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Drosophila Dpp Signaling Is Mediated by the *punt* Gene Product: A Dual Ligand-Binding Type II Receptor of the TGFβ Receptor Family

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Summary

Signaling by TGFβ-related factors requires ligand-induced association between type I and type II transmembrane serine/threonine kinases. In Drosophila, the *saxophone* (*sax*) and *thick veins* (*tkv*) genes encode type I receptors that mediate signaling by decapentaplegic (dpp), a member of the bone morphogenetic protein (BMP) subgroup of TGFβ-type factors. In this report, we demonstrate that the Drosophila *punt* gene encodes atr-II, a previously described type II receptor that on its own is able to bind activin but not BMP2, a vertebrate ortholog of dpp. Mutations in *punt* produce phenotypes similar to those exhibited by *tkv*, *sax*, and *dpp* mutants. Furthermore, punt will bind BMP2 in concert with tkv or sax, forming complexes with these receptors. We suggest that punt functions as a type II receptor for dpp and propose that BMP signaling in vertebrates may also involve sharing of type II receptors by diverse ligands.

Introduction

Cytokines of the transforming growth factor β (TGFβ) superfamily control a wide range of developmental and physiological functions in higher eukaryotes (reviewed by Massagué et al., 1994). This diverse group of effector molecules modulates immune and endocrine activities; controls tissue specification, growth, and repair processes; and mediates axial patterning events during early embryogenesis. The highest degree of sequence conservation among various family members is found in the C-terminal domain and is centered about a set of similarly spaced cysteine residues. Based on structural and biological similarities, these factors have been traditionally subdivided into at least three distinct subgroups, which include the TGFβs, the activins, and the decapentaplegic (dpp)/bone morphogenetic protein (BMP) family (reviewed by Kingsley, 1994).

In Drosophila, the products of the *dpp*, *screw* (*scw*), and *60A* genes have been shown to be members of the dpp/BMP subgroup (Padgett et al., 1987; Wharton et al., 1991; Doctor et al., 1992; Arora et al., 1994). *dpp* is the best characterized of the three and has served as a paradigm for studying the mechanism of BMP signaling. The dpp ligand plays a number of different roles during Drosophila development. In the early embryo, it appears to act as a morphogen in specifying differential cell fate along the dorsal–ventral axis (Ferguson and Anderson, 1992; Wharton et al., 1993). Later in embryogenesis, *dpp* controls aspects of dorsal mesoderm specification and endoderm morphogenesis (Immergluck et al., 1990; Panganiban et al., 1990; Hursh et al., 1993; Staehling-Hampton et al., 1994). During larval stages, it controls the outgrowth of imaginal tissue and is a key molecule in specifying proximal–distal patterning of adult appendages (Spencer et al., 1982; Basler and Struhl, 1994). The molecular components of the dpp signal transduction machinery are likely to be conserved during evolution. Dpp is ~75% identical to the mammalian BMP2 and BMP4 at the amino acid sequence level, and BMP4 can functionally substitute for dpp (Padgett et al., 1993; Sampath et al., 1993). Therefore, studies of the dpp signaling system are likely to have broad implications for understanding the general principles governing TGFβ signaling.

How cells respond to a diverse set of factors, such as the TGFβ-type ligands, remains a central question in the field. The identification of specific sets of cell surface receptors that signal in response to TGFβ-type ligands represents a crucial first step toward elucidating the mode of action of these factors (reviewed by Attisano et al., 1994). The dimeric ligand appears to associate with two types of transmembrane serine/threonine kinases, known as type I and II receptors, forming complexes whose stoichiometry has not been completely characterized (see Chen and Derynck, 1994; Yamashita et al., 1994). Both receptor components appear to be required for signal transduction since cell lines lacking one or the other receptor for TGFβ or activin do not respond to the ligand unless transfected with the missing receptor (Wrana et al., 1992; Attisano et al., 1993; Carcamo et al., 1994). Phylogenetic comparisons of all receptor serine/threonine kinase sequences reveal that most cluster into two groups. The type I receptors exhibit at least 60% kinase sequence identity among different members. In addition, they share a similar spac-

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ing of cysteine residues in the extracellular domain as well as a highly conserved region rich in glycine and serine residues (the GS box) located just N-terminal to the kinase domain (Wrana et al., 1994b). Type II receptors show more divergence in both kinase sequence and extracellular cysteine spacing and, in contrast with type I receptors, exhibit a C-terminal extension distal to the kinase domain that is rich in serine and threonine residues.

Recent studies on the TGFβ receptor complex suggest that the following sequence of events initiates the TGFβ signal transduction cascade (Wrana et al., 1994a). Receptor II is a constitutively active kinase that appears to be the primary determinant of ligand selection. Once bound to receptor II, TGFβ is recognized by receptor I, which is recruited into the complex and phosphorylated by receptor II at serine and threonine residues within the GS domain. Thus, receptor II is the primary TGFβ receptor, and receptor I is its substrate and downstream signaling component. Ligand-bound receptor II can interact with several different type I isoforms, thereby generating the potential for a multivalent response to a given ligand. Consistent with this model is the observation that, within a given cell type, different biological responses are signaled depending on the particular type I isoform that is engaged in the receptor complex (Carcamo et al., 1994).

At present it is not known whether all aspects of this model will apply to other TGFβ family members. It is likely that the activins follow a parallel mode of receptor activation since their ligand–receptor interaction properties are similar to those described for TGFβ. In the case of the dpp/BMP family, the situation is less clear. Ligand-induced receptor complexes with the potential to signal appropriate biological responses have not yet been identified.

