TGF-β/BMP superfamily members, Gbb-60A and Dpp, cooperate to provide pattern information and establish cell identity in the Drosophila wing

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SUMMARY

Within a developing organism, cells receive many signals which control their proliferation, fate specification and differentiation. One group of such proteins is the TGF-β/BMP class of related signaling molecules. Based on expression studies, multiple members of this class of ligands must impinge upon the same cells of a developing tissue; however, the role that multiple TGF-β/BMP ligands may play in directing the development of such a tissue is not understood. Here we provide evidence that multiple BMPs are required for growth and patterning of the Drosophila wing. The Drosophila BMP gene, gbb-60A, exhibits a requirement in wing morphogenesis distinct from that shown previously for dpp, a well-characterized Drosophila BMP member. gbb-60A mutants exhibit a loss of pattern elements from the wing, particularly those derived from cells in the posterior compartment, consistent with the gbb-60A RNA and protein expression pattern.

Based on genetic analysis and expression studies, we conclude that Gbb-60A must signal primarily as a homodimer to provide patterning information in the wing imaginal disc. We demonstrate that gbb-60A and dpp genetically interact and that specific aspects of this interaction are synergistic while others are antagonistic. We propose that the positional information received by a cell at a particular location within the wing imaginal disc depends on the balance of Dpp to Gbb-60A signaling. Furthermore, the critical ratio of Gbb-60A to Dpp signaling appears to be mediated by both Tkv and Sax type I receptors.

Key words: gbb-60A, dpp, decapentaplegic, TGF-β, BMP.

INTRODUCTION

Intercellular signaling is essential for the proper manifestation of cell growth, fate specification and differentiation during the development of multicellular organisms. The transforming growth factor-β (TGF-β) superfamily of signaling proteins are secreted as dimers and mediate intercellular communication through their association with heteromeric integral membrane receptor complexes consisting of type I and type II serine/threonine kinase receptor proteins (Massagué, 1996). Ligand binding sets off a cascade of biochemical events, which culminates in a change to the target cell ranging from alterations in cell proliferation to the acquisition of specific cell fates (reviewed in Massagué, 1996). Given the plethora of data substantiating the importance of these multifunctional molecules in regulating diverse developmental events in a number of different species (Roberts and Sporn, 1990; Kingsley, 1994a; Hogan, 1996), elucidation of the mechanisms by which TGF-β signaling proceeds and how cells interpret and distinguish between different signals is crucial to our understanding of development.

The mature dimer or ligand can exist as a heterodimer as well as a homodimer, consisting of two different TGF-β-related monomers. The diversity of biological processes regulated by TGF-β signaling has often been attributed to the multitude of molecular interactions possible in this signaling pathway, including the potential for different monomer combinations, various associations of multiple type I to type II receptors (Yamashita et al., 1994), and a variety of interactions between an increasing number of intracellular proteins, eg. the Smad proteins (Derynck and Zhang, 1996; Massagué et al., 1997). An additional level of complexity that must be considered is that in many developing tissues multiple TGF-β signals must impinge upon a single cell and possibly signal through the same pathway. It has been observed that a number of members exhibit overlapping expression patterns (e.g. Dudley and Robertson, 1997; Lyons et al., 1995) and the question arises as to whether a cell can distinguish between different TGF-β signals and, if so, how is that information interpreted. During mouse development some degree of functional interchangability has been proposed for different BMP proteins, the largest group of TGF-β superfamily members (Dudley and Robertson, 1997). However, the generality of functional interchangability in other systems has not been determined. The possibility remains that more than one TGF-β signal contributes to a single developmental process and,
furthermore, that multiple ligands bind to receptors on the same cell in some way eliciting a different response in that cell. Here we present evidence indicating that Gbb-60A and Dpp, two Drosophila BMP members, are both required for proper wing morphogenesis and that they cooperate to direct normal development.

Three BMPs have been identified in Drosophila: Dpp, 60A and Scw (Padgett et al., 1987; Wharton et al., 1991; Doctor et al., 1992; Arora et al., 1994). Dpp and 60A each define a separate subfamily exhibiting greatest sequence similarity to vertebrate BMP2 and BMP4, and BMP5, BMP6, BMP7 and BMP8, respectively (Wharton et al., 1991; Kingsley, 1994a; Hogan, 1996). Dpp has been well-characterized and is known to act as a morphogen during patterning events that take place at multiple stages of development including embryogenesis and imaginal disc development. Cells have been shown to respond to the Dpp signal in a graded manner during the specification of dorsal embryonic cell fates (Ferguson and Anderson, 1992; Wharton et al., 1993) and in the specification of positional values along the anteroposterior (A/P) axis of the future adult wing (Lee et al., 1996; Nellen et al., 1996). The precursor cells of imaginal structures are set aside during embryogenesis as separate groups, or discs, of cells that actively proliferate during the larval period (Cohen, 1993). Patterning of the imaginal discs occurs during larval development and differentiation of imaginal structures takes place during the pupal stage. In the wing imaginal disc, dpp RNA is expressed in a localized pattern within a stripe of cells lying just anterior to the anterior/posterior (A/P) compartment boundary (Gelbart, 1989; Masucci et al., 1990; Posakony et al., 1991). Support for the proposal that cells adopt different fates within the wing disc in response to varied levels of a gradient of Dpp signal emanating from the A/P boundary has been provided by analysis of receptor mutants and downstream target genes (Burke and Basler, 1996; Lee et al., 1996; Nellen et al., 1996; Singer et al., 1997). In Drosophila, the BMP type I receptors encoded by thick veins (tkv) and saxophone (sax) have been shown to mediate various aspects of Dpp signaling (Affolter et al., 1994; Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Xie et al., 1994).

