Similarities between trunk and spätzle, putative extracellular ligands specifying body pattern in *Drosophila*

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The basic body plan of *Drosophila* is specified by four determinant systems that organize pattern along the anteroposterior and dorsoventral axes. Two of these systems (anterior and posterior) depend on localized mRNAs. In contrast, the other two (ventral and terminal) require locally generated extracellular ligands that are transduced, respectively, by the transmembrane receptors Toll and torso (tor). The ligand for the Toll receptor is thought to be spätzle (spz), a secreted protein that is activated by proteolytic cleavage. Here we report that *trunk* (*trk*), a gene required for activity of the tor receptor, encodes a protein that resembles spz in several respects. In particular, the sequence suggests that *trk* is a secreted protein and that it contains an internal site for proteolytic cleavage. Furthermore, the carboxy-terminal domain of *trk* has a similar arrangement of cysteines to that of spz. We propose that *trk* encodes an extracellular ligand involved in specifying terminal body pattern and suggest by analogy with spz that a cleaved form of *trk* constitutes the ligand for the tor receptor.

[Key Words: *Drosophila*; pattern formation; extracellular ligands; *trunk*; *spätzle*; signal transduction; *torso*]

Received August 10, 1995; revised version accepted September 5, 1995.

The early development of the *Drosophila* embryo is governed by localized determinants that are generated during oogenesis and then activated upon fertilization. These determinants fall into two classes: localized RNAs, such as *bicoid* (*bcd*) and *nanos* (*nos*), which are deposited at the anterior and posterior poles of the egg cell, and poorly understood extracellular components that appear to be associated with the vitelline membrane surrounding the egg cell, either at the poles or along the ventral surface. Following fertilization, *bcd* and *nos* RNAs are translated and their protein products diffuse through the embryo, controlling most aspects of anteroposterior pattern by regulating the transcription of zygotic gap genes such as *giant*, *hunchback*, *Krüppel*, and *knirps*. At the same time, the extracellular components along the ventral surface and at the poles appear to give rise to distinct ventral and terminal ligands that diffuse in the perivitelline fluid layer surrounding the embryo until they are bound and sequestered by the transmembrane receptors Toll and torso (tor). These receptors then transduce the ventral and terminal signals by regulating the transcription of dorsoventral genes (e.g., *twist*, *snail*, and *zen*) and additional gap genes (*tailless* and *huckebein*) (for review, see Nüsslein-Volhard et al. 1987; St. Johnston and Nüsslein-Volhard 1992).

The local generation of extracellular ligands in the perivitelline fluid layer is best understood for the dorsoventral system. In this case, a series of gene products

have been identified that appear to constitute a processing system for generating the ligand. Some of these products [encoded by the *nudel* (*ndl*), *windbeutel* (*wind*), and *pipe* (*pip*) genes] appear to be made in the follicle cells that surround the egg cell and synthesize the vitelline membrane. Others are made by the oocyte or early embryo and appear to constitute both a putative ligand [encoded by the *spätzle* (*spz*) gene] and a protease cascade (encoded by the *snake*, *easter*, and *gastrulation defective* genes) that allows the processing and activation of the ligand in response to the local activities of *ndl*, *wind*, and *pip* gene products (for a review of the dorsoventral system, see Chasan and Anderson 1993).

The situation for generating the terminal signal is less well understood. Two genes, *torso-like* (*tsl*) and *trunk* (*trk*), have been identified that are essential for activity of the receptor, encoded by the *tor* gene. (Casanova and Struhl 1989; Sprenger et al. 1989; Stevens et al. 1990). *tsl* appears to be expressed by specialized follicle cells at each end of the maturing oocyte (Savant-Bhonsale and Montell 1993; Martin et al. 1994) and is found at each end of the early embryo after fertilization (Martin et al. 1994). Moreover, its restricted expression in these follicle cells appears to be critical to generate a localized terminal signal in the embryo because ectopic *tsl* expression during oogenesis causes central portions of the early embryo to develop terminal structures (Savant-Bhonsale and Montell 1993; Martin et al. 1994). In

contrast, *trk* activity appears to be required in the female germ line (Schüpbach and Wieschaus 1986b). As in the case of dorsoventral signal, the tor receptor appears to play a significant role both in sequestering and transducing a putative extracellular ligand that is locally generated (Sprenger and Nüsslein-Volhard 1992; Casanova and Struhl 1993), presumably as a consequence of *tsl* activity. Hence, *trk* could encode the ligand or a component that facilitates its processing or subsequent interactions with the receptor.