In Drosophila, the products of the *saxophone* (*sax*) and *thick veins* (*tkv*) genes have been implicated in the propagation of the *dpp* signal (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Xie et al., 1994). Mutations in both genes show a similar set of genetic interactions with reduced-function *dpp* mutations, such as maternal enhancement of dorsal patterning defects during early embryogenesis and zygotic enhancement of adult appendage defects. Both genes encode products with the structural properties of type I receptors, and under appropriate conditions, each can bind dpp or BMP2, a functional ortholog of dpp (Brummel et al., 1994; Penton et al., 1994). In addition, like tkv, the mouse Brk-IA and human ALK6 and ALK3 BMP receptors exhibit the unusual property of being able to bind BMP2 in the absence of a type II receptor (Koenig et al., 1994; Penton et al., 1994; ten Dijke et al., 1994). In contrast with the receptor interactions mediated by TGFβ and activin, this observation raises the possibility that formation of some BMP receptor complexes might be guided by the ability of the type I receptor to select ligand.

In this report, we demonstrate that the previously characterized Drosophila *atr-II* (for activin type II receptor) gene, which codes for a type II receptor that by itself binds activin but not BMP2, corresponds to the *punt* gene. Furthermore, we demonstrate that *punt* mutants exhibit phenotypes similar to those exhibited by *sax*, *tkv*, and *dpp* mutations and that the *punt* gene product will bind BMP2 in the presence of tkv or sax. We propose that *punt* is normally involved in dpp signaling and suggest that other activin type II receptors may also have dual ligand binding properties that could enable them to mediate signaling by BMPs as well as activins. Our observation that type II receptors have diverse ligand binding abilities provides an additional mechanism by which cytokines of the TGFβ family can elicit a wide variety of biological responses.

Results

The Atr-II Receptor Is Encoded by *punt*

The *punt* gene was initially identified in a screen for third chromosome zygotic lethal mutations that disrupt embryonic cuticular patterning (Jürgens et al., 1984). The single allele recovered, *punt*^{135}, exhibits a dorsal open phenotype very reminiscent of that produced by certain *tkv* alleles (Nüsslein-Volhard et al., 1984; Terracol and Lengyel, 1994). Deficiency mapping has previously localized *punt* within the 88C3-E2 interval on the right arm of the third chromosome (Jürgens et al., 1984). More recently, we mapped the gene for atr-II, a type II serine/threonine kinase receptor capable of binding activin, to the same region of the chromosome (Childs et al., 1993). Despite the inability of atr-II to bind BMP2, the fact that the *punt* phenotype is very similar to that produced by *tkv*, which encodes a type I BMP receptor (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994), led us to examine whether *punt* was equivalent to *atr-II*. From a collection of single P element insert lines isolated by A. C. Spradling and colleagues of the Drosophila Genome Project, we identified one line, l(3)1046, that contains a P element in the 88C-D interval. Several lines of evidence demonstrate that the P insertion in this line has disrupted the *punt* gene. First, animals homozygous for the P element die with a phenotype similar to *punt*^{135} mutants. Second, the P element insertion fails to complement the *punt*^{135} allele. Third, mobilization of the P element resulted in multiple independent excision lines that are homozygous viable and fertile, indicating that the lethal phenotype of l(3)1046 was caused by the insertion and not by a secondary lesion on the chromosome.

We employed plasmid rescue to clone the P insertion and a small amount of flanking genomic DNA. Probes specific for the flanking sequences were used to establish a walk in the region, and nearby transcripts were identified by Northern blot analysis and cDNA library screening. To identify precisely the point of P insertion, we sequenced from the ends of the P element into flanking DNA. We found that the P element was inserted in the untranslated leader sequence of the *atr-II* transcript 2 bp from the 5′ end (defined as the longest cDNA; Childs et al., 1993). To confirm the identity between the two genes, we generated germline transformants in which the *atr-II* cDNA was driven by the ubiquitin promoter (Brummel et al., 1994). Ubiquitous expression of *atr-II* is able to rescue the lethality of several combinations of *punt* alleles to full viability and renders the females fertile (see Experimental Procedures). Taken together, these data indicate that *atr-II* is *punt*, and we will henceforth refer to the atr-II receptor as punt.

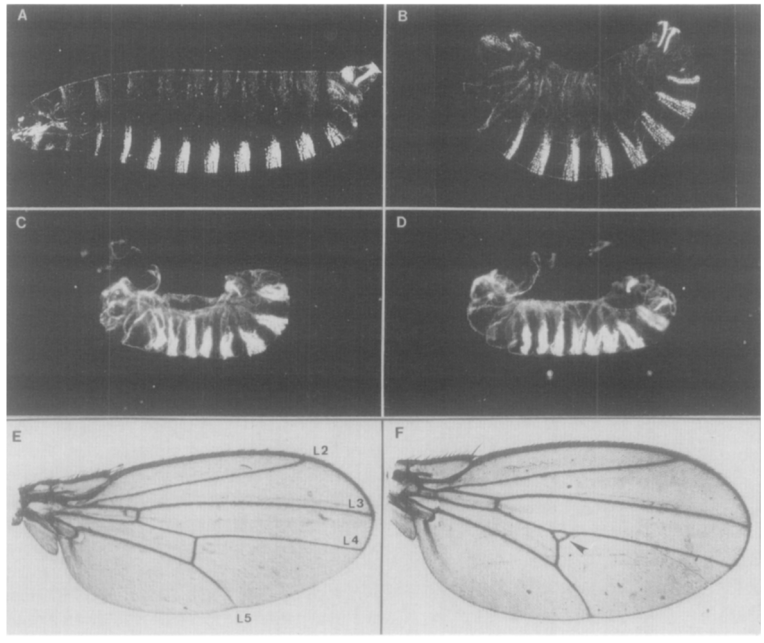

Figure 1. Zygotic Mutations in *punt* Affect Embryonic Patterning and Wing Venation