Interestingly, the mutant phenotypes associated with mutations in dpp and the receptor genes, sax and tkv are not equivalent (Brummel et al., 1994; Twombly et al., 1996; Singer et al., 1997), as would be expected if a receptor specifically transmitted a signal unique to that ligand. These discrepancies could be explained by the possibility that Sax and Tkv receptors are activated by a TGF-β family member other than Dpp. A loss of receptor function could then reflect the loss in signaling triggered by more than one ligand, either as a heterodimer or as two homodimers that each bind to the same receptor. Therefore, the function of a particular TGF-β ligand cannot be determined based solely on the examination of receptor function and/or activation of downstream elements, as such results could incorrectly attribute functions to that ligand alone, as well as confound the actual molecular mechanism of signal transduction. As yet it has not been definitively demonstrated that Sax and Tkv receptors can mediate other signals such as those elicited by Scw or 60A. However, mutant analysis and the coexpression of dpp and scw in the Drosophila embryo has led to the proposal that Scw signals as a Dpp/Scw heterodimer with a higher ‘activity’ than the Dpp homodimer (Arora et al., 1994; Raftery et al., 1995) but data indicating through which receptor such a signal is mediated has not been reported.

In this paper, we provide evidence that 60A (henceforth referred to as gbb-60A) is required for the growth of imaginal tissues and for patterning of the adult wing. We examine the expression of gbb-60A in the imaginal discs and show that high levels of Gbb-60A protein expression is in a pattern complementary to dpp expression. We demonstrate that Gbb-60A and Dpp signal together to accomplish the wild-type pattern of the adult Drosophila wing. The Tkv and Sax receptors are implicated as mediators of this synergistic signaling. Furthermore, several pieces of evidence suggest that Tkv may directly mediate the Gbb-60A signal. These data provide the first functional evidence that concerted signaling from two different TGF-β/BMP ligands is required in the control of a specific developmental process.

**MATERIALS AND METHODS**

**Drosophila melanogaster strains and crosses**

Mutant alleles used in this study are described in Flybase (1996), gbb-60A is a null allele and gbb-60A is a hypomorphic allele (K. A. W., K. deCastro, E. Borod and J. Cook, unpublished data). Recombinant lines were generated that removed a secondary mutation present on the original gbb-60A chromosome. All lines behave similarly; line 10 (gbb-60A R10) was used in experiments presented here. All recombinants between alleles of dpp, sax, tkv and gbb-60A were tested for the presence of each allele independently. All crosses were done on a cornmeal-sugar media at 25°C. Percent viability was determined by dividing the number of individuals in the Cy+ class by half of those in the Cy balancer class and multiplying by 100. Wings and legs were mounted in DPX mountant (EM Sciences).

**Generation of gbb-60A mutant clones**

Clones of genetically marked cells were made using the FLP/FRT system (Golic and Lindquist, 1989; Xu and Harrison, 1994). gbb-60A null clones were generated in the progeny of a cross of: yw1118/Y; P{>whs}G13aG13a1 sha1 gbb-60A1/SM6a males to yw1118/Y; P{>whs}G13aG13a1 P{hsFLP}; P{>whs}G13aG13a1 P{y+} sha+ gbb-60A+ females. Adults were transferred every 12 hours. FLP recombinase was induced by heat shock (37°C) for 50 minutes at 36 hours, 48 hours and 60 hours after egg lay (AEL). Clones were identified based on the shavenoid (sha) recessive marker, sha1, which removes or reduces the tricomes on the wing blade (Lawrence et al., 1986). A total of 158 wings were examined.

**Construction of UAS-gbb-60A transgenic lines**

A 1.6 kb EcoRI fragment from cDNA16 which contains the entire gbb-60A coding region (Wharton et al., 1991) was cloned into the Apol site of pUAST (Brand and Perrimon, 1993). Four transgenic lines were established by injection of UAS-gbb-60A and, when driven by GAL4 26B and hsGAL4 (Brand and Perrimon, 1993), each line was tested for its ability to rescue gbb-60A mutant phenotypes.

**RNA in situ hybridization and immunohistochemistry**

Whole-mount in situ hybridization to RNA in imaginal discs using digoxigenin-labeled RNA probes was done as described in Tautz and Pfeifle (1989) with the following modifications. Tissues were treated with protease K (50 μg/ml) for 3.5 minutes at room temperature and samples were hybridized ON at 55°C. Sense and antisense RNA probes were made using cDNA16 (Wharton et al., 1991) as a template. Immunohistochemistry with polyclonal rabbit anti-60A (Doctor et al., 1992) and monoclonal mouse anti-β-galactosidase (Sigma) was done.
RESULTS

Mutations in gbb-60A result in reduced imaginal discs

In order to gain a full understanding of the function of the 60A gene during development and to investigate the potential for combined signaling from two different TGF-ß/BMP family members, we had previously isolated mutations in the 60A gene (see Materials and Methods). Individuals homozygous for null alleles of the 60A gene, such as gbb-60A1, exhibit embryonic defects in gut morphogenesis and result in early larval lethality (K. A. W., K. deCastro, E. Borod and J. Cook, unpublished data). Allelic combinations between a hypomorphic 60A allele, gbb-60A4, and a null allele or a deficiency deleting the 60A locus result in later larval lethality with only rare adult escapers (<1%). Third instar larvae of such a genotype (gbb-60A4/gbb-60A1) appear transparent and smaller than wild-type larvae (Fig. 1A,B). These larvae develop more slowly than wild type and never attain wild-type size. The transparency appears to be due to a defect in both the quantity and quality of the fat body, as well as a dramatic reduction in imaginal tissues (Fig. 1C,D). In addition to a reduction in imaginal disc tissue, other tissues that normally proliferate during larval development, such as areas of the brain (Fig. 1D), are also reduced. The 60A locus has been named glass bottom boat (gbb) in light of the remarkable transparency of the mutant larvae and we will refer to it as gbb-60A in this paper.

