the presence of excess extracellular domains likely sequesters DPP and impedes its diffusion. To further test this idea, we restricted the expression of dominant negative TKV to the A/P boundary using the ptc-Gal4 driver. This line expresses GAL4 in a narrow stripe of cells in the anterior compartment that overlaps with the dpp-expressing cells. The anterior boundary of the ptc domain is positioned just posterior of where L3 forms (Sturtevant et al., 1997). Consistent with the model that excess extracellular domains restrict DPP diffusion, we found that such localized expression of dominant negative TKV leads to non-cell-autonomous, dominant negative effects (Fig. 5B). Similar to ubiquitous expression of wild-type or dominant negative TKV, these effects include a reduction in the OMB and SAL domains as well as the loss of L4, the cross veins and a partial loss of L3 in adult wings. In contrast to TKV, expression of dominant negative SAX using ptc-Gal4 does not result in such non-cell-autonomous effects. Even at almost lethal copy numbers, dominant negative SAX does not reduce SAL or OMB expression and only prevents the formation of the anterior crossvein at the A/P boundary (data not shown). This result indicates that SAX is far less potent than TKV to interact with DPP and to interfere with its diffusion. The same is also true for the DPP type II receptor PUT. Like SAX, overexpression of dominant negative PUT with ptc-Gal4 results only in the loss of the anterior crossvein (data not shown). Consistent with previous findings showing that PUT has low affinity for BMPs in the absence of type I receptor (Letsou et al., 1995), this observation indicates that excess PUT extracellular domains do not interfere with DPP diffusion.

To ensure that these effects were not caused by secondary events such as dpp down-regulation, we also expressed wild-type TKV in the posterior compartment using en-Gal4. As mentioned earlier, en-Gal4 is not expressed in the DPP-secreting cells and expression of wild-type TKV is therefore unlikely to affect DPP expression. We find that, similar to A9-Gal4, expression of TKV-WT with en-Gal4 has very little effects on the size of the SAL domain. Expression of OMB, however, is clearly altered in the posterior compartment (Fig. 5C). A stripe of enhanced OMB expression is observed posterior of the DPP-secreting cell. Further away, posterior of that stripe, OMB expression is significantly reduced. At later stages, adult wings show truncations of distal L4 and lack most of L5. Taken together, these effects are consistent with the
model that an increased concentration of wild-type TKV outside of the DPP expression domain sequesters DPP and limits DPP diffusion, which results in increased signaling leading to enhanced OMB expression close to the source, but in reduced signaling and OMB expression further away from the source.

Finally, we examined the effects of DPP signaling on dpp expression by in situ hybridization in third instar discs. We found that overexpression of dominant negative or wild-type TKV and SAX with A9-Gal4 showed no noticeable effect on dpp expression (data not shown). We noted, however, that high-level expression of dominant negative TKV at the A/P boundary (using ptc-Gal4) results in an expansion of the dpp expression domain to roughly twice its normal size (Fig. 5D). On the other hand, ubiquitous expression of activated TKV was found to down-regulate dpp expression. These results show that dpp is negatively regulated by TKV signaling in the wing disc. They also show that dominant negative effects are not caused by dpp down-regulation, since overexpression of dominant negative TKV results in more and not less DPP expression.

DISCUSSION

DPP and GBB preferentially signal through different receptors during wing development

Previous genetic analysis has implicated both the SAX and TKV receptors in mediating DPP signaling. The data provided in this paper argue that these receptors actually respond to at least one more ligand, which is GBB. Our results imply that DPP signals preferentially through TKV and that the function of GBB signaling primarially through SAX is to enhance the DPP/TKV signal. Our argument rests on several observations. First, expression of dominant negative TKV and SAX result in qualitatively different effects. While ubiquitous expression of dominant negative TKV affects the whole wing and particularly interferes with the formation of the central region, dominant negative SAX mainly affects the formation of peripheral vein and intervein tissue. If both receptors were simply acting as DPP receptors, one would expect that expression of either dominant negative SAX or TKV would lead to a very similar phenotypic outcome. However, we also found that localized expression of dominant negative TKV at the A/P boundary using ptc-Gal4 results in non-autonomous effects that are most likely due to DPP sequestration, while in contrast expression of dominant negative SAX in the same way only results in the cell autonomous loss of the anterior crossvein. Taken together, these results indicate that SAX is not likely to function as a primary DPP receptor.

Since dominant negative SAX expression results in phenotypes different from TKV, we examined the role of other potential ligands in wing development. We focused on GBB because mutant phenotypes indicate that it is required for wing patterning (Khalsa et al., 1998). We found that, like dominant negative TKV receptor expression phenocopies certain dpp hypomorphic combinations, dominant negative SAX resembles gbb hypomorphic mutations.

Further support for DPP signaling primarily through TKV and GBB through SAX comes from phenotypic analysis of ligand and dominant negative receptor coexpression experiments. We found that dominant negative SAX can almost completely suppress the GBB overexpression phenotype, while TKV can only partially suppress. In contrast, DPP overexpression phenotypes cannot be suppressed by dominant negative SAX, but are reversed quite well by dominant negative TKV.