Anterior is to the left and dorsal side is up in (A)–(D). *punt* alleles show temperature-sensitive defects in embryonic patterning. Cuticle of a wild-type embryo (A) compared with a homozygous $punt^{P1}$ mutant at 22°C (B) and a homozygous $punt^{135}$ mutant at 29°C (C). The larva in (B) shows defects in head involution, such that the anterior segments often remain attached to the thorax on the dorsal side. At nonpermissive temperatures these embryos show the dorsal open phenotype (C). The dorsal cuticle is incomplete, and the gut extrudes through the dorsal opening. Similar defects in embryonic patterning are caused by zygotic loss of *tkv*, illustrated by the tkv^{8} embryo in (D). Escaper adults of the genotype $punt^{135}/punt^{88}$ recovered at 18°C (F) show ectopic wing venation compared with wild-type wings in (E).

punt Is Required for Proper Embryonic Patterning and Adult Development

Wild-type embryos secrete cuticle that displays a characteristic array of dorsal and ventral pattern elements (Figure 1A). Cuticular analysis of $punt^{135}/punt^{135}$ and $punt^{P1}/punt^{P1}$ (insertion mutation in l(3)1046) mutants at varying temperatures revealed that *punt* lethality is temperature sensitive. Although defects in dorsal cuticle were observed at several temperatures, the large holes characteristic of dorsal closure mutants such as *tkv* were observed at high frequency only at temperatures of 25°C or greater (Figures 1C and 1D). In contrast, defects in head involution coupled with smaller holes in the anterior dorsal region of the cuticle were observed at 22°C (Figure 1B). At 18°C, the lethal phenotype consisted of only minor defects in the head skeleton.

Several *punt* alleles, including $punt^{51}$ and $punt^{88}$, were isolated in a screen for lethal excisions of the P element in l(3)1046 (M. Horner and A. L., unpublished data). Southern blot analysis revealed that both lines were associated with internal deletions of the P element (data not shown). At 22°C, heteroallelic combinations of $punt^{P1}$ or $punt^{135}$ with the new *punt* alleles results in the embryonic lethal phenotype depicted in Figure 1B. At 18°C, it is possible to recover heteroallelic $punt^{88}/punt^{135}$ individuals at close to the expected Mendelian frequency (Table 1). These adults are nearly indistinguishable from wild type. However, a closer examination of $punt^{88}/punt^{135}$ adults raised at 18°C revealed that 10% (12 of 119) display ectopic wing venation in the region of the posterior cross vein (Figures 2E and 2F). At higher temperatures (22°C–23°C), viability of $punt^{88}/punt^{135}$ flies was vastly reduced, and their phenotype was often grossly abnormal. Aberrant phenotypes included a reduction in wing size and the presence of cleft notums similar to those produced by certain *tkv* and dpp^{d} alleles (Spencer et al., 1982; Segal and Gelbart, 1985; Terracol and Lengyel, 1994). No $punt^{88}/punt^{135}$ adults were recovered at 25°C. The viability of *punt* heteroallelic adults at low temperature was not allele specific. Similar results were obtained when we measured the viability of $punt^{51}/$

Table 1. Temperature Sensitivity of *punt* Heteroalleleic Combinations

Temperature	$punt^{88}/TM3, Ser$	$punt^{135}/MKRS$	$punt^{88}/punt^{135}$	Number Counted
18°C	236 (40)[a]	152 (26)	196 (33)	584
20°C	41 (39)	26 (25)	38 (36)	105
22°C	253 (45)	235 (42)	74 (13)	562
23°C	949 (59)	650 (41)	3 (<1)	1602
25°C	99 (50)	100 (50)	0 (0)	584
	$punt^{51}/TM3, Ser$	$punt^{135}/MKRS$	$punt^{51}/punt^{135}$	
18°C	129 (45)	48 (17)	110 (33)	287
22°C	39 (56)	25 (36)	6 (18)	70

[a] The numbers in parenthesis indicate the percentage of the total progeny. Because the *TM3, Ser/MKRS* progeny are not viable, the expected frequency for each class is 33.3%.

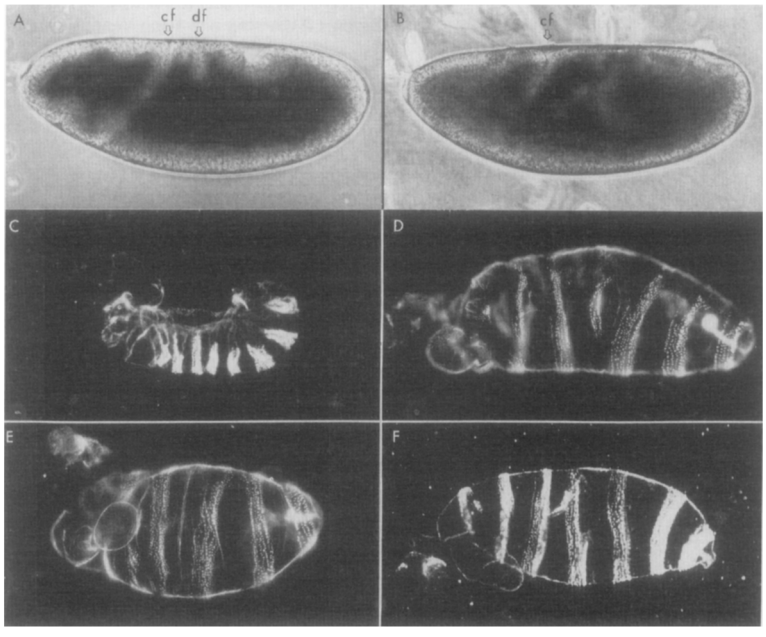

Figure 2. Loss of Maternal *punt* Activity Results in a Ventralized Phenotype

(A and B) Anterior is to the left. Phase-contrast optics of wild-type (A) and mutant (B) embryos during early stages of germband extension (stage 7; Campos-Ortega and Hartenstein, 1985). Embryos lacking maternal *punt* function were derived from escaper females of the genotype $punt^{135}/punt^{88}$ crossed to $punt^{135}/punt^{88}$ males. In the mutants (B), the cephalic furrow (cf) is shifted dorsally, the dorsal folds (df) are reduced or missing, and the germband extends beneath the surface of the embryo. These features are characteristic of ventralized embryos. (C–F) Dark-field images of mutant larvae. The embryos in (D) and (E) were generated by inducing *punt* mutant germline clones in an ovo^{D} background by γ-irradiation. Embryos from $punt^{88}$ germline clones fertilized with a wild-type sperm have a partially ventralized phenotype (D) compared with the dorsal open zygotic phenotype of *punt* mutants (C). Embryos from $punt^{88}$ clones fertilized by *punt* mutant sperm (E) show a severe ventralized phenotype also exhibited by dpp^{H46}-null mutants (F). The loss of dorsal pattern is accompanied by an expansion of ventral denticle belts. The terminal segments are displaced into the interior of the embryo owing to defects in germband movement.