Mutations in gbb-60A affect imaginal disc derivatives

The rare adult escapers of the genotype gbb-60A4/gbb-60A1 exhibit small misshapen wings, which lack the posterior cross vein (PCV), much of longitudinal vein L5, distal portions of L4 and the posterior half of the anterior cross vein (ACV) (Fig. 2C). The mutant wings are small, narrow and pointed with only rare adult escapers (<1%). Third instar larvae of such a genotype (gbb-60A4/gbb-60A1) appear transparent and smaller than wild-type larvae (Fig. 1A,B). These larvae develop more slowly than wild type and never attain wild-type size. The transparency appears to be due to a defect in both the quantity and quality of the fat body, as well as a dramatic reduction in imaginal tissues (Fig. 1C,D). In addition to a reduction in imaginal disc tissue, other tissues that normally proliferate during larval development, such as areas of the brain (Fig. 1D), are also reduced. The 60A locus has been named glass bottom boat (gbb) in light of the remarkable transparency of the mutant larvae and we will refer to it as gbb-60A in this paper.

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Subsequent to our initial observations of the lethality and phenotype of the gbb-60A hypomorphic allele, gbb-60A4, we have removed by recombination a secondary mutation present on the gbb-60A4 chromosome (see Materials and Methods). This secondary mutation exacerbated the lethality associated with the gbb-60A4 mutation and had no effect on adult phenotypes. Once the secondary mutation was removed, it was possible to produce viable adults that were homozygous for the gbb-60A4 mutation at a significantly higher frequency than that observed for the gbb-60A4/gbb-60A1 null genotype (see Fig. 5I). We have verified by sequence analysis that the viable adults are indeed homozygous for the gbb-60A4 mutation (data not shown). The mutant wing phenotype of the gbb-60A4 homozygous adult is similar to, but less severe than, that observed for the rarer gbb-60A4/Df or gbb-60A4/gbb-60A1 adults (Fig. 2B,C). In gbb-60A4 homozygotes, the overall shape of the wing is broader and less pointed, more like wild type. The ACV is often complete (54% of gbb-60A4 individuals versus 0% of gbb-60A4/gbb-60A1) and longitudinal veins L4 and L5 are longer.

Given that gbb-60A4 retains some gbb-60A function, we examined the effect of complete loss of gbb-60A function on wing patterning and morphogenesis through the production of
null clones. The FLP-FRT-mediated recombination system (Golic and Lindquist, 1989; Xu and Harrison, 1994) was used to generate clones of cells null for gbb-60A function in the imaginal discs (Fig. 2D-G). Consistent with a potential role for gbb-60A in proliferation, gbb-60A null clones are never recovered in the adult wing if induced during the early period of cell proliferation (before 36 hours AEL); however, clones in all parts of the wing were recovered from later inductions. Clones located in both the anterior and posterior compartments, as well as clones limited to the posterior compartment, exhibited defects in the same pattern elements affected in hypomorphic wings: loss of the PCV, and portions of L5 and L4 (Fig. 2D,E). Clones often also resulted in the loss of distal L3 at the margin. The majority (>80%) of the clones that exhibited a mutant phenotype covered a portion of the wing where vein material would normally be found. Clones strictly limited to the anterior compartment or along the A/P boundary exhibited no wing defects with the exception of very small clones that were located near the ACV (7% of the total clones restricted to the anterior compartment). These clones resulted in ectopic vein material anterior to L3 (Fig. 2F). In several wings vein loss was observed in wild-type tissue at a distance from the gbb-60A+/− patch (Fig. 2G). In each case that this non-autonomous effect was found, multiple clones were present in the wing, one in the posterior compartment and another along the A/P boundary, sites that otherwise did not produce abnormalities. This type of vein loss was never observed in the generation of control clones.

Expression of gbb-60A in the imaginal discs is complementary to that of dpp
The expression of gbb-60A in imaginal discs was examined by both whole-mount RNA in situ hybridizations (Fig. 3A,C,E) and antibody staining using a Gbb-60A antibody (Fig. 3B,D,F). gbb-60A RNA is expressed in the wing disc (Fig. 3A) mainly in the posterior compartment in the pteropleural and medial regions extending into the progenitors of the scutellum. High levels are found within the wing pouch in cells belonging to both the posterior and anterior compartments with higher levels in the posterior. A small amount of expression is found within the hinge region. In the eye/antennal disc, gbb-60A RNA is highest anterior to the morphogenetic furrow and in the medial regions with lower levels of expression posterior to the morphogenetic furrow (Fig. 3C). gbb-60A is expressed throughout the posterior compartment of the leg imaginal discs and within the ventral anterior compartment (Fig. 3E).