Here we report the cloning and sequencing of the *trk* gene as well as an analysis of *trk* transcription and the lesions associated with a series of *trk* mutations. We find that *trk* encodes a protein that is likely to be secreted and that resembles spz in two notable respects. First, it contains a domain that appears similar to a well-characterized proteolytic cleavage site in complement component 3 (Müller-Eberhard 1988). Although spz does not contain a similar site, there is compelling evidence that it is activated by proteolytic cleavage (Morisato and Anderson 1994). Second, the portion of *trk* carboxy-terminal to the putative cleavage site contains a similar arrangement of cysteines to that found in spz (Morisato and Anderson 1994). This arrangement is reminiscent of the cystine knot motif found in other families of extracellular ligands (McDonald and Hendrickson 1993). Finally, our sequence analysis of several *trk* mutations suggests that both the putative cleavage site as well as the conserved cysteines in the carboxy-terminal domain are essential for *trk* protein activity. We therefore postulate that *trk* encodes an extracellular ligand involved in specifying terminal body pattern and propose by analogy with spz that a cleaved form of *trk* constitutes the ligand for the tor receptor.

Results

Cloning of the *trk* gene

We have cloned the *trk* gene using a P-element-induced mutation (*trk*^{V67-1}) provided by T. Schüpbach (Princeton University, NJ). P-element-containing phage were selected from a library made with genomic DNA from *trk*^{V67-1} mutant embryos, and a P-element-containing phage from the *trk* locus was identified by cross-hybridization with cosmids from a chromosomal walk in the 31BC chromosomal interval provided by I. Whitehead and N. Clegg. Genomic DNA from this phage was then used as a hybridization probe to recover a corresponding phage from a wild-type genomic library. Southern blot analysis allowed the detection of differences between genomic DNAs derived from wild-type, *trk*^{V67-1}, and *trk*^{V67-1} revertant stocks, and the region containing the P element was subcloned and sequenced to determine the site of insertion. Genomic DNAs from the *trk* region were inserted into the P(*ry*⁺) transformation vector C20, returned to the *Drosophila* genome by P-element-mediated transformation, and assayed for their ability to rescue the maternal effect phenotype associated with the *trk*¹ mutation. The transformation experiments showed

that *trk* is contained within a 3-Kb segment of genomic DNA that fully rescues the terminal patterning defects caused by loss-of-function mutations in the *trk* gene (Fig. 1). This rescuing genomic fragment includes sequences homologous to embryonic mRNAs derived from three transcription units, one of which we infer encodes the *trk* protein because it is the only unit of the three that (1) initiates within the rescuing DNA fragment (2) is entirely contained within this DNA fragment, and (3) gives rise to a transcript that is altered (shortened) by insertion of the P element associated with the *trk*^{V67-1} mutation (see Fig. 1). Furthermore, we have determined the sequences of four EMS-induced point mutations in *trk* and each is associated with a single-amino-acid change in the putative coding sequence (see legend to Fig. 2).

Seven *trk* cDNAs were obtained and all are colinear with the genomic DNA, indicating that there are no introns in the *trk* gene. *trk* cDNA probes detect a transcript of ~0.8 kb on a Northern blot (Fig. 1), similar to the size of the longest cDNAs, as well as a smaller transcript of ~0.6 kb, which appears to represent a prematurely terminated form of the 0.8-kb transcript (Figs. 1 and 2). These transcripts are present in unfertilized eggs and early embryos but are not detected in embryos aged >2 hr after egg laying. In situ hybridization shows that these RNAs are present in the nurse cells, but not in the surrounding follicle cells, in agreement with the *trk* germ-line requirement (Schüpbach and Wieschaus 1986b); we also detect uniform expression of *trk* transcripts in early embryos (Fig. 1C).

The predicted *trk* protein

The *trk* cDNAs contain part or all of a single open reading frame (ORF) that could be initiated at either of two start codons at nucleotides 1998 and 2025 to generate proteins of 235 or 226 amino acids, respectively (Fig. 2); however, the sequence around the ATG at 2025 is a better match with the *Drosophila* consensus sequence for translational initiation (Cavener 1987). In both cases, hydropathy profiles reveal a short hydrophobic stretch at the amino terminus that obeys the rules defining a signal sequence (Fig. 2; von Heijne 1985). No other potential transmembrane domains are predicted in the ORF, suggesting that the *trk* protein is secreted; however, we note the presence of a short hydrophobic stretch at the carboxyl terminus that is also present in spz and other growth factors with the cystine knot motif. A noncanonical polyadenylation signal is present at position 2725. In five of the seven cDNAs sequenced, a stretch of poly(A) is found 23 or 39 nucleotides downstream from this site. We note the presence of two canonical polyadenylation sites beginning at nucleotides 2549 and 2588 (dotted underline), upstream of the stop codon beginning at nucleotide 2703. These sequences appear to function in vivo because we found single cDNAs that terminated after these sequences (one of each type out of a total of seven cDNAs sequenced) and because we observe a higher mobility band in our Northern blot analysis (Fig. 1B). These smaller transcripts could not code for the intact protein;