$punt^{135}$ adults at 18°C (Table 1). Since all the alleles we have tested exhibit a temperature-sensitive phenotype, it is possible that the *punt* gene product is involved in an inherently temperature-sensitive pathway. Alternatively, it is possible that the alleles examined represent regulatory mutants that retain residual *punt* activity at low temperature.

Maternal and Zygotic Loss of *punt* Ventralizes the Embryo

We have previously demonstrated that the *punt* transcript is supplied maternally as well as zygotically (Childs et al., 1993). To examine whether maternal and zygotic loss of *punt* results in more severe embryonic patterning defects than zygotic loss alone, we analyzed eggs laid by *punt* homozygous females recovered at 18°C, and we also employed mitotic recombination to induce *punt* germline clones. Both analyses demonstrated a requirement for maternal *punt* gene product in dorsal–ventral patterning. When $punt^{88}/punt^{135}$ females recovered at 18°C are mated with $punt^{88}/punt^{135}$ males and subsequently shifted to the restrictive temperature (25°C), they produced severely ventralized embryos. In contrast with wild type, embryos lacking both the maternal and zygotic *punt* gene product gastrulate abnormally (Figures 2A and 2B). In embryos derived from $punt^{135}/punt^{88}$ matings, the dorsal folds are reduced in size and number, the cephalic furrow is shifted dorsally, and germband extension is defective. Similar gastrulation defects are exhibited by a number of mutants in dorsal–ventral patterning genes, including *sax*, a type I receptor (Xie et al., 1994). Such defects result from a transformation of the dorsal amnioserosa cells to more ventral fates (Arora and Nüsslein-Volhard, 1992). To confirm that *punt* is required maternally for specification of amnioserosa, we employed antibodies directed against the amnioserosal cell marker Krüppel. No cross-reactivity was detected in mutant embryos derived from $punt^{135}/punt^{88}$ parents, indicating that these embryos lack the dorsal-most tissue (data not shown). Loss of amnioserosa is also a feature of mutations in *dpp*, *sax*, and *tkv* (Wharton et al., 1993; Brummel et al., 1994; Nellen et al., 1994).

Analysis of germline clones revealed that only about 50% of the embryos derived from a female carrying a $punt^{88}$ clone that was mated with $punt^{88}$ heterozygous males showed a severely ventralized cuticle, as evidenced by the circumferential presence of ventral dentical belts (Figures 2E). These cuticles are similar to those secreted by *dpp*-null (Figure 2F) and *tkv*-null mutant embryos (Nellen et al., 1994; Terracol and Lengyel, 1994). The remaining embryos showed a moderately ventralized cuticle that is nevertheless stronger than the zygotic dorsal open phenotype (Figures 2C and 2D). It is likely that the most severe phenotype results from eggs fertilized by *punt* mutant sperm, while the moderate phenotype represents the eggs fertilized by $punt^{+}$ sperm. This was confirmed by examining embryos derived from females carrying a $punt^{88}$ germline clone mated with (+/+) males. All embryos showed a moderately ventralized phenotype.

The *punt* Gene Is Required for Dpp Signaling

In addition to its function at other stages of development, *dpp* plays a well-characterized role in regulating the morphogenesis of the embryonic midgut. Expression of *dpp* at two locations in the gut mesoderm (parasegments 3 and 7 [PS3 and PS7]) is subject to positive autoregulation and thus provides a sensitive assay for disruptions in dpp signaling (Figure 3A; Panganiban et al., 1990; Hursh et

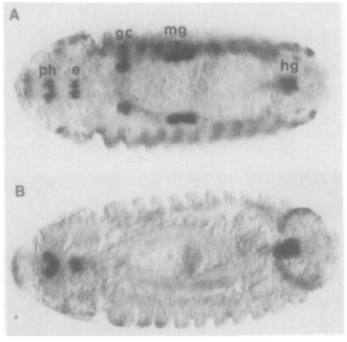

Figure 3. Mutations in *punt* Affect *dpp* Autoregulation

Wild-type (A) and $punt^{135}$ homozygous embryos raised at 22°C (B) stained with *dpp* riboprobes. *dpp* RNA was detected in discrete domains along the developing gut tube in the pharynx (ph), esophagus (e), gastric caeca primordia (gc), PS7 region of the midgut (mg), and hindgut (hg) in wild-type embryos (A). In $punt^{135}/punt^{135}$ embryos, *dpp* RNA was not detected in the gastric caeca or in the midgut, but could be seen in the pharynx, esophagus, and hindgut.

al., 1993; Capovilla et al., 1994; Staehling-Hampton and Hoffmann, 1994). In *punt* mutants, *dpp* transcripts were not detected either in the primordia of the gastric caeca (PS3) or in the PS7 region of the midgut mesoderm (Figure 3B). *dpp* expression in PS7 is necessary for the expression of *labial* (*lab*) in the endodermal cells that lie beneath the visceral mesoderm (Immergluck et al., 1990; Panganiban et al., 1990; Reuter et al., 1990). The level of lab protein in the endoderm of *punt* mutants is considerably reduced (data not shown). Despite the absence of *dpp* transcripts in PS3 and PS7, *dpp* expression in regions of the embryo that are not subject to autoregulation were unaffected. Mutant alleles of *tkv* and *sax* also affect gut development and show similar alterations in the expression of *dpp* and *lab* (Affolter et al., 1994; Penton et al., 1994; Nellen et al., 1994).