Gbb-60A protein is generally detected (Fig. 3B,D,F) at locations coincident with gbb-60A RNA with one major exception: the presence of a stripe of markedly lower Gbb-60A protein running through the middle of the wing pouch (Fig. 3B). This 'stripe' of reduced expression is coincident with the prominent stripe of dpp RNA expression in the cells anterior to the anterior/posterior (A/P) boundary of the wing imaginal disc (Fig. 4A-C). We also observe a weak but consistent reduction in Gbb-60A expression along the dorsal/ventral boundary from which the cells of the wing margin are derived (Fig. 4A). High levels of Gbb-60A expression exists in a pattern complementary to the localized expression of dpp in the other imaginal discs as well. For example, Gbb-60A expression is absent or at very low levels in the morphogenetic furrow of the eye disc, the site of dpp expression (Figs 3, 4D-F). In addition, the regions of low or absent Gbb-60A expression in the antennal and leg imaginal discs are the sites of dpp expression (Fig. 4D-F; data not shown). It seems
therefore appropriate that the abbreviation of *glass bottom boat* (*gbb*) is a mirror image of the letters *dpp*, thus reflecting the observed complementary pattern of *gbb-60A* and *dpp* expression. We do not intend to suggest that *gbb-60A* and *dpp* expressing cells are mutually exclusive, especially since the reagents available to assay for the presence of these BMP ligands do not allow such resolution. However, based on the reagents available our data indicate that the predominant RNA and protein expression of *gbb-60A* and *dpp* appear to be in different regions of the imaginal discs with some cells in the overlapping areas in which the co-expression of *gbb-60A* and *dpp* may occur.

While in most discs the expression of *gbb-60A* RNA and protein does not differ significantly, we cannot exclude the possibility that in the wing disc the apparent absence of Gbb-60A along the A/P boundary reflects the inability of a Gbb-60A polyclonal antibody to recognize the Gbb-60A protein in these cells perhaps due to some particular structural conformation of Gbb-60A, such as a heterodimer, in these cells. We believe this is very unlikely since a second polyclonal antibody directed at a different portion of the protein, the amino-terminal pro domain as opposed to the ligand domain (Doctor et al., 1992), also reveals the same reduced staining along the A/P boundary (data not shown).

**Dpp and Gbb-60A signaling are required together for normal wing morphogenesis**

The structures in the wing that are affected most dramatically by mutations in *gbb-60A*, the PCV and L5 are those that are least sensitive to the reduction or absence of *dpp* (Posakony et al., 1991; deCelis, 1997; Sturtevant et al., 1997). The ACV and longitudinal veins L2 and L4, lying on either side of the A/P boundary, have been shown to be most sensitive to the loss of Dpp signaling (Segal and Gelbart, 1985; Posakony et al., 1991; deCelis, 1997) consistent with the proposal that Dpp organizes wing pattern via a morphogen gradient emanating from the A/P boundary. (It has been proposed that longitudinal vein L3 is fated by a different mechanism and as a result is not sensitive to the level of Dpp signaling (Sturtevant, 1997).)
Since mutations in the gbb-60A locus indicate that gbb-60A is essential for establishing cell identity in the wing, especially within the posterior compartment, we investigated the possibility that Gbb-60A and Dpp signal together to provide positional information for the entire wing. No dominant genetic interactions were observed between alleles of gbb-60A and dpp, therefore, recombinant chromosomes were constructed using alleles of dpp which result in an overall lowering of dpp expression in the imaginal discs (dpp<sup>d5</sup>, dpp<sup>d6</sup> and dpp<sup>d12</sup>) (Masucci et al., 1990), and gbb-60A alleles, gbb-60A<sup>d</sup> and gbb-60A<sup>l</sup>. Individuals heterozygous for dpp<sup>d</sup> alleles (dpp<sup>d</sup>+/+) are phenotypically wild type. As homozygotes or in various heteroallelic combinations, these dpp alleles can be lethal or can generate adults with appendage defects ranging from minor loss of vein material to truncations of the entire appendage (Spencer et al., 1982).

An individual heterozygous for a dpp<sup>d</sup> allele and homozygous or transheterozygous for gbb-60A alleles (dpp<sup>d</sup> gbb-60A<sup>d</sup> + gbb-60A<sup>d</sup> or dpp<sup>d</sup> gbb-60A<sup>d</sup> + gbb-60A<sup>l</sup>) exhibits wing phenotypes qualitatively different than that observed in the gbb-60A mutant alone (Fig. 5). gbb-60A mutants heterozygous for dpp<sup>d</sup> exhibit a significant loss of L4 (>60% lack more than half of L4) and a more frequent loss of the ACV. gbb-60A mutants heterozygous for a more severe dpp allele, dpp<sup>d12</sup>, not only show a greater loss of L4, ACV and L2 vein material (>90% lack more than half of L4 and 100% lack the entire ACV including a thinning of L2) but also a loss of intervein tissue, especially between L2/L3 and L4/L5 as exhibited by a reduction in the overall size of the wing (Fig. 5B,D). In a small number of individuals (3%), a gap at the distal end of L3 is apparent. Interestingly, in the dpp gbb-60A/ + gbb-60A genotypes, we do not observe a greater loss of L5 but in fact a reduction in the loss of this posteriormost vein. 100% of dpp<sup>d12</sup> gbb-60A<sup>d</sup> + gbb-60A<sup>l</sup> exhibit only a gap in

