Receptor serine/threonine kinases implicated in the control of Drosophila body pattern by decapentaplegic

Nellen, D; Affolter, M; Basler, K
Receptor serine/threonine kinases implicated in the control of Drosophila body pattern by decapentaplegic

Abstract

Members of the TGF beta superfamily of secreted signaling molecules regulate growth and cellular patterning during development and interact with specific type I and type II membrane receptors possessing a cytoplasmic serine/threonine kinase domain. We describe two members of the type I receptor family in Drosophila and demonstrate that they are encoded by the genes saxophone (sax) and thick veins (tkv). Further, we show that mutations that abolish sax or tkv activity cause phenotypes similar to partial or complete loss of activity, respectively, of the TGF beta homolog decapentaplegic (dpp). We propose that specification of distinct cell fates in response to different concentrations of dpp may be achieved combinatorially by the sax and tkv receptors.
Receptor Serine/Threonine Kinases Implicated in the Control of Drosophila Body Pattern by decapentaplegic

Denise Nellen,* Markus Affolter,1 and Konrad Basler*
*Zoologisches Institut
Universität Zürich
8057 Zürich
Switzerland
1Biozentrum
Universität Basel
4056 Basel
Switzerland

Summary

Members of the TGFβ superfamily of secreted signaling molecules regulate growth and cellular patterning during development and interact with specific type I and type II membrane receptors possessing a cytoplasmic serine/threonine kinase domain. We describe two members of the type I receptor family in Drosophila and demonstrate that they are encoded by the genes saxophone (sax) and thick veins (tkv). Further, we show that mutations that abolish sax or tkv activity cause phenotypes similar to partial or complete loss of activity, respectively, of the TGFβ homolog decapentaplegic (dpp). We propose that specification of distinct cell fates in response to different concentrations of dpp may be achieved combinatorially by the sax and tkv receptors.

Introduction

During development, single cells or groups of cells adopt distinct fates that depend on their position within a larger cell population. One way spatial information could be established is for a regulatory substance (or morphogen) to be distributed in a graded manner such that cells at different positions are exposed to different concentrations of that substance (Crick, 1970; Lawrence, 1972; Tickle et al., 1995). Such a gradient mechanism requires that cells measure the local concentration of the morphogen and respond appropriately.

The best described model system in which gradients organize body pattern is the subdivision of the anteroposterior axis of the early Drosophila embryo (reviewed by St Johnston and Niisslein-Volhard, 1992). The bicoid and hunchback proteins, for example, are distributed in a graded manner such that cells at different positions are exposed to different concentrations of that substance (Crick, 1970; Lawrence, 1972; Tickle et al., 1995). Such a gradient mechanism requires that cells measure the local concentration of the morphogen and respond appropriately.

To understand how cells discriminate among different concentrations of dpp, we have sought to define components of the dpp signal transduction pathway. In particular,
we have concentrated on identifying putative receptors for dpp, based on the assumption that they would be members of the same kinase gene family as the recently cloned TGFβ and activin receptors. We describe the identification of two transmembrane STKs in Drosophila that are structurally homologous to TGFβ and activin type I receptors and show that they are encoded by the previously defined genes saxophone (sax) and thick veins (tkv). Further, we show that sax is required for the specification of the dorsal-most structure of the embryo, the amnioserosa, whereas tkv is required for patterning the entire domain of the presumptive embryonic ectoderm normally specified by dpp. These results suggest that both kinases may function in vivo as type I dpp receptors. Moreover, the differential requirement for the two receptor STKs suggests that the interpretation of different dpp activity levels may be achieved by the combinatorial use of both receptors.

Results

Two Novel Receptor STKs Are Encoded by tkv and sax

To isolate Drosophila genes encoding STK receptors, we used degenerate oligonucleotides directed against conserved sequences present in the mouse activin type II receptor (Mathews and Vale, 1991) and the C. elegans Daf-1 product (Georgi et al., 1990; see Experimental Procedures). cDNA synthesized from imaginal disc poly(A)+ RNA served as template to amplify DNA fragments by the polymerase chain reaction (PCR). Products were screened by sequence analysis and subsequently used to isolate corresponding cDNA clones from an imaginal disc cDNA library. Two classes of cDNA clones were found with open reading frames encoding for transmembrane STKs designated STK-A and STK-B (Figure 1). Both conceptual proteins exhibit an N-terminal signal sequence and a second stretch of hydrophobic residues that could serve as a transmembrane sequence. A comparison with the type II receptors for activin and TGFβ as well as with the Daf-1 product indicates a high degree of similarity in sequence and domain organization (Figure 1), suggesting that STK-A and STK-B serve as transmembrane receptors for a ligand of the TGFβ superfamily. Recently, a sequence comparison between receptors of the type I and type II subfamilies has revealed a region of similarity among type I receptors that is located immediately upstream of the kinase domain (Wrama et al., 1994). This region of 30 amino acids (containing a characteristic SGSGS sequence) is also present in STK-A and STK-B (Figure 1), suggesting that both of them belong to the type I subfamily of receptors.