Figure 1. The *trk* transcription unit. (A) A partial restriction map of the 3004-bp *PvuII*–*SalI* fragment that rescues the *trk* phenotype and, hence, contains the *trk* gene. (A) *AvaI*; (N) *NsiI*; (Ps) *PstI*; (Pv) *PvuII*; (S) *SalI* (see Fig. 2 for DNA sequence). Three transcription units overlap with or are contained within this region, as shown by arrows. Note that the *trk* transcript is the only one that initiates and is entirely contained within the rescuing DNA. The insertion site of the P element associated with the *trk*^{V67-1} mutation is also indicated. (B) Expression of *trk* RNA. Northern blots of RNAs from both wild-type and *trk*^{V67-1} mutant embryos probed with labeled DNA derived from the *NsiI*–*AvaI* genomic fragment. Note that *trk* transcripts are present predominantly in embryos aged 0–2 hr following fertilization and that the size of the *trk* transcript is reduced in mutant embryos (the blot on the left was also probed for *rp49* transcripts to confirm the integrity of each RNA sample). A smaller transcript of ~0.6 kb is observed in wild-type embryos, which appears to be a product of premature termination (see legend to Fig. 2). (C) In situ hybridization of the nurse cell/oocyte complex (top) and early embryo (bottom) with labeled DNA complementary to the *NsiI*–*AvaI* genomic fragment. Note that *trk* mRNA can be detected in the nurse cells, but not the follicle cells, during oogenesis and that the transcripts appear to be uniformly distributed in early embryos.

1992), are also conserved in *trk*). The similarity between *trk* and C3 is confined to this domain; hence, *trk* does not appear to be a *Drosophila* C3 homolog. Nevertheless, the presence of this domain suggests that *trk* protein is proteolytically cleaved. We note that the conserved arginine immediately upstream of the putative cleavage site in *trk* (arrow, Fig. 3) is changed to a glutamine in the *trk*⁶ mutation. This substitution causes a complete loss of *trk* gene function, consistent with the possibility that cleavage is essential for *trk* activity.

Another feature of the *trk* protein is the arrangement of cysteine residues in its carboxyl terminus, which resembles that found in the carboxy-terminal portion of the *spz* protein (Fig. 4; Morisato and Anderson 1994). It is this portion of *spz* that is thought to be activated by proteolytic cleavage and to constitute the ligand for the Toll receptor (Morisato and Anderson 1994; Schneider et al. 1994). The arrangement of carboxy-terminal cysteines in *trk* and *spz* is also reminiscent of that of the cystine knot motif found in several growth factors and extracellular ligands (McDonald and Hendrickson 1993; Murray-Rust et al. 1993). Other conserved features of this motif, however, are not present in either *spz* or *trk* (Murray-Rust et al. 1993; N. McDonald, pers. comm.), suggesting that the arrangement of cysteines in *spz* and *trk* may define a novel structural motif.

Molecular characterization of *trk* mutant alleles

To gain a better understanding of the function of the *trk* protein we have sequenced the *trk*-coding region of four *trk* alleles that behave genetically as amorphs. In the *trk*³ mutation, the tryptophan at position 89 is changed to a stop codon and thus should result in a truncated protein. In the *trk*⁴ mutation, another tryptophan at position 211

hence, we believe that they are nonfunctional. We have also found a genomic polymorphism between different strains at position 2313, where the sequence GTA-GAGAAGAA is replaced by GTGGAGAA, giving rise to a predicted polypeptide with two lysines beginning at position 99 instead of the three present in the reported sequence.

Analysis of the predicted *trk* protein sequence reveals a stretch of 31 amino acids (amino acids 62–92) with similarity to a portion of complement component 3 (C3), which includes the site for its proteolytic cleavage to forms C3a and C3b (Müller-Eberhard 1988). For example, as shown in Figure 3, 13/31 residues are identical between this portion of *trk* and mouse C3 (Wetsel et al. 1984); moreover, highly conserved amino acids, which include the putative protease cleavage and binding sites (subdomains A and B), as well as the length of the intervening spacer region (S) between them (Mathias et al.

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Genes Dev. 1995, **9**:

Access the most recent version at doi:[10.1101/gad.9.20.2539](https://doi.org/10.1101/gad.9.20.2539)

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