**Fig. 5.** gbb-60A and dpp interact synergistically. (A-D) Genetic combinations involving the dpp<sup>d</sup> alleles, dpp<sup>d5</sup> and dpp<sup>d12</sup>, with gbb-60A<sup>d</sup> homozygotes or the heteroallelic gbb-60A<sup>d</sup> gbb-60A<sup>l</sup> result in enhanced wing phenotypes. (A) dpp<sup>d5</sup> gbb-60A<sup>d</sup> + gbb-60A<sup>d</sup>R10. (B) dpp<sup>d12</sup> gbb-60A<sup>d</sup> + gbb-60A<sup>l</sup>R10. The complete loss of the ACV is indicated by an arrow. (C) dpp<sup>d5</sup> gbb-60A<sup>d</sup> + gbb-60A<sup>d</sup>R10. (D) dpp<sup>d12</sup> gbb-60A<sup>d</sup> + gbb-60A<sup>l</sup>R10. (E) Wild-type male prothoracic leg. (F) Male prothoracic leg from dpp<sup>d12</sup> gbb-60A<sup>d</sup> + gbb-60A<sup>l</sup>R10. The five tarsal segments (bracket) in the wild-type male prothoracic leg are indicated (E). Loss of the ACV (arrow) in the dpp heteroallelic combination dpp<sup>d5</sup> dpp<sup>d6</sup> (G) is suppressed by gbb-60A null allele, dpp<sup>s4</sup> + / dpp<sup>d6</sup> gbb-60A<sup>1</sup> (H). All wings were taken from females and photographed at the same magnification. (I) The percentage viability of dpp<sup>d</sup> gbb-60A/ + gbb-60A genotypes is indicated. gbb-60A is abbreviated gbb. The total number of individuals scored in the viability crosses is shown in parenthesis.
 mutations alone was also observed (Fig. 5I). The adult viability of expected as compared to control crosses). These results combinations (Fig. 5A-D). However, similar to the other vein material as seen above with other in a restoration of the ACV (Fig. 5H) not a further loss of other regulatory These phenotypes are not only observed with but rather as the ratio of changing the level of one ligand we should be able to generate. Different relative levels of Gbb-60A to Dpp signaling would result in different positional information. At some points within the developing wing, the readout may be synergistic while at other sites antagonistic.

Correct balance of Gbb-60A and Dpp signaling is essential to normal patterning
If cells across the wing disc indeed respond to different levels of Gbb-60A versus Dpp signaling, we reasoned that by changing the level of one ligand we should be able to generate a patterning mutant phenotype. We should then be able to potentially rescue that phenotype by changing the level of the other ligand in an attempt to restore the balance of signaling elicited by the two ligands to that found in wild type. To test this hypothesis, we first made a UAS-Gbb-60A construct which contained the entire gbb-60A coding region and generated multiple transgenic lines. We have demonstrated that these UAS-Gbb-60A lines produce functional Gbb-60A since gbb-60A mutations can be rescued to adulthood when the expression of UAS-Gbb-60A is driven by hsGAL4 or by GAL4-24B (Table 1). GAL4-24B directs expression mainly in the embryonic mesoderm (Brand and Perrimon, 1993), the site of high levels of gbb-60A expression in the embryo (Doctor, 1992; K. A. W., unpublished data). In addition to the rescue of lethality, the mutant wing phenotype associated with gbb-60A viable mutants (Fig. 2) was rescued when hsGAL4 was used to direct expression. Using the same experimental conditions, we were unable to rescue gbb-60A mutants (lethality or wing phenotype) with previously reported UAS-60A lines (Staehling-Hampton et al., 1994).

Since Gbb-60A is normally expressed at a very reduced level along the A/P boundary, we investigated the effect of overexpressing Gbb-60A in this region. Wings from UAS-Gbb-60A/ptc-GAL4 exhibit ectopic vein material anterior to the A/P boundary (Fig. 6B). If dpp is also mis-expressed (UAS-gbb-60A/UAS-dpp/ptc-GAL4) this mutant phenotype is rescued (Fig. 6C). The effect of raising Gbb-60A signaling along the A/P boundary where Dpp is normally very high results in mispattering. If the original balance of higher Dpp to Gbb-60A along the A/P boundary is re-established by adding more Dpp the wild-type positional information is restored. When ectopically expressed, the UAS-dpp (gift from Mike O’Connor) used in these studies fails to produce any mutant phenotypes. Other examples of the effects of manipulating the relative levels of Gbb-60A and Dpp is shown when ectopic expression is more widespread using 71B-GAL4 (Fig. 6E,F) or A9-GAL4 (Fig. 6H). In each case somewhere but not all of the defects in pattern elements caused by ectopic expression of gbb-60A are rescued by also expressing dpp. Given that our loss-of-function interaction data fail to demonstrate a simple antagonistic relationship between gbb-60A and dpp (indeed quite the opposite), we must conclude that these ectopic expressions reflect a change in the balance of dpp to gbb-60A activity or signaling. Which specific elements of the ectopic Gbb-60A mutant phenotype are rescued clearly depends on the pattern of ectopic expression during the entire period of wing patterning as well as the endogenous levels of Dpp and Gbb-60A.

gbb-60A alleles genetically interact with mutations in BMP type I receptor genes, tkv and sax
The Dpp signal has been shown to be mediated by two different BMP type I receptors, Tkv and Sax, during wing morphogenesis as well as during other stages of development (Xie et al., 1994; Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Twombly et al., 1996; Singer et al., 1997). To address whether either or both of these receptors are important in mediating the signals resulting from the actions of