Genetic Interactions between Type I and Type II Receptor Mutants

The phenotypic analysis strongly suggests that *punt* encodes a type II dpp receptor. In an effort to determine whether punt acts in concert with either sax or tkv, two dpp type I receptors, we examined *trans*-heterozygous combinations of *punt* and *tkv* or *punt* and *sax* mutants to look for enhanced phenotypes. Several phenotypic observations support a possible functional interaction between *punt* and *sax*. First, the introduction of one mutant *punt* allele into a *sax* mutant background causes wing venation defects in adults (Figures 4A–4C). In 35%–50% of these individuals, the wings lack the posterior cross vein (Figure 4B), and in 10% of the cases, the longitudinal wing vein L4 fails to reach the wing margin (Figure 4C). Second, progeny of some *sax–punt* transheterozygous females (*sax/+; punt/+*) exhibit a high degree of lethality when crossed to wild-type males. Progeny of females heterozygous for the $punt^{135}$ allele in combination with either sax^1 or sax^2, when crossed to wild-type males, exhibit 64% and 87% lethality, respectively (Figure 4D). Of this lethality, 75% is larval, and the phenotypes vary from apparently

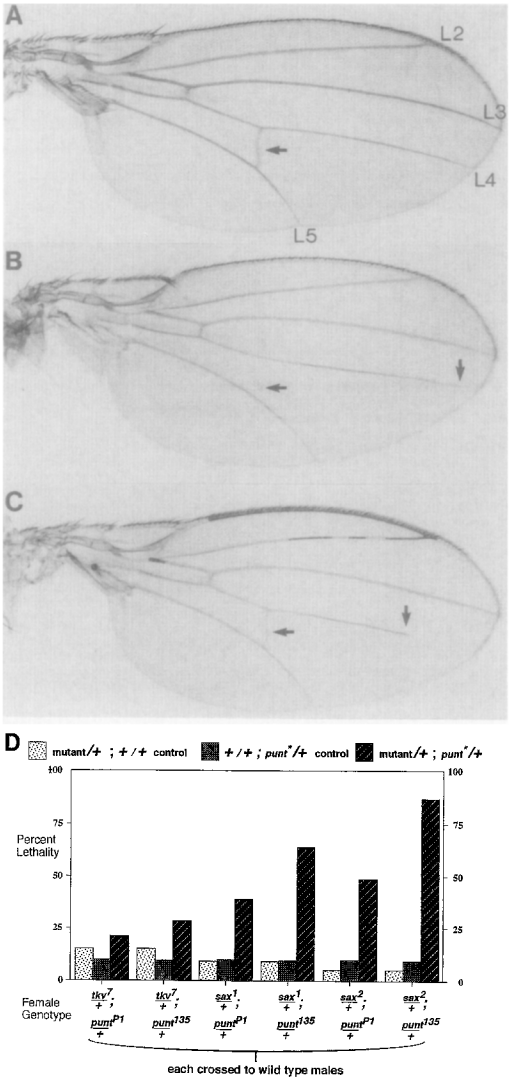

Figure 4. Genetic Interactions between *punt* and *sax*

(A) sax^1/sax^2 individuals exhibit nearly wild-type wings. A low frequency (~1%) of these adults display a mild reduction in the posterior cross vein (horizontal arrow). The longitudinal wing veins L2–L5 are indicated.

(B and C) Of sax^1/sax^2; $punt^{135}/+$ individuals, 35%–50% lack the posterior cross vein (horizontal arrow), and in 10% of the individuals, L4 fails to reach the wing margin (vertical arrows).

(D) Progeny of females double heterozygous for *sax* and *punt* mutations exhibit significant lethality. Females of the genotype listed below the bar graph triplets were crossed to wild-type males, and the progeny were scored for viability. The first bar in the triplet (stippled) represents the lethality observed in progeny of heterozygous type I receptor mutant females; the second bar (cross-hatched) is the lethality observed in progeny of heterozygous *punt* mutant females; the third bar (hatched) is the lethality observed in progeny of double heterozygous mutant females (type I receptor mutant/+; *punt/+*).

wild-type to individuals with disorganized trachea and defective imaginal discs (data not shown). Control crosses clearly indicate that this lethality is not merely an additive effect of the *sax* and *punt* mutations. These effects are only seen in combinations with the sax^1 or sax^2 alleles, which contain lesions in their kinase domains, and were

induced on different parental chromosomes. The same *punt* allele displays a much weaker interaction in combination with either a *sax* deficiency or a null allele (*Df(2R)H23* or sax^{1rv5}) and exhibits 9% and 27% lethality, respectively. Since the sax^{1rv5} allele was derived from sax^{1}, we conclude that the interaction is specific for *sax* and is not caused by another lesion on the sax^{1} chromosome. In this assay, the tkv^{7} allele does not display any significant interaction with *punt* mutants. While the above observations do not provide direct evidence of protein–protein interaction, the results are consistent with the formation of a heteromeric dpp receptor complex containing the punt and sax products.

BMP2 Promotes Formation of Heteromeric Receptor Complexes between Punt and Tkv or Punt and Sax

To determine whether punt binds dpp and forms complexes with tkv or sax, we expressed receptors transiently in monkey COS1 cells. To facilitate the identification and purification of receptor complexes, punt was tagged with a hexahistidine sequence (His) while tkv and sax were tagged with the hemagglutinin epitope (HA). His sequences bind efficiently to Ni^{2+}–NTA resin, while the HA epitope is specifically recognized by the monoclonal antibody 12CA5. Cells transfected with tagged receptors alone or in combination were incubated with ^{125}I–BMP2, the mammalian dpp ortholog, and receptor-bound ligand cross-linked using disuccinimidyl suberate.