The cytological map position of STK-A is 25D on the left arm of chromosome 2, a region that has been saturated for conserved sequences present in the mouse activin type II receptor (Mathews and Vale, 1991) and the C. elegans Daf-1 product (Georgi et al., 1990; see Experimental Procedures). cDNA synthesized from imaginal disc poly(A)+ RNA served as template to amplify DNA fragments by the polymerase chain reaction (PCR). Products were screened by sequence analysis and subsequently used to isolate corresponding cDNA clones from an imaginal disc cDNA library. Two classes of cDNA clones were found with open reading frames encoding for transmembrane STKs designated STK-A and STK-B (Figure 1). Both conceptual proteins exhibit an N-terminal signal sequence and a second stretch of hydrophobic residues that could serve as a transmembrane sequence. A comparison with the type II receptors for activin and TGFβ as well as with the Daf-1 product indicates a high degree of similarity in sequence and domain organization (Figure 1), suggesting that STK-A and STK-B serve as transmembrane receptors for a ligand of the TGFβ superfamily. Recently, a sequence comparison between receptors of the type I and type II subfamilies has revealed a region of similarity among type I receptors that is located immediately upstream of the kinase domain (Wrama et al., 1994). This region of 30 amino acids (containing a characteristic SGSGS sequence) is also present in STK-A and STK-B (Figure 1), suggesting that both of them belong to the type I subfamily of receptors.

The cytological map position of STK-A is 25D on the left arm of chromosome 2, a region that has been saturated for lethal complementation groups by Szidonya and Reuter (1988). In situ hybridization to deficiency-bearing chromosomes that have breakpoints at 25D identified the tkv locus as the only possible candidate for STK-A among the complementation groups in the chromosomal interval 25D (see Table 1 in Experimental Procedures). tkv, originally identified by adult viable alleles that affect the wing vein pattern.
was later found to correspond to a recessive lethal function allelic to the embryonic lethal slater (str) isolated by Nüsslein-Volhard et al. (1984) (Szidonya and Reuter, 1988). To verify that tkv encodes STK-A, we sequenced the two embryonic lethal genes tkv<sup>str</sup> and tkv<sup>str</sup>. Both these alleles were ethyl methanesulfonate (EMS) induced on the same isogenized parental chromosome (Nijsslein-Volhard et al., 1984), therefore each of them could serve as a polymorphism-free control for the other one (see Experimental Procedures). We discovered a point mutation in the allele tkv<sup>str</sup> that changes the conserved glutamate residue 528 into a lysine at amino acid position 144 resulting in a predicted protein that terminates immediately N-terminal of the conserved cysteine cluster in the extracellular domain of the STK-A protein (Figure 2). The tkv<sup>str</sup> sequence has a stop codon at amino acid position 144, indicative of type I receptors (Wrana et al., 1994). Amino acid exchanges found in tkv and sax mutants are indicated. Truncations caused by nonsense mutation or P element insertion are shown schematically below the wild-type products.

Figure 2. Schematic Representation of Wild-Type and Mutant tkv and sax Gene Products

Shown are the putative signal sequences (small open box), the extracellular cysteine residues (vertical bars), the transmembrane region (closed box), the STK domain (large open box), and the GS domain preceding the kinase domain (hatched). The presence of a GS box is indicative of type I receptors (Wrama et al., 1994). Amino acid exchanges found in tkv and sax mutants are indicated. Truncations caused by nonsense mutation or P element insertion are shown schematically below the wild-type products.

Confirmation that sax corresponds to the gene encoding STK-B was obtained by introducing an 8.9 kb genomic EcoRI fragment encompassing the STK-B open reading frame (with 3 kb of 5' and 2 kb of 3' flanking regions) into the germine by P element–mediated transformation. This fragment is able to rescue the sterility of sax mutant females. embryos derived from sax<sup>+/+</sup> females carrying the STK-B transgene are viable and exhibit a wild-type cuticle pattern (see Figure 4C). To exclude the possibility that another gene within the genomic rescue fragment is responsible for the rescue of the sax mutant phenotype and to determine whether kinase activity is required for the function of sax protein, we introduced a single point mutation into the genomic rescue fragment replacing the invariably conserved ATP-binding site Lys-291 with a Met. This mutated transgene is unable to rescue the sterility of sax<sup>+/+</sup> mutant females.

These results show that the two previously identified genes tkv and sax both encode proteins belonging to the type I receptor STK family. Recently, Xie et al. (1994) provided independent evidence that sax encodes a type I STK receptor.

Early Ventralization Phenotypes Caused by Complete Loss of tkv or dpp Gene Function Are Indistinguishable

Homozygous tkv mutant embryos have severe defects resulting in lethality. Most notably, the dorsal epidermis of tkv homozygous embryos fails to close, leading to a protrusion of the gut in stage 15 and 16 embryos and a large hole on the dorsal side in the cuticle (Nüsslein-Volhard et al., 1984). In most other respects, however, dorsoventral patterning occurs normally in zygotically mutant embryos. For example, Krüppel (Kr) expression in amnioserosa cells (Ray et al., 1991) is apparently normal, as is the refinement of the zerknüllt (Rushlow and Levine, 1990; Ray et al., 1991) and pannier (Ramain et al., 1993; Winick et al., 1993) expression patterns during cellularization (Affolter et al., submitted).