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<th>UAS-gbb-60A</th>
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<td>GAL4 24B</td>
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Gbb-60A and Dpp, we investigated the possibility of a genetic interaction between alleles of gbb-60A and alleles of tkv or sax. Recombinants were constructed between gbb-60A and several alleles of tkv and sax. The addition of Df(2L)tkv2, a chromosomal deficiency that removes the tkv locus (Penton et al., 1994), into a gbb mutant background results in a severe mutant wing phenotype with a dramatic loss of both the PCV and ACV and most of L4 and L5 (Fig. 7A, B). In addition, distal gaps are present in L2 and L3. A less extreme phenotype is seen with tkv6, a hypomorphic allele that retains significant receptor function (data not shown). Interestingly, the more severe phenotype of tkv2 gbb-60A1+/gbb-60A4 has qualities that are distinct from those observed in dpp gbb/ + gbb individuals, notably the effect on L5. Furthermore, unlike the genetic interactions observed with dpp alleles, the viability of tkv gbb-60A1+/gbb-60A4 is not reduced but in fact is considerably increased when compared to gbb-60A4/gbb-60A4 (Fig. 7E). These data indicate that the interaction that we observe between tkv and gbb-60A cannot be explained solely as a secondary consequence of lowering Dpp signaling readout by mutating a receptor that mediates Dpp signaling. These data suggest that Tkv is able to mediate Gbb-60A signaling and that it may do so in different ways at different times during development.

We also analysed the effect of reducing the gbb-60A copy number in an individual compromised for functional Tkv receptor. tkv427 behaves as a hypomorphic allele. As a homozygote, tkv427 is phenotypically wild type and exhibits no defects in the wings; however, in trans to a null, Df(2L)tkv2, a slight thickening of wing veins is observed (Fig. 7C). This ectopic vein phenotype is thought to reflect a feedback loop in which dpp fails to be downregulated properly by tkv in the vein primordia resulting in the response of boundary intervein cells to the vein-promoting Dpp signal, and thus the formation of vein outside of the vein proper (deCelis, 1997). We observe that reducing gbb-60A in this tkv mutant background (tkv427+/Df(2L)tkv2 gbb-60A1/) produces a further thickening of wing veins (Fig. 7D). This result suggests that gbb-60A may play a role in vein differentiation itself and/or in the tkv/dpp feedback loop important in defining the boundaries of the vein.

Genetic combinations used to investigate the potential interaction between gbb-60A and sax alleles indicate a reduction in viability for gbb-60A mutant genotypes containing a single copy of a sax null allele (sax gbb-60A1+/gbb-60A4) (Fig. 7E). This reduction in viability suggests that lowering both gbb-60A and sax compromises development. The wing phenotype of the few viable adults recovered was similar to a very severe gbb-60A mutant wing phenotype shown in Fig. 2C, with a substantial loss of L5, complete loss of the PCV and ACV and loss of half of L4. Clearly the levels of Gbb-60A signaling and Sax function are dependent on one another.

**DISCUSSION**

**gbb-60A is required for growth and patterning of imaginal tissues**

Our gbb-60A mutant analysis indicates that the gbb-60A gene is required for proper wing morphogenesis, a process requiring
both growth and patterning of wing imaginal tissues. The requirement for gbb-60A in growth of imaginal discs is exemplified by the dramatic reduction of imaginal tissues observed in gbb-60A mutant larvae (Fig. 1D), which contributes to a transparent phenotype for which we have named the 60A gene glass bottom boat (gbb). Our mutant analysis further demonstrates that lowering gbb-60A function results in the reduction of the size of the adult wing (Fig. 2). The reduction in wing tissue is suggestive of a failure of imaginal cell proliferation. These results are similar to what has been found for mutations in dpp, as well as in a number of other genes that encode BMP signaling components in Drosophila (Spencer et al., 1982; Raftery et al., 1995; Brummel et al., 1994; Burke and Basler, 1996; Singer et al., 1997). These data taken together suggest that BMP signaling in Drosophila plays a role in the positive control of cell proliferation, opposite to the negative effect on cell growth attributed to TGF-β itself (Massagué et al., 1990; Roberts and Sporn, 1990). Studies in progress will delineate in which cells and at what times during development gbb-60A is required for proliferation, as well as how the signal is mediated.

With the identification of a gbb-60A hypomorphic allele, we have been able to partially bypass the requirements for gbb-60A in early stages of development which when not met lead to lethality. Thus, we have been able to elucidate a requirement for gbb-60A in wing morphogenesis (Fig. 2). The generation of clones null for gbb-60A function reaffirmed this requirement for gbb-60A in the specification of pattern elements within the wing. Our analysis of loss-of-function mutations demonstrates that the posterior portion of the wing is most sensitive to the level of gbb-60A function with the PCV and vein L5 most sensitive to a reduction of gbb-60A, followed by L4, the ACV, L3 and least sensitive, L2 (Fig. 2). In addition, the non-autonomous effect of gbb-60A− clones suggests that disrupting gbb-60A levels in one part of the disc must alter how cells in other regions of the disc interpret their position (Fig. 2G).