As previously described (Penton et al., 1994), immunoprecipitates obtained from affinity-labeled cells transfected with HA-tagged tkv contained a product of ~80 kDa that corresponds to ^{125}I–BMP2 cross-linked to HA-tagged tkv (Figure 5A). Analysis of lysates from cells cotransfected with His-tagged punt and HA-tagged tkv revealed the presence of an additional affinity-labeled product of 75 kDa that coprecipitated with HA-tagged tkv and corresponded to the expected size for affinity-labeled His-tagged punt. Furthermore, both of these affinity-labeled products were also isolated when lysates from cotransfected cells were incubated with Ni^{2+}–NTA–agarose to purify His-tagged punt specifically. Consistent with our previous observations (Childs et al., 1993), no products affinity labeled with BMP2 could be isolated from cells transfected with punt alone. This suggests that punt alone has low affinity for BMP2 but binds BMP2 efficiently when engaged in a heteromeric complex with tkv.

To determine whether punt also interacts with sax to form a heteromeric BMP receptor complex, we performed similar experiments using HA-tagged sax (Figure 5B). As shown previously, sax expressed alone in COS cells bound BMP2 only weakly (Brummel et al., 1994). However, when coexpressed with punt, anti-HA immunoprecipitates revealed increased affinity labeling to HA-tagged sax. Since His-tagged punt and HA-tagged sax were incompletely resolved on SDS–polyacrylamide gels, we purified His-tagged punt from lysates of cells affinity labeled with ^{125}I–BMP2 using Ni^{2+}–NTA–agarose. Analysis of these purified extracts clearly revealed the presence of affinity-labeled His-tagged punt in cells cotransfected with both punt and sax. Thus, punt and sax, which separately display low affinity for BMP2, can cooperate to form a high affinity receptor complex. Together these data indicate that punt functions as a dpp/BMP2 receptor when it is coexpressed with either tkv or sax and that punt forms a heteromeric complex with either of the dpp type I receptors, tkv or sax.

Discussion

punt May Act as a Dual Specificity Receptor

We originally cloned *punt* (*atr-II*) by low stringency hybridization to ActR-II, a mouse activin receptor (Childs et al., 1993). Sequence and phylogenetic comparisons between type II receptors initially suggested that punt was an activin receptor. Within the kinase domain, punt shows >60% identity to other activin type II receptors. In contrast, it shows <40% identity in the kinase region to TGFβ type II receptors or to DAF-4, a previously identified type II receptor capable of binding BMP2 (Estevez et al., 1993). In addition, punt also behaves as an activin type II receptor with respect to its ligand binding properties. In the absence of other receptors, punt is able to bind activin A with a specificity and affinity comparable to mammalian activin type II receptors (Childs et al., 1993). In the presence of activin, punt is also able to form a heteromeric complex

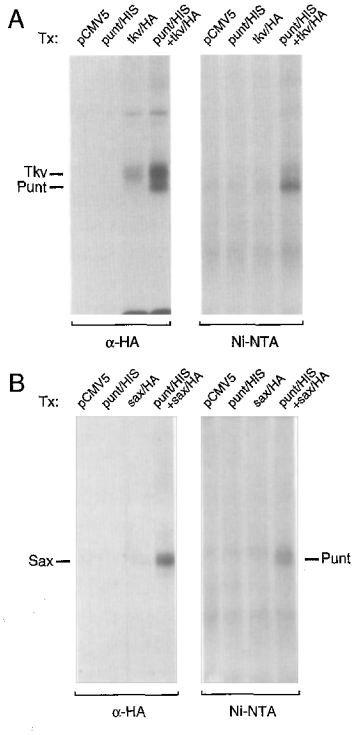

Figure 5. The Punt Receptor Binds BMP2 and Forms Heteromeric Complexes with Tkv and Sax

COS cells were transiently transfected with the indicated cDNAs and affinity labeled by sequential incubation with 500 pM ^{125}I–BMP2 and disuccinimidyl suberate. Aliquots of cell lysate were subjected to immunoprecipitation with the anti-HA antibody 12CA5 (α-HA) or purified with Ni^{2+}–NTA–agarose (Ni–NTA) followed by SDS–polyacrylamide gel electrophoresis and autoradiography. The position of the affinity-labeled products tkv and punt are indicated in (A), while in (B) punt and sax are indicated.

with the atr-I product, a Drosophila type I receptor of unknown function (Wrana et al., 1994b). A striking aspect of its ligand binding properties, however, is the inability of punt to bind BMP2 in the absence of other receptors. This is in direct contrast with DAF-4, which is able to bind BMP2 quite efficiently in the absence of a type I partner (Estevez et al., 1993).

The sequence similarities and ligand binding properties of punt initially led us to suggest that punt was an activin receptor (Childs et al., 1993). The results presented in this paper suggest that in vivo ligands for punt include dpp and perhaps other members of the BMP family. Our reasons are several fold. First, punt will bind BMP2 when combined with an appropriate type I receptor. Second, mutations in *punt* interfere with aspects of Drosophila development that require dpp signaling. In particular, maternal and zygotic loss of *punt* results in severely ventralized embryos like those produced by null *dpp* mutations. These observations indicate a requirement for punt in patterning the ectoderm and strongly suggest that dpp is a functional ligand for punt. This is consistent with the observation that zygotic loss of *punt* interferes with *dpp* transcription in the gastric caeca and in PS7 of the midgut. In these regions, *dpp* transcription is subject to positive autoregulatory feedback (Immergluck et al., 1990; Panganiban et al., 1990; Reuter et al., 1990; Capovilla et al., 1994; Hursh et al., 1993; Staehling-Hampton and Hoffmann, 1994). Another zygotic consequence of *punt* mutations is an inability to undergo proper dorsal closure. This phenotype is identical to that of zygotic mutations in *tkv*, a type I dpp receptor (Penton et al., 1994; Terracol and Lengyel, 1994). Presumably maternal contribution of both *punt* and *tkv* allows the completion of early cell fate specification events that are essential for dorsal development, and it is only when the maternal products are depleted that additional requirements for dpp function during late embryogenesis are revealed. Another event compromised by both *punt* and *dpp* mutations is the patterning of imaginal disc derivatives. Homozygous *punt* mutants that develop at semipermissive temperature produce adults with wing venation and thoracic defects similar to those produced by certain alleles of *tkv* (Terracol and Lengyel, 1994) and *dpp* (Spencer et al., 1982). Finally, the genetic interactions observed between *punt* and *sax* mutants are consistent with the involvement of punt in mediating the dpp signal.