It is important to recognize that we do not imply that GBB only signals through SAX and that DPP exclusively signals through TKV. This follows from our observation that expression of activated SAX can never achieve the same level of signaling, as measured by SAL and OMB expansion, as can overexpression of GBB-60A. This is readily explainable if GBB signals through TKV when expressed at high levels and accounts for why dominant negative TKV will partially suppress the GBB overexpression phenotype. The interactions of DPP and GBB with TKV and SAX might reflect relative differences in binding affinities (with GBB having a preference for SAX and DPP for TKV) or in the effectiveness of certain ligand-receptor complexes at propagating a downstream signal.

Expression and diffusion of BMPs is controlled by the concentration of receptors

It has been recently shown that high levels of the HH receptor PTC function as a barrier that helps confine the action of HH to a limited region (Chen and Struhl, 1996). We have found that TKV likely plays a similar role in third instar imaginal discs. A striking feature of the tkv mRNA expression pattern in wing discs is its notable absence in the vicinity of the DPP source at the A/P compartment boundary and its enriched expression at the disc periphery. We have found that supplying additional wild-type TKV in the wing pouch results in dominant negative phenotypes such as reduction of the OMB domain. We infer that such effects result from inhibition of diffusion by sequestration of ligand near the A/P boundary, since the similar phenotypes can be generated by expression of dominant negative TKV along the A/P boundary or by expression of wild-type TKV in cells posterior of the DPP domain. The phenotype that results from ubiquitous overexpression of wild-type TKV, however, is likely not just the result of ligand sequestration, but also due to negative autoregulation. Although we found no visible change of dpp expression when one copy of wild-type TKV is expressed using A9-Gal4, partial repression is seen using ptc-Gal4 (T. Haerry, unpublished observation) and complete repression occurs when high levels of activated TKV are ubiquitously expressed. Taken together, we suggest that the low level of tkv expression seen in the wing pouch is required to both maintain high levels of dpp expression and to prevent interference with long-range DPP diffusion.

The observation that TKV likely limits the diffusion of DPP also offers an explanation for a puzzling feature of the OMB expression pattern. Previously Lecuit et al. (1996) found that when the level of DPP is enhanced along the A/P boundary within its normal domain, the size of the disc and SAL expression is expanded while OMB is not. This is unexpected if OMB activation requires a lower signaling threshold than SAL, but can be understood if high levels of TKV surrounding the OMB expression domain act as a sink to sequester excess DPP and thereby help limit the size of the OMB domain (Fig. 6). It remains unclear what regulates the TKV expression during larval development. Although very high levels of DPP
(multiple copies expressed with A9-Gal4) reduce tkv transcription to some level (T. Haerry, unpublished data), moderate levels of DPP and/or GBB that are sufficient to induce SAL and OMB do not alter the tkv expression pattern. Understanding the mode of TKV regulation is important for future studies since it constitutes yet another pathway that indirectly controls DPP morphogen activity by regulating the expression of its primary receptor.

**Biological significance of multiple ligands and receptor synergism**

It has been previously proposed that a shallow gradient of DPP directly induces different response thresholds in the wing disc with SAL requiring the highest levels and OMB moderate to low levels (Nellen et al., 1996). Based on our finding that both dominant negative sax expression and gbb hypomorphic loss-of-function mutants show normal SAL expression but exhibit a reduced domain of OMB expression, we propose that the DPP signaling gradient through TKV alone is too steep to induce OMB transcription outside of the SAL domain (Fig. 6). In order for OMB to be activated at the proper distance from the A/P boundary, we propose two mechanisms that help to expand the effective range of the DPP activity gradient in wing discs. The first is the down-regulation of TKV within the OMB domain by an unknown pathway. As a consequence, DPP diffusion is enhanced, but
due to low receptor concentrations it becomes more difficult to deliver a robust signal and influence tissue fates in distal regions. We therefore propose that a relatively uniform level of GBB signaling through SAX synergistically enhances the DPP/TKV signaling gradient above the threshold required for OMB induction (Fig. 6). Although our findings have shown that the synergistic interaction of SAX and TKV signaling affects primarily OMB expression, the same mechanism is likely to influence SAL expression as well. Previous clonal analysis has shown that sax is required for activation of SAL at the border of its domain (Singer et al., 1996). It is important to note that expression of dominant negative receptors and hypomorphic gbb alleles only result in a partial loss-of-function situation and therefore may not be strong enough to affect SAL expression. We also would like to note that the synergistic effects of GBB/SAX on the DPP signaling gradient may be more complicated than indicated in Fig. 6. Our finding that dpp expression is down-regulated if signaling levels are too high together with the observation that the GBB protein concentration is reduced in DPP-secreting cells (K. A. W., unpublished data), suggest that receptor synergy mainly enhances low signaling levels but has very little effect on the peak levels, which as a consequence may be lower than indicated.