Complementary expression pattern of gbb-60A and dpp suggest these ligands exist primarily as homodimers

The expression of gbb-60A RNA and protein are consistent with the requirement that we have identified for gbb-60A in the wing. The pattern of Gbb-60A protein expression in the wing imaginal disc as revealed by a Gbb-60A polyclonal antibody (Fig. 3B) is intriguing in light of the highly localized expression of dpp RNA in the cells just anterior to the A/P boundary of the wing imaginal disc (Gelbart, 1989; Masucci et al., 1990; Posakony et al., 1991). The highest levels of expression observed for these two BMPs not only complement one another in the wing imaginal disc but also in the leg and eye/antennal discs (Figs 3, 4). One implication of the mainly non-overlapping expression domains of gbb-60A and dpp is that these two BMPs may exist predominately as homodimers. Heterodimer formation would only take place in cells where Gbb-60A and Dpp were co-expressed and given the localized expression of dpp RNA this could only occur along the A/P boundary. Further evidence of a role for Gbb-60A as a homodimer is provided by the defects that we observe in gbb-60A null clones in posterior cells, which do not express dpp and do not exhibit a mutant phenotype when null for dpp. The loss of pattern elements within these clones indicates that Gbb-60A must act as a homodimer in these cells.

Ratio of Gbb-60A to Dpp signaling is critical to normal wing morphogenesis

An increasing number of TGF-β/BMP family members have been implicated as key players in directing a number of developmental processes including cell proliferation, patterning and differentiation. Given that multiple TGF-β/BMP members have been found to be expressed within the same developing tissue (see Hogan, 1996; Kingsley, 1994b), it is of interest to understand the specific function of each of these members as well as the mechanism by which they signal. Thus
far, we know very little about the role of more than one ligand in a tissue. In some cases the expression domains overlap, raising the possibility of heterodimer formation and therefore the potential for eliciting a different response than that achieved by the homodimer. Varied responses within a tissue may not only result from homom/heterodimer differences but also from multiple homodimers signaling in an additive or perhaps antagonistic manner. Furthermore, should the signaling from multiple homodimers be synergistic, other responses could be achieved. Empirical evidence to support this possibility has not previously been presented. Rather, in the mouse, it has been suggested that the loss of BMP7 can be compensated for by BMP2 which is expressed in the same tissue (Dudley and Robertson, 1997). In this paper, we provide data indicating that, in addition to the fact that Gbb-60A has a role in wing morphogenesis distinct from that of Dpp, Gbb-60A and Dpp signal together to pattern the adult wing. Both genes are also required earlier in larval development and again the process appears to depend on the presence of both ligands.

In order to investigate the potential interplay between Gbb-60A and Dpp signaling, we examined the possibility that alleles of both genes may genetically interact. The genetic interaction that we observed between dpp and gbb-60A resulted in a change in viability as well as a change in the presence or absence of pattern elements in the adult. Given the nature of the molecules encoded by these genes, the genetic interaction that we observe could be interpreted in several ways.

One interpretation is that gbb-60A somehow modulates dpp activity such that in the presence of Gbb-60A the output of Dpp signaling is boosted. Given the dramatic morphogenetic organizing properties exhibited by dpp and thus far not observed for gbb-60A, we could speculate that the role of gbb-60A may be to merely fine tune the effectiveness of Dpp signaling. A simplistic model to explain such a modulation of Dpp signaling could either be that a different response is attained by the Dpp/Gbb-60A heterodimer versus the Dpp homodimer. Alternatively, the two ligands could signal independently as homodimers and Gbb-60A signaling somehow facilitates Dpp signaling, in which case we would expect the functions of dpp and gbb-60A to be additive.

Another interpretation is that for the most part Dpp and Gbb-60A signal independently and where the signals overlap cells are able to differentiate between the relative levels of these signals, read the relative levels of these two ligands and respond accordingly. The important issue being that the response elicited is dependent on the balance of Dpp to Gbb-60A signaling at that location in the developing tissue. This model would allow for both homodimer and heterodimer signaling as well as the complex response that we observe in the interactions between dpp and gbb-60A loss-of-function alleles, which exhibits aspects of both synergy and antagonism. Dpp and Gbb-60A signaling could be additive in some cells but more importantly a shift in the balance of these ligands would provide new or different positional information.

Our data provide strongest support for the second model based on the following reasons. Genetic interactions between alleles of gbb-60A and dpp were identified by mutant wing phenotypes, which include the loss of pattern elements not normally absent in phenotypes produced by mutations in either gene alone or by the copy number of that mutation alone (Fig. 5). In addition to the more severe wing phenotype, we observed an increase in the degree of lethality of dpp gbb-60A1 + gbb-60A genotypes (Fig. 5I) beyond what would be expected if their signaling were additive. The effect of dpp+ on the percent viability observed for gbb-60A mutants is not limited to alleles of dpp that alter dpp transcriptional levels but is also apparent in genotypes where the reduction of Dpp protein activity is affected (S. S.-T., unpublished data). Therefore, lowering the level of Dpp signaling in a gbb-60A mutant background results in an exacerbation of the degree of lethality normally observed for either genotype alone. The qualitative changes in the wing phenotype of dpp/ gbb-60A interacting genotypes is indicative of the fact that the role of gbb-60A is not merely to enhance the Dpp signal in an additive manner but that the relationship between Dpp and Gbb-60A signaling must be much more complex. If Dpp and Gbb-60A signaling were simply additive, we would predict that in a gbb-60A mutant the effective activity of Dpp would just be lowered and thus the first pattern element to be affected would be those that are normally most sensitive to the loss of dpp, such as the ACV, L2 and L4. This is not the case, as the pattern elements most sensitive to the lowering of gbb-60A are the PCV, L5 and then L4. In addition, as the levels of both dpp and gbb-60A are lowered, we see a restoration of L5 indicating that the ratio of Gbb-60A to Dpp is the important criteria not the absolute levels of both ligands. Furthermore, if Gbb-60A enhanced the Dpp signal, we would expect that increasing both dpp and gbb-60A would lead to phenotypes associated with elevated Dpp signaling. Instead, phenotypes induced by ectopic expression of gbb-60A are partially suppressed by ectopic expression of dpp (Fig. 6) further supporting our proposal that the balance of Gbb-60A and Dpp signaling activity is the mitigating factor in establishing positional information.