To test whether maternal expression of tkv is required for the correct establishment of dorsoventral polarity in homozygous tkv mutant embryos, we generated mosaic females to produce progeny devoid of maternal tkv product. Mosaic females were generated by two methods. First, the FLP/FRT technique was used to catalyze mitotic recombination leading to homozygous tkv<sup>-</sup> clones in tkv<sup>+/+</sup> flies (Golic, 1991; Xu and Rubin, 1993; see Experimental Procedures for alleles used). Such females were fertilized either with tkv<sup>+</sup> males or with wild-type males, and the resulting progeny that did not hatch were analyzed for cuticle phenotypes. The second method made use of a dominant female sterile mutation on the left arm of chromosome 2 (an ovo<sup>str</sup> transgene, Chou et al., 1993). tkv<sup>P[ovo<sup>str</sup>]</sup> larvae were X-ray irradiated; under these conditions, only females bearing a homozygous tkv<sup>+/+</sup> germine clone are

---

**Figure 2:** Schematic Representation of Wild-Type and Mutant tkv and sax Gene Products.

Shown are the putative signal sequences (small open box), the extracellular cysteine residues (vertical bars), the transmembrane region (closed box), the STK domain (large open box), and the GS domain preceding the kinase domain (hatched). The presence of a GS box is indicative of type I receptors (Wrama et al., 1994). Amino acid exchanges found in tkv and sax mutants are indicated. Truncations caused by nonsense mutation or P element insertion are shown schematically below the wild-type products.
Figure 3. Removal of Maternal tkv Activity in Germline Clones

(A and B) Embryos from tkv<sup>-/-</sup>tkv<sup>++</sup> germline clones that have a paternal wild-type copy of tkv are partially ventralized, similar to homoygous dpp<sup>++</sup> embryos (see Figure 4D). The embryo shown in (A) has been generated by FLP/H1-induced somatic recombination using y,w,hs-FLP, tkv<sup>-/-</sup> FRT[40,neo<sup>+</sup>]/Pw FRT[40,neo<sup>-</sup>] females fertilized with wild-type males (see Experimental Procedures). The same phenotype was observed (B) when X-rays were used to generate somatic clones in females transheterozygous for tkv<sup>++</sup> and an autosomal P[ovoDl] insertion. All embryos (n = 20) of such females that have been mated with tkv<sup>++</sup>/Cyo mutant males showed either this phenotype (B) or the complete ventralization phenotype shown in (D). Removal of maternal and zygotic tkv activity with the FLP/FRT system also produced completely ventralized embryos (n > 200) (C). Denticles extend over the entire circumference of these embryos. They have no dorsal structures, and Filzkörper and head skeleton are also missing. The identical phenotype is found in embryos homozygous mutant for a dpp null allele (dpp<sup>++</sup>) as shown in (E).

Figure 4. Embryos from sax Mutant Females Are Partially Ventralized and Resemble Embryos with a Reduced Dosage of dpp

(A), a sax<sup>/sax+</sup> mutant female (3) or a P element rescued sax<sup>/sax+</sup> female (C) are compared with embryos homoygous mutant for the weak dpp allele dpp<sup>++</sup> (D) or heterozygous mutant for the dpp null allele dpp<sup>++</sup> (E) or an embryo derived from a sax<sup>/sax+</sup> mutant female (F). (A) Cuticle of a wild-type embryo. Dorsal is to the right and anterior is up. Chitinized mouthparts and head skeleton are visible anteriorly, the Filzkörper posteriorly. (B) Embryos derived from sax<sup>/sax+</sup> mothers show deletions of anterodorsal cuticular structures of the head, in particular the labrum, the epistomal plate bearing the labral sense organ, and the dorsal arms and the dorsal bridge of the cephalopharyngeal skeleton. These structures are affected in weak dpp alleles (Wharton et al. 1993), and they map to the anterodorsal part of the blastoderm fate map (Jurgen et al., 1988). The remaining parts of the cephalopharyngeal skeleton, the ventral arms and the vertical plate, and the wall of the pharynx are often forced out of the body cavity. The two ventral halves of the head skeleton are not connected. The mouth hooks, the antennomaxillary complex, and the cirri are present. Dorsal structures of the thoracic and abdominal segments are not affected, and no expansions of the ventral denticle belts are observed. In the tail region, all the structures posterior to A7 are internalized. (C) Embryos derived from P element rescued sax<sup>/sax+</sup> females produce a wild-type cuticle pattern, and they develop into phenotypically wild-type adults. In homozygous dpp<sup>++</sup> embryos (D) and heterozygous dpp<sup>++</sup> embryos (E) the cephalopharyngeal skeleton is similarly affected as in embryos from sax<sup>/sax+</sup> females. The same structures as in embryos derived from sax<sup>/sax+</sup> mothers (B) are affected, although the average phenotype produced is slightly milder.
Figure 5. Maternal sex Activity Is Required to Specify the Dorsal-Most Pattern Element, the Amnioserosa

The Kr-/lacZ marker is expressed in all amnioserosa cells of wild-type embryos (A and E) but is continuously absent in embryos derived from sax+/ sax° mutant females (B). Shown are stage 14 embryos that have been stained using an antibody against β-galactosidase (A and B) or with X-Gal (C-F).

