

Variation in the number of activated torso receptors correlates with differential gene expression

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SUMMARY

Activation of receptor tyrosine kinases triggers many developmental decisions, yet we do not understand how activation of a single receptor can be transduced into different cell responses. The torso pathway in *Drosophila* provides a model to address this issue since it generates more than one response in the embryo. The torso receptor tyrosine kinase is activated at the embryonic poles under the control of trunk, a protein with similarities to several types of extracellular growth factors. Activation of torso is responsible for the development of a variety of structures, whose appearance can be correlated with activation of at least two different genes along the terminal region. In this study we have analyzed mutations in *torso* and *trunk* that

express low levels of the respective proteins. We show that different amounts of torso or trunk molecules correlate with the expression of different zygotic genes, implicating changes in the number of activated torso molecules as one of the mechanisms defining differential gene expression. We suggest that variation in the number of activated receptors at the cell surface is a general mechanism that leads to differential gene expression and thus the generation of different cell responses.

Key words: torso, trunk, signal transduction, gene expression, *Drosophila*, receptor tyrosinase kinase

INTRODUCTION

Receptor tyrosine kinases are involved in many developmental processes where they can elicit a variety of responses. A basic question in trying to understand their role in development is how activation of a single receptor can be transduced into different cell responses in terms of gene activation (for a review, see Marshall, 1995). The torso pathway in *Drosophila* is a particularly appropriate model with which to address this issue owing to its ability to generate more than one response in the embryo. The torso receptor tyrosine kinase is distributed along all the surface of the *Drosophila* embryo but is only activated at the poles, where it specifies development of its terminal structures (Schüpbach and Wieschaus, 1986; Klinger et al., 1988; Casanova and Struhl, 1989; Sprenger et al., 1989). Activation of the tor receptor has been shown to depend on a diffusible extracellular ligand generated at each pole that is trapped by the receptor, thereby impeding further diffusion (Sprenger and Nüsslein-Volhard, 1992; Casanova and Struhl, 1993). Recently, we have cloned *trunk* (*trk*), one of the genes required for tor activation, and have shown that it codes for a protein with similarities to several types of extracellular growth factors, which is likely to be the torso ligand (Casanova et al., 1995).

Activation of the torso signal transduction pathway is responsible for the activation of *tailless* (*tll*) and *huckebein* (*hkb*) at the posterior end of the embryo (Weigel et al., 1990). However, while *tll* is expressed in all this region, *hkb* expression is activated only in its most terminal part (Pignoni

et al., 1990; Brönner and Jäckle, 1991). One mechanism allowing differential gene expression could be an instructive torso activity gradient. Using mutations in *torso* in which activation is ligand-independent it has been possible to correlate distinct levels of tor signaling with differential gene activation (Casanova and Struhl, 1989): low levels of the constitutive torso activity are able to activate *tll* gene expression whereas only high levels can activate *hkb* expression. However, it remains an open question whether such a mechanism is also used during normal development when wild-type torso is only activated at the poles. Alternatively, a gradient of tor receptor activity may not be responsible for organizing the terminal pattern. Instead, a sharp step function of active tor receptor at the cell surface could generate one or more gradients of activated intracellular substrates that could diffuse within the cytoplasm and specify the distinct elements of the terminal pattern (Casanova and Struhl, 1989).

Extra copies of either the *tor* gene or the *trk* gene do not alter the embryonic pattern, as judged by monitoring the expression of zygotic genes (Casanova and Struhl, 1989; our own unpublished observations), indicating that in