**BMP type I receptors, Tkv and Sax, mediate signaling**

We investigated the possibility that BMP type I receptors, Tkv and Sax, mediate the signaling by both Gbb-60A and Dpp. Heteroallelic combinations sax gbb-60A1/+ gbb-60A4 or tkv gbb-60A1/+ gbb-60A4 exhibit more extreme mutant phenotypes than those observed in gbb-60A genotypes alone (Fig. 7). Since Dpp and Gbb-60A signaling is linked in some cases, the tkv/gbb-60A or sax/gbb-60A interactions could reflect an indirect effect of compromising the efficiency of Dpp signaling by lowering the pool of receptors that are known to mediate the Dpp signal. Several aspects of the tkv/gbb-60A genetic interactions suggest that this is not wholly the explanation for the enhanced phenotypes. First, in Df(2L)tkv2 gbb-60A1/+ gbb-60A4 individuals, we observe a severe loss of veins including L3, a pattern element that is never affected in dpp mutants and has been postulated to be determined independently of Dpp signaling (Sturtevant et al., 1997). Second, if the interaction that we observe is a secondary consequence of reducing Dpp signaling, we would expect the viability of the Df(2L)tkv2 gbb-60A1/+ gbb-60A4 genotype to be much reduced as is seen in dpp gbb-60A1/+ gbb-60A4 genotypes (Fig. 5I), yet it is significantly increased. Third, a similar enhancement of the tkv+/Df(2L)tkv2 vein thickening phenotype as induced by gbb-60A1/+ has been observed by increasing, not decreasing, dpp+ through the addition of a dpp transgene (Penton et al., 1994). Thus, different levels of Gbb-60A and Dpp affect signaling through Tkv in dramatically
different ways at different stages of development. These pieces of data do not negate the possibility that Gbb-60A boosts or enhances the Dpp signal at some level and that this is mediated through Tkv in some fashion, perhaps as a heterodimer with higher affinity or by some other as yet unknown mechanism. This possibility is consistent with data suggesting that Tkv mediates a higher level of Dpp signaling (Singer et al., 1997). However, our results indicate that, for some developmental events, it is likely that Tkv mediates the Gbb-60A signal alone and may be responsible for mediating the antagonism between these two ligands observed in some aspects of their genetic interaction. The mechanism by which this differential mediation of signal remains to be elucidated.

Despite the unique roles that dpp and gbb-60A each play, the observed genetic interactions indicate that Dpp and Gbb-60A signaling can be dependent upon one another and that they must signal together in a complex manner involving both synergy and antagonism to achieve normal wing morphogenesis as well as viability. gbb-60A, like dpp, has multiple requirements during development, in addition to its role in growth, patterning and vein promotion in the developing wing. Given the expression pattern of gbb-60A and dpp in other imaginal discs and the phenotypic consequences of their genetic interaction in the adult leg (Fig. 5F), it is likely that gbb-60A and dpp function together in other tissues as well, to direct various developmental programs. The molecular implications of the functional data presented here can be quite straightforward. The complex signaling of gbb-60A and dpp must be mainly accomplished by Gbb-60A and Dpp homodimers, however, some degree of Gbb-60A/Dpp heterodimer signaling can not be eliminated by our data. Regardless, cells in the developing wing are clearly able to interpret different levels of Gbb-60A versus Dpp. Numerous protein/protein interactions at different points in the transduction of TGF-β/BMP signals contribute to the diversity of effects elicited by these signaling molecules. The mechanism responsible for the interpretation of different ligands is unknown and remains to be examined. However, at one level both Tkv and Sax receptors appear to mediate Gbb-60A and Dpp signals and in one scenario a difference in binding affinities for the two different ligands could account for different signaling readouts. Experiments using activated and dominant negative receptors support the notion that Tkv and Sax mediate Dpp and Gbb-60A signaling at differential levels (T. E. Haerry, O. K., M. B. O’Connor and K. A. W., unpublished data). TGF-β receptors are thought to form tetrameric signaling complexes consisting of two type I and two type II receptors, however, as yet it is not known whether two different type I receptors can both contribute to that complex. If this were the case, it is conceivable that Gbb-60A and Dpp could signal through complexes containing both Tkv and Sax. Therefore, various combinations of these ligands and type I receptors could form to elicit different types of output. When considering ligand/receptor interactions it is important to remember that the presence of proteases and other binding proteins may dictate the availability of the ligand to bind to a receptor complex, as well as with what affinity it binds (Lopez-Casillas et al., 1994; Nakato et al., 1995). Having demonstrated that two different BMPs are both required for a single developmental process, we can now begin to dissect the signaling network by which these two ligands accomplish their task.

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