The lack of dorsal Kr-/lacZ-positive amnioserosa cells can be rescued by injection of sax mRNA into the dorsal side of mutant preblastoderm embryos (C). However, in contrast with ventral injection of dpp mRNA (Ferguson and Anderson, 1992b), ventral deposition of sax mRNA does not transform lateral or ventrolateral cells into Kr-/lacZ-expressing amnioserosa cells, neither in maternal sex mutant (D) nor in wild-type embryos (data not shown).

Kr-/lacZ-positive cells observed in wild-type embryos (E) are absent in embryos laid by the rare females homozygous mutant for the sax null allele sax° (F). All embryos shown carry a single Kr-/lacZ transgene except for embryos shown in (E), which are homozygous for the Kr-/lacZ insertion.

The phenotype of these embryos is very similar to embryos homozygous mutant for the weak allele dpp°° (St Johnston et al., 1990) and that represent the strongest ventralizing phenotypes caused by mutations in a zygotic gene (Arora and Nüsslein-Volhard, 1992).
indistinguishable from that of complete loss of dpp gene function provides strong support for the possibility that tkv encodes a dpp receptor.

**Complete Loss of sax Activity Causes Embryonic Phenotypes Indistinguishable from Partial Loss of dpp Function**

Females transheterozygous for the two sax alleles isolated by Schüpbach and Wieschaus (1989) are fully viable and do not show any abnormalities except for their sterility. To analyze the cause for this sterility, we examined the cuticular phenotypes of embryos derived from such females. Irrespective of whether these embryos received paternally a mutant or a wild-type sax allele, two characteristic defects are observed: deletions of anterodorsal head structures and an internalization of the Filzkörper and the seventh and eighth abdominal segments (Figure 4B, see legend for a more detailed description of head defects). The head defects and the terminal defects are similar to those of dpp Function.

The head defects and the terminal defects are similar to the phenotypes described for embryos homozygous for weak alleles of dpp or heterozygous for a null dpp mutation (Figures 4D and 4E; Wharton et al., 1993). The abnormal internal position of the telson and the last abdominal segments has been proposed to be a consequence of a defect in germ band retraction due to the loss of amnioserosa (Ferguson and Anderson, 1992a; Wharton et al., 1993). The amnioserosa is derived from the dorsal-most cells of the blastoderm embryo and has been shown to be the first dorsal pattern element to be deleted by a fate map shift in ventralizing mutants (Arora and Nüsslein-Volhard, 1992). To monitor the differentiation of amnioserosa cells, we used a Kr-/lacZ marker gene that specifically labels these cells between stages 8 and 15 of embryogenesis (Ferguson and Anderson, 1992b). As shown in Figure 5, wild-type stage 14 embryos exhibit a large number of dorsally situated amnioserosa cells, whereas embryos of the same age derived from sax+/sax2 mothers have only a few if any Kr-/lacZ-positive cells. Kr-/lacZ-expressing cells are also absent at earlier stages, indicating that amnioserosa differentiation never takes place in embryos from sax mutant females.

Since the alleles sax1 and sax2 have been isolated in a screen for female sterile mutations, these alleles were selected to allow homozygous females to survive. It is possible, therefore, that they are not null alleles. Indeed, sequencing of the two alleles revealed that each of them exhibits a single point mutation altering a highly conserved amino acid residue of the kinase domain (see Figure 2). Furthermore, we found genetic evidence that these two mutations are not null alleles (see below). To analyze the null phenotype of sax, we used a sax allele that carried a P element insertion in the sax coding region (provided by I. Kiss; Török et al., 1993). The P[lacW] insertion disrupts the sax open reading frame after the first 36 N-terminal amino acid residues and thus likely constitutes a null allele (Figure 2). In contrast with the sax1 and sax2 alleles, mutants homozygous for saxdio during larval stages. However, in stocks in which sax2 is balanced with Sm6a, we occasionally observe homozygous adults. Homozygous saxd2 females are less viable and fertile than their heterozygous siblings. The rare embryos that were obtained from such females lack Kr-/lacZ-positive amnioserosa cells (Figure 5F) and show a cuticular phenotype similar to that of sax+/sax2-derived embryos (Figure 4F), although it is generally milder and more variable (irrespective of their zygotic sax genotype). As we note below, the less extreme phenotype associated with this sax null mutation indicates that the sax1 and sax2 mutant proteins are dominant negative forms of the protein that may interfere with other dpp-dependent receptor complexes.

In contrast with embryos devoid of tkv activity, embryos lacking sax activity show no expansion of their ventral denticle belts, nor do they fail to differentiate dorsal ectoderm in the trunk region, as judged by the normally patterned dorsal hairs (data not shown). The patterning defect caused by lack of sax activity is therefore limited to the dorsal-most structure, the amnioserosa.

It has been demonstrated that high levels of dpp activity are required in the dorsal-most region of the early embryo for the specification of amnioserosa cells (Ferguson and Anderson, 1992a; Wharton et al., 1993). If sax participates in interpreting the dpp signal for amnioserosa development, it should also be required autonomously in dorsal but not ventral cells. To test this, we prepared sax RNA in vitro and injected it at either the dorsal or the ventral side of embryos derived from sax+/sax2 mothers. Dorsally injected sax RNA was able to restore Kr-/lacZ-positive cells (Figure 5C), whereas RNA injected at the ventral side or H2O injected dorsally had no effect (Figure 5D). Hence, the sax gene product appears to be required in dorsal cells for the generation of amnioserosa cells. Conversely, sax expression in ventral cells is not sufficient to specify amnioserosa.