Although the above observations argue that *punt* is involved in mediating certain aspects of dpp signaling, we cannot exclude the possibility that *punt* may also mediate signaling by other members of the TGFβ family, including an as-yet-unidentified activin-like factor. While there is no suggestion from the *punt* mutant phenotype for involvement of another ligand, we have not determined whether any of the *punt* alleles are null. Preliminary molecular analysis of mutant alleles suggests that they may be regulatory mutants. It is possible that these alleles still retain some function and only exhibit *dpp*-like phenotypes because the *dpp* pathway is the most sensitive to reduction in receptor activity. The only other identified TGFβ-type ligands in Drosophila, the products of the *60A* and *scw* genes, are both members of the BMP subfamily (Wharton et al., 1991; Doctor et al., 1992; Arora et al., 1994). Like *dpp*, mutations in *scw* also disrupt dorsal patterning (Arora and Nüsslein-Volhard, 1992; Arora et al., 1994). It is possible that punt may also mediate signaling by scw or perhaps a heterodimer of scw and dpp. We think it is unlikely that punt acts solely as a scw receptor since the loss of maternal and zygotic *punt* function results in a more severe phenotype than that of null *scw* mutations.

Dpp Receptor Complexes: Implications for BMP and Activin Signaling

An interesting feature of the Drosophila dpp receptor system that may extend to BMP signaling in other organisms is the partial reversal of ligand binding roles for the type I and type II receptors compared with the TGFβ and activin receptors. Type II receptors for TGFβ and activin can bind ligand directly (Mathews and Vale, 1991; Attisano et al., 1992) whereas punt binds BMP2 efficiently only when coexpressed with sax or tkv. Likewise, type I TGFβ receptors can contact ligand bound to type II receptors but not free ligand, while tkv, a BMP type I receptor, can bind free ligand directly in the absence of a type II receptor. This property is also shared by the mammalian BMP type I receptors BMPR-IA/Brk-I and BMPR-IB, which bind BMP2, BMP4, and BMP7 in the absence of a cotransfected type II receptor (Koenig et al., 1994; Penton et al., 1994; ten Dijke et al., 1994), and suggests that the reversal of ligand binding properties of type I and type II BMP receptors may not be peculiar to Drosophila.

Despite these differences, the present results clearly show a dependence of dpp type I receptors on punt for binding ligand. Coexpression of punt enhances ligand binding to tkv and is essential for ligand binding to sax. Our phenotypic studies are also consistent with the possiblity that punt interacts physiologically with sax and tkv. In the TGFβ and activin receptor systems, the type II receptors are constitutively active kinases that phosphorylate the GS domain of type I receptors in the ligand-induced complex, initiating a signaling pathway in which the type I receptors are downstream components. This mode of activation may also apply to dpp/BMP receptors except that, in this case, both receptors may be required to generate a high affinity binding site whereas activin and TGFβ binding is specified by their respective type II receptors. It may be that for many BMP family members, ligand selection is principally dictated by the type I receptor or shared by both receptors, as illustrated by the sax and punt combination, neither of which will bind BMP2 efficiently on their own.

The ability of DAF-4 to bind BMP2 on its own suggests that not all BMP receptors have reversed binding characteristics relative to the TGFβ paradigm. However, it should be noted that the physiological ligand for DAF-4 and its normal receptor I partner are currently unknown, making it difficult to establish the relevance of this interaction. We have found that although DAF-4 is able to complex with tkv and sax in the presence of BMP2 in tissue culture cells (Brummel et al., 1994; Penton et al., 1994), in transgenic flies it is unable to rescue *punt* mutations to viability (G. Marqués and M. B. O., unpublished data). This result may

indicate that the type of complex formed among BMP2, DAF-4, and various type I BMP receptors is nonfunctional and emphasizes the necessity for correlating inferences based on sequence homologies and in vitro binding data with in vivo function.

A third type I receptor identified in Drosophila, atr-I, also interacts with punt in vitro. Both isoforms of atr-I have binding properties of typical activin type I receptors and form activin-induced complexes with punt (Wrana et al., 1994b). The possibility of a similar interaction in vivo has not been confirmed since neither the ligand nor *atr-I* mutations are available. The ligand is probably not dpp, since BMP2 is not able to induce complex formation between punt and atr-I (J. L. W. and J. M., unpublished data). Nonetheless, the ability of punt to bind activin in the absence of a type I receptor suggests that some of the previously identified activin type II receptors could actually function as type II receptors for BMPs or perhaps be components common to signaling by both classes of ligands. Based on amino acid sequence similarity, the members of the TGFβ superfamily fall into small clusters of closely related isoforms progressively divergent from the dpp/BMP2 cluster. Although the BMPs, activins, and TGFβs conceptually represent distinct subfamilies, some degree of cross-reactivity in their receptor interactions is conceivable. If some type I and type II receptors have the potential to function in several pathways, then the ensemble of possible receptor–ligand complexes can become increasingly elaborate. Such heterogeneity may help explain how a multitude of cellular responses can be elicited by various members of the TGFβ family.