Our studies suggest that two BMP-type ligands synergistically signal through different type I receptors and control the expression of at least one common downstream target gene. That SAX and TKV signaling might be interconnected is supported by the finding that ubiquitous low-level expression of the TKV isoform can rescue a fraction of  

Fig. 6. Model for threshold formation by DPP signaling through TKV and GBB through SAX. A steep DPP/TKV activity gradient induces SAL and OMB in almost completely overlapping domains. Synergistic interaction of ubiquitous GBB/SAX signaling shifts the DPP/TKV activity gradient above the lower threshold. Consequently, OMB is ubiquitously activated, while SAL remains restricted. Elevated levels of TKV limit DPP diffusion in the periphery, which results in the decline of the gradient and the restriction of the OMB domain.

Fig. 7. Models for the synergistic signaling of TKV and SAX. GBB/SAX and DPP/TKV may signal in parallel pathways and interact downstream, for instance through different SMADs. Alternatively, TKV and SAX may be present in the same complex in which SAX may phosphorylate TKV and stimulate the TKV-kinase activity.
sax mutations to full viability (Brummel et al., 1995). In addition, we have recently found that a chimeric receptor composed of the SAX extracellular domain and the TKV kinase is able to rescue SAX mutations to full viability and fertility (G. Marques and M. B. O’C., unpublished data). These results imply that the TKV kinase is able to signal to all of the relevant SAX downstream targets. This situation may be different from what has recently been described for the mammalian BMPR-IB and IA receptors, which appear to be dedicated to specific functions during vertebrate limb development (Zou et al., 1997).

While our studies indicate that activated SAX synergizes with activated TKV in wing discs, recent mRNA injection experiments have shown that the same synergistic mechanism helps to promote formation of the amnioserosa at the blastoderm stage (J. Neul and C. Ferguson, personal communication). The mutant phenotypes of sax and gbb indicate that GBB is not the major ligand for SAX at that stage. However the SCW ligand, which exhibits a mutant phenotype similar to sax, is expressed at that time. Thus, in the early embryo, it is likely that SCW signaling through SAX acts to augment DPP activity.

Although synergistic signaling provides a means of enhancing the response to a graded ligand, its molecular mechanism remains obscure. One possibility is that this effect is due to synergistic interaction of downstream components of TKV and SAX signaling (Fig. 7). It has been shown that SMAD proteins are directly phosphorylated downstream components of BMP and other TGF-β-type signaling pathways. In cell culture experiments, synergistic interactions have been described between different SMADs (Zhang et al., 1996). In Drosophila, phosphorylated MAD moves to the nucleus in response to DPP signaling, where it can bind directly to DNA to activate downstream targets (Kim et al., 1997). In other cases SMADs associate with different cofactors to activate transcription (Chen et al., 1997). Therefore, one possible mechanism involves the phosphorylation by TKV and SAX of two different SMAD-like proteins, or a SMAD and a second transcription factor associated with it (Fig. 7). Such a mechanism could lead to the synergistic activation of a target gene like omb.

Another scheme to explain synergism is that SMADs might have multiple phosphorylation sites that are preferentially modified by the two different receptors and, when both modifications are present, a higher level of transcriptional activity results. Precedence for different phosphorylation sites influencing SMAD activity has recently come from the finding that the ERK kinase can phosphorylate SMAD1 and attenuate the activity of the TGF-β-stimulated phosphorylation (Kretzschmar et al., 1997).

A third possibility is that SAX could act through TKV (Fig. 7). In this scenario, SAX and TKV, along with their respective type II partners, are components of a common receptor complex. Previous biochemical experiments indicate that TGF-β-type receptor complexes are likely tetrameric in composition, consisting of two type I and two type II components (reviewed by Heldin et al., 1997). It is not clear whether heterologous mixing of different type I and II partners can take place in the same complex. If so, then it is possible that SAX may hyperactivate TKV, perhaps through a direct phosphorylation step, or that ligands are more efficiently recruited into a signaling complex containing both SAX and TKV than into homomeric complexes.

We are very grateful to Reinhard Schuh, Stefan Grimm and Gert Pflugfelder for the anti-SAL and anti-OMB antibodies. We thank S. Park for injections of some of the constructs and Ted Brummel for stimulating discussions. This manuscript was improved by the thoughtful comments of L. Raftey and K. Arora. This work was supported by PHS grant GM47462 to M.B.O and NSF grant IBN-9604769 to K. A. W. T. H. was supported from fellowships from the Ciba-Geigy Jubiläumsstiftung and the American Cancer Society-California Division. O. K. was supported in part by NIH GM 07601 training grant to fulfill requirements for a PhD degree. K. A. W. was supported by an Established Investigatorship from the American Heart Association with funds contributed in part by the AHA, Maine Affiliate. M. B. O. is an Associate Investigator of the Howard Hughes Medical Institute.

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