**Involvement of sax In Other dpp-Dependent Patterning Events**

As mentioned above, homozygous saxd2 individuals derived from heterozygous females can, in certain situations, survive to adulthood and show only minor defects (see below; the same phenotypes are seen in escapers that are transheterozygous for saxd2 and a deficiency for sax (saxd2ID(2R)NCX9)). Hence, if sax participates in the reception of the dpp signal, it cannot be required for mediating all of the responses normally specified by dpp. As we describe below, the correlation of sax phenotypes with known requirements for dpp suggests that sax is required for dpp-dependent signaling in many tissues, but that it might be required only for cells to interpret maximal levels of dpp, as in the early embryo.

**dpp expression in parasegment 7 of the embryonic visceral mesoderm has been found to be required for midgut morphogenesis, presumably by controlling the spatially restricted expression of several genes in the visceral mesoderm (e.g., Ultrathorax, wingless; Immerglück et al., 1990; Panganiban et al., 1990; reviewed by Bienz, 1994). Loss of dpp expression results in the lack of the second midgut constriction and blocks the expression of the homeotic gene labial (lab) in neighboring endodermal cells of the central portion of the midgut (Immerglück et al., 1990; Panganiban et al., 1990). In embryos homozy-
Body Patterning by TGFβ Receptor Homologs tkv and sax

231

Figure 6. Zygotic sax Activity Is Required for Morphogenesis of the Embryonic Midgut and Patterning Imaginal Disc Derivatives

In wild-type embryos (A), three constrictions form in the midgut during stage 16 (arrow heads). In homozygous sax alleles (C), the second midgut constriction is missing, as it is in homozygous dpp alleles (Segal and Gelbart, 1985) mutants (B). In addition, whole-mount in situ hybridizations demonstrate that lab transcripts are consistently present at reduced levels in homozygous sax embryos (F) when compared with wild type (D). No lab transcription is induced in the central midgut of dpp mutants (E).

(G and H) Altered wing vein pattern in saxP/saxP adults. Shown is a wing from a homozygous saxP mutant female (H). A wild-type wing is shown for comparison (G). Ectopic vein material is observed along longitudinal vein 2 and the anterior crossvein is consistently absent (arrow).

ngs for the sax allele, the second constriction is completely absent, a phenotype very similar to that observed in embryos homozygous for the dpp allele (Figures 6B and 6C). However, and in contrast with the complete removal of dpp activity, lab induction still occurs after midgut fusion albeit at reduced levels (compare Figure 6D with 6F).

Later in development, dpp plays a crucial role in growth and patterning of the imaginal discs (Spencer et al, 1982; Posakony et al., 1990). We noticed that various disc derivatives (wings, legs, eyes) of sax/sax escapers exhibit defects. For example, in the wings of females, the anterior crossvein (Figure 6H) between longitudinal veins 3 and 4 is always absent. This crossvein is positioned near the anteroposterior compartment boundary (Garcia-Bellido et al., 1973), along which cells express and presumably require maximal levels of dpp in the wing imaginal disc (Posakony et al., 1990; Raftery et al., 1991; Basler and Struhl, 1994). Similarly, both sexes have eyes that are smaller than the wild type (data not shown). Semithin sections of eyes from sax homozygotes revealed that only the number but not the pattern of the individual ommatidia are affected. The smaller eyes of sax homozygotes could therefore be the result of an impaired movement of the morphogenetic furrow in the eye imaginal disc (Heberlein et al., 1993; Ma et al., 1993). Similar defects are associated with the partial loss of dpp function in the eye (Spencer et al., 1982; Heberlein et al., 1993), supporting the view that sax function is required for mediating the response to peak levels of dpp.

Consistent with the dorsoventral pattern defects of sax mutant embryos, it appears therefore that sax is not an absolute prerequisite for dpp signaling, but that it is only required for cells to respond correctly to maximal levels of dpp activity.

Interactions among tkv, sax, and dpp

As shown above, loss of function mutations in tkv or sax cause dorsoventral patterning phenotypes similar to complete or partial loss of function phenotypes for dpp. Thus, the transmembrane STK5s encoded by tkv and sax may function as type I receptors for dpp. As described below, we find tkv and sax alleles that encode full-length proteins with point mutations in their kinase domains enhance the
Dpp receptors.

Manner, consistent with a direct role for tkv and sax as alleles sax+ or tkv-/+ survived with almost the same frequency as progeny from wild-type females and did not exhibit signs of ventralization. For the sax', sax2', and tkv-/- alleles, however, we observed greatly reduced survival rates (with dpp+/ males) or even complete lethality (with dpp+/ males) (Figure 7A). Analysis of the cuticular phenotypes of embryos generated in these crosses confirmed this result: dpp+/ embryos derived from sax'/+, sax2'/+, or tkv-/-/+ females show ventralized phenotypes (Figure 8B) similar to those observed in embryos homozgyous for dpp+/ or heterozygous for a complete null allele of dpp. This dominant behavior of the sax', sax2', and tkv-/- alleles is strictly maternal: no interaction was observed when these sax and tkv alleles were introduced paternally (Figure 7B). We also find that females transheterozygous for sax' and tkv-/- are virtually sterile and produce weakly ventralized embryos with head defects and internalized telson even when these embryos carry two wild-type alleles of dpp (Figures 7B and 8D).