Experimental Procedures

Drosophila Strains

Drosophila strains were cultured on standard cornmeal yeast extract dextrose medium. Wild type is Canton S or *cn; ry*. The P element insertion mutation $punt^{P1}$ in l(3)1046 was generated in a screen for P element lethals (G. Karpen and A. C. Spradling of the Drosophila Genome Project). Additional *punt* mutants were isolated in a screen for lethal excisions of the P element transposon in l(3)1046 (M. Horner and A. L., unpublished data). The $rosy^+$-marked P element in l(3)1046 was mobilized with an external source of transposase (Robertson et al., 1988). P element excision lines were identified by the absence of the $rosy^+$ eye color marker. Two of the excision lines that failed to complement $punt^{135}$ were designated $punt^{51}$ and $punt^{88}$. sax^{1rv5} was generated by V. T. and W. M. G. (unpublished data). All other strains have been described in Lindsley and Zimm (1992).

DNA Isolation and Sequencing

P element rescue was performed as described in Ashburner (1989). Genomic DNA was isolated from the l(3)1046 fly line, digested with XbaI, and ligated. Recombinant plasmids were selected on kanamycin media. Phage clones spanning the insertion site were isolated using standard techniques (Maniatis et al., 1982). Drosophila genomic libraries were provided by Dr. J. Tamkun (University of California, Santa Cruz) and purchased from Stratagene. cDNAs were isolated from a 8–12 hr embryonic library (Brown and Kafatos, 1988).

dpp Transcript Localization

Distribution of *dpp* message in embryos was determined using antisense riboprobes labeled with digoxigenin–UTP (Boehringer Mannheim) as described previously (Arora et al., 1994).

P Element Construction and Transformation Rescue

The P[*ubi–punt*] plasmid was constructed by filling in the SacII–HindIII fragment of *punt* and blunt end ligating it to the unique XbaI site in P[*ubi–CaSpeR*] (Brummel et al., 1994). $Df(1)w, yw^{67c23}$ stock was used as a recipient for germline transformation (Rubin and Spradling, 1982). For rescue experiments, strains carrying a transgene on the X or the II chromosome were used. Both lines were male sterile. Female flies of the genotype $punt^{P1}/TM2$ carrying a single copy of the transgene were crossed to $punt^{P1}/TM2$, $punt^{135}/TM3$, or $punt^{51}/TM2$ males. Progeny from this cross were scored for the survival of *punt/punt* homozygous adults at 25°C. Observed survival was 116%–152% of expected and depended on the transgenic line used.

Mammalian Cell Transfections

For transient transfection assays, monkey COS1 cells were transfected with receptor cDNAs subcloned into the mammalian transfection vector pCMV5 using DEAE–dextran as previously described (Attisano et al., 1992). Cells were trypsinized 24 hr after transfection and reseeded into 60 mm dishes and subsequently assayed for ligand binding 48 hr posttransfection. An influenza virus HA epitope (amino acids YDVPDYASL) in sax and tkv and a His tag in punt were introduced at the C-terminus as previously described (Attisano et al., 1993).

Binding and Affinity Labeling

Human recombinant BMP2 (gift from J. Wozney, Genetics Institute) was iodinated by the chloramine T method (Frolik et al., 1984). Transfected COS1 cell monolayers were affinity labeled with ^{125}I–BMP2 and disuccinimidyl suberate (Pierce Chemical) as in Attisano et al. (1992). Labeled cell monolayers were solubilized in lysis buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 0.5% Triton X-100) in the presence of protease inhibitors at 4°C for 20–30 min and centrifuged to remove debris. For nickel chromatography, cell extracts were made to 20 mM imidazole and incubated with Ni^{2+}–NTA–agarose (Qiagen) for 1 hr at 4°C and were rinsed briefly three times, then twice for 5 min in 20 mM imidazole in lysis buffer, and twice in 20 mM Tris (pH 7.4). Bound proteins were eluted by boiling in SDS sample buffer. For immunoprecipitations, cell extracts were incubated with monoclonal antibody 12CA5 (BABCO) for 1–2 hr at 4°C and collected on protein A–Sepharose beads (Pharmacia). The immunoprecipitates were washed five times in cold lysis buffer and then resuspended in sample buffer for analysis by SDS–PAGE and autoradiography.

Germline Clones

Homozygous mutant germline clones were generated in the background of a dominant female sterile ovo^D mutation. *yw;* P[ovo^{D1}] C13X3/*TM3* males (Chou et al., 1993) were crossed to $punt^{88}/MKRS$ females. Second instar larvae from this cross were harvested and irradiated with 1000 rads. Virgin females of the genotype $punt^{88}$/P[ovo^{D1}] C13X3 were mated to $punt^{88}/MKRS$ or $punt^{P1}/MKRS$ males. Females carrying a single copy of ovo^{D1} do not lay eggs unless a mitotic recombination event in the germline results in a homozygous mutant clone. A total of nine egg-laying females were recovered from among 500 females screened. Of these, one was a distal recombination event and therefore excluded from analysis. The remaining eight females carried a $punt^{88}/punt^{88}$ germline clone.

Lethal Phase Studies

Two sets of crosses were done. In one set, virgin females heterozygous for *punt* and either *sax* or *tkv* were crossed to Canton S males. In a second set, virgin females heterozygous for either *sax* or *tkv* were crossed to mutant *punt* males. Virgin females of the genotype described in Figure 4D were collected from the progeny, and 3-day-old virgins were crossed to *cn; ry* males. Embryos were harvested after 12 hr of egg laying, gridded on food medium bottles, and then placed at 25°C. The number of fertilized eggs examined were as follows: sax^2, n = 254; tkv^7/+; $punt^{P1}$/+ and tkv^7/+; $punt^{135}$/+, n ⩾ 421; sax^2/+; $punt^{P1}$/+ and sax^2/+; $punt^{135}$/+, n ⩾ 676; and for the remainder of genotypes, n ⩾ 1237. Embryonic viability was assessed at 36 hr after egg laying and larval viability at 10–15 days after egg laying. Adult eclosion was assessed for up to 18 days after egg laying.

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