Thus, the presence of the sax1', sax2', or tkv-/- mutant proteins in early embryos appears to compromise their ability to respond to dpp by differentiating dorsal tissue. Because these mutant proteins each contain a single amino acid substitution in a highly conserved portion of the kinase domain, these dominant negative phenotypes could be due to the absence of the mutant proteins to interact with and sequester limiting amounts of dpp protein on the outside of the cells without being able to activate the kinase domain within the cell.

Discussion

Tkv and Sax Encode Putative Type I Receptors for the Dpp Protein

Here, we describe two novel members of the type I receptor STK family in Drosophila and show that they are encoded by the previously defined genes tkv and sax. Although we have no biochemical evidence for a direct interaction of dpp protein with either the tkv or the sax gene products, we propose that these two transmembrane proteins are receptors involved in the dpp signal transduction pathway based on the following lines of evidence.

First, type I and type II receptors for TGFβ1 and for activin have been identified and shown to encode transmembrane STKs with characteristic structural features (Mathews and Vale, 1991; Lin et al., 1992; Attisano et al., 1993; Ebner et al., 1993, Franzen et al., 1993). The dpp gene products, we propose that these two transmembrane proteins are receptors involved in the dpp signal transduction pathway based on the following lines of evidence.

Phenotype of weak dpp mutations in a dominant negative manner, consistent with a direct role for tkv and sax as dpp receptors.

Females heterozygous for each of the sax or tkv alleles were crossed to males heterozygous for a weak hypomorphic dpp allele (dpp+/ or dpp+/ Wharton et al., 1993). Progeny derived from females heterozygous for the null alleles sax' or tkv-/- survived with almost the same frequency as progeny from wild-type females and did not exhibit signs of ventralization. For the sax', sax2', and tkv-/- alleles, however, we observed greatly reduced survival rates (with dpp+/ males) or even complete lethality (with dpp+/ males) (Figure 7A). Analysis of the cuticular phenotypes of embryos generated in these crosses confirmed this result: dpp+/ embryos derived from sax'/+, sax2'/+, or tkv-/-/+ females show ventralized phenotypes (Figure 8B) similar to those observed in embryos homozgyous for dpp+/ or heterozygous for a complete null allele of dpp. This dominant behavior of the sax', sax2', and tkv-/- alleles is strictly maternal: no interaction was observed when these sax and tkv alleles were introduced paternally (Figure 7B). We also find that females transheterozygous for sax' and tkv-/- are virtually sterile and produce weakly ventralized embryos with head defects and internalized telson even when these embryos carry two wild-type alleles of dpp (Figures 7B and 8D).

Thus, the presence of the sax1', sax2', or tkv-/- mutant proteins in early embryos appears to compromise their ability to respond to dpp by differentiating dorsal tissue. Because these mutant proteins each contain a single amino acid substitution in a highly conserved portion of the kinase domain, these dominant negative phenotypes could be due to the absence of the mutant proteins to interact with and sequester limiting amounts of dpp protein on the outside of the cells without being able to activate the kinase domain within the cell.

Discussion

Tkv and Sax Encode Putative Type I Receptors for the Dpp Protein

Here, we describe two novel members of the type I receptor STK family in Drosophila and show that they are encoded by the previously defined genes tkv and sax. Although we have no biochemical evidence for a direct interaction of dpp protein with either the tkv or the sax gene products, we propose that these two transmembrane proteins are receptors involved in the dpp signal transduction pathway based on the following lines of evidence.

First, type I and type II receptors for TGFβ1 and for activin have been identified and shown to encode transmembrane STKs with characteristic structural features (Mathews and Vale, 1991; Lin et al., 1992; Attisano et al., 1993; Ebner et al., 1993, Franzen et al., 1993). The dpp protein shows extensive sequence homology to both TGFβ1 and activin. The receptors for dpp are most likely membrane STKs with characteristic structural features (Mathews and Vale, 1991; Lin et al., 1992; Attisano et al., 1993; Ebner et al., 1993, Franzen et al., 1993). The dpp protein shows extensive sequence homology to both TGFβ1 and activin. The receptors for dpp are most likely therefore members of the same family of transmembrane proteins defined by the TGFβ1 and activin receptors.

Second, mutations that abolish sax and tkv activity cause phenotypes that are indistinguishable, respectively, from partial or complete loss of function mutations in the dpp gene. In the case of sax, loss of gene function blocks specification of the amnioserosa. This is the dorsal-most derivative on the blastoderm fate map and appears to be specified by peak levels of dpp activity (Wharton et al., 1993). The zygotic phenotypes of sax can similarly be correlated with requirements for high levels of dpp. More dramatically in the case of the tkv gene, complete loss of early
interacting with a ubiquitous ligand. For example, sax could be required specifically for amnioserosa development because maternal sax protein is only present in the dorsal-most region of the wild-type embryo. However, such a mechanism is unlikely. In particular, we have found that the site of injection of sax mRNA does not determine where amnioserosa cells arise. Furthermore, the maternal RNA gene products of tkv and sax are uniformly distributed in early embryos (M. A., D. N., and K. B., unpublished data). And finally, dpp is known to be expressed asymmetrically, in dorsally positioned cells (St Johnston and Gelbart, 1987; Ray et al., 1991), and this early dorsal expression is not affected by tkv or sax mutations (unpublished data). dpp has been implicated as a gradient morphogen in the control of dorsal pattern in the epidermis (Ferguson and Anderson, 1992a, 1992b). Thus, the tkv and sax proteins appear to constitute ubiquitously expressed receptors that respond to a spatially restricted ligand, dpp.

Further support for our hypothesis that sax and tkv act as type I receptors for dpp comes from the observation that the presence of the sax¹, sax², and tkv² mutant proteins can compromise the ability of dpp to specify dorsoventral pattern. These alleles encode full-length transmembrane proteins that have single amino acid substitutions of conserved residues in their kinase domain. We therefore interpret their effects as a dominant negative interaction with components of the dpp signaling system. In principle, these defective sax and tkv proteins could simply compete with wild-type kinases for substrate binding, or form homodimers with the corresponding wild-type receptors and thereby hinder their participation in signal transduction. We note however that the stronger ventralizing phenotype of embryos derived from sax¹Isax²+ or sax²Isax² females compared with embryos devoid of any maternal sax protein (from sax%axP females) indicates that the mutant sax proteins are more likely to perturb the function of a different receptor component. This is consistent with the finding that the type I receptor for TGFβ is a component of a heterodimeric signaling receptor complex that also consists of the type II receptor (Wrana et al., 1992).
A likely target therefore of the dominant negative effect of sax\(^{1}\), sax\(^{2}\), and tkv\(^{+}\) proteins are type II receptors for dpp, which have not been identified so far.

**How Can Varying Levels of dpp Activity Specify Different Portions of the Dorsoventral Pattern?**

Our results indicate that one strategy cells may use to interpret different levels of dpp activity might be the employment of multiple receptors. We suggest that at least two type I receptors are involved in pattern formation. One of these two receptors, together with a type II receptor, could explain how blastoderm cells adopt one of three distinct dorsoventral cell fates, depending on their exposure to dpp. In dorsal regions, where dpp levels appear to be highest, activation of both the sax and tkv receptors would lead to the aminoserosa fate. In more lateral regions, where the level of dpp activity is lower, only the tkv receptor kinase may be stimulated leading to the specification of dorsal epidermis. Finally, in more ventral regions that are not exposed to dpp, neither the tkv nor the sax kinases are active, leading by default to the specification of ventral epidermis. This model (Figure 9) is based on at least two assumptions. First, that sax activity is only triggered at high concentrations of dpp, for example due to a lower affinity of the sax protein for its ligand; and second, that the different kinases provide cells with qualitatively different information. Similar combinatorial models involving multiple type I STK receptors could account for the concentration dependent responses of cells to TGFB proteins observed in other systems.

**Experimental Procedures**

**RNA Preparation, PCR Amplification, and cDNA Isolation**

Poly(A\(^{+}\)) RNA was isolated from a large scale imaginal disc preparation (provided by the Fristrom lab) and used for primary cDNA synthesis as described previously (Basler et al., 1993). PCR was carried out using the same conditions as in Basler et al. (1993) with the following two pairs of degenerate primers: 5'G-TGGAGCATCTGACGATCG-3' and 5'GAGATCCATTAGATCTGAG-3' and 5'GAGGATCCAGCATCGAG-3' and S'-GAGGATCCAGCATCGAG-3', in which degenerate positions are in parenthesis and restriction sites underlined. The sequence within and adjacent to the long open reading frame was determined on both strands by the dideoxy chain termination method (Sanger et al., 1977) using Sequenase (U. S. Biochemical). Sequencing of Mutant Alleles

**RNA Injections**

To generate a template for STK-B transcripts, the STK-B cDNA pk7 was mutagenized by PCR to contain a Ncol site at the ATG start codon such that it could be fused to the \(\beta\)-globin leader of the plasmid pDH021 (Sprenger and Nüsslein-Volhard, 1992). Linearized plasmid DNA was transcribed with SP6 polymerase as described previously (Krieg and Melton, 1988). The concentration of the transcribed RNA was determined spectrophotometrically, and transcript integrity was checked by agarose gel electrophoresis. RNA was injected into preblastoderm embryos and deposited dorsally or ventrally as illustrated in Figure 14 of Chasan and Anderson (1993). All experiments to discriminate between dorsal and ventral injections were done with RNA from a single transcription reaction. For the experiment shown in Figure 5 we used STK-B RNA at a concentration of 100 \(\mu\)g/ml.

**Sequencing of Mutant Alleles**

The sax alleles sax\(^{1}\) and sax\(^{2}\) could be amplified by PCR from genomic DNA isolated from homozygous or hemizygous adult flies. Amplified fragments covering the entire coding region were subcloned and sequenced using the various primers available from sequencing the cDNAs; each of the alleles served as a polymorphism free control for the other one. For the two tkv\(^{+}\) alleles, we amplified a genomic fragment from heterozygous adults using a primer corresponding to the 3' end of the first Intron 197 bp downstream from the STK-B ATG start codon (5'C-GAGATTTCGAG(A)-3') and a second primer derived from sequences 3' of the TAA stop codon (5'CAGAGCTACAAACAGCGAG-3'). The 2 kb DNA fragments covering almost the entire STK-B coding region were subcloned. Since 50% of the amplified fragments correspond to the wild-type STK-B gene on the CyO chromosome, and to avoid PCR-derived artifacts, at least five independently cloned PCR fragments were sequenced for each allele over the entire length.

**Southern and In Situ Hybridizations**

In situ hybridization to polytene chromosomes was done as described previously, using digoxigenin-11-dUTP-labeled DNA probes (de Frutos et al., 1990). Probes were derived from plasmids harboring cDNAs of STK-A and STK-B. For quantitative Southern blot analysis, we used EcoRI or BamHI digested DNA isolated in parallel from equal amounts of heterozygous adult flies and hybridized the blots to digoxi-

- Genomic Constructs

A genomic library from D(2R)K\(^{+}\)/CyO flies in \(\lambda\)EMBL4 (Baumgartner, 1993) was screened for sax function and molecularly for the presence or absence of a tkv or sax hybridization signal (Table 1).

The deficiency stocks for tkv are described by Szodynia and Reuter (1968). Deficiencies D(2L)lac\(^{1}\) and D(2L)lac\(^{2}\) of the tkv locus could not be tested with quantitative Southern hybridizations because these stocks also carry the duplications Dp(2;3)B19, Dp(2;3)rc\(^{+}\) and Dp(3;2)B10, respectively. Stocks with rearranged chromosomes for sax were obtained from the Bloomington Stock Center or from P. Heitzler (Heitzler et al., 1993).

**Germline Clones**

The tkv alleles tkv\(^{+}\) and tkv\(^{+}\) were recombined onto chromosomes carrying a M{rs-neo}; FRT101 at cytological position 40A (Xu and Huben, 1993). Females carrying these recombinant chromosomes were crossed with y,w hs-FLP, P[y+]; FRT104, P{hs-neo}; FRT104 males, and the resulting progeny were heat shocked during third instar larval pe-

- Genomc Constructs
et al. (1987) was screened with a sax cDNA probe to isolate wild-type sax genomic DNA. An 8.4 kb EcoRI fragment from a hybridizing phage clone was subcloned into the transformation vector pCaSpeR4 (Pirrotta, 1988) for P element–mediated germine transformation (Spradling and Rubin, 1982).

To introduce the Lys to Met exchange at amino acid position 291, the sequence encoding codon numbers 291–297 was changed to 5'-ATGATATTCTTCAGTCGCGAC-3' using PCR. The mutated sequence was confirmed by sequencing and stepwise reassembled into the genomic 6.9 kb DNA fragment.

In Situ Hybridization, X-Gal and Antibody Staining

In situ hybridization to whole-mount embryos was performed as described by Tautz and Pfeifle (1989) with minor modifications (Affolter et al., 1993). To detect lab transcripts, the entire insert of the cDNA clone 241 was used (Mlodzik et al., 1988). To assay for \( \beta \)-galactosidase expression, injected embryos carrying the \( Kr-lacZ \) construct on the third chromosome were aged until stage 14 at 18°C, fixed in a mixture of 4% formaldehyde and heptane, washed and transferred to staining solution (10 mM [pH 7.0] sodium phosphate buffer with 0.1% Triton, 333 mM each of K3Fe(CN)6 and K4Fe(CN)6·3H2O, 1 mM MgCl2, and 150 mM NaCl). Stained embryos were directly photographed under DIC optics without devitellinization.

Immunostaining of embryos was done according to standard procedures (Ashburner, 1989), followed by a secondary antibody conjugated with biotin. Secondary antibodies were revealed using the HRP ABC kit (Vectastain).

Genetic Interactions

To test for genetic interactions among dpp and tkv and sax, adult progeny of appropriate crosses (see Figure 7A) were scored to determine the number of Cy and Cy' flies. The percentage viability was then calculated by dividing the number of Cy' flies by the number of Cy flies (if one of the parents was wild type) or by half the number of Cy flies (if both parents were Cy). To assay the cuticular embryonic phenotypes, crosses identical to those used for viability studies were performed, and progeny of appropriate crosses (see Figure 7A) were scored to determine the number of Cy and Cy' flies. The percentage viability was calculated by dividing the number of Cy flies by the number of Cy' flies (if one of the parents was wild type) or by half the number of Cy flies (if both parents were Cy).

Acknowledgments

We thank M. Keggenhoff for excellent technical assistance and P. Heitzler, J. Nüsslein-Volhard, P. Poddgett, J. Perrimon, G. Reuter, T. Schüpbach, G. Struhl, I. Szidonya, and especially I. Kiss for providing us with fly stocks. We also thank B. Dickson, A. Furley, E. Hafen, M. Piasecz, and particularly G. Struhl for helpful comments on the manuscript and L. Attisano and M. Hoffmann for discussions. This work was supported by the Howard Hughes Medical Institute and grants from the Swiss National Science Foundation.

Received April 0, 1994; revised May 31, 1994.

References


Driever, W., and Nüsslein-Volhard, C. (1988b). The bicoid protein de-
terminates position in the Drosophila embryo in a concentration-dependent manner. Cell 64, 96–104.


GenBank Accession Numbers

The accession numbers for the sequences reported in this paper are L33475 (tkv) and L33476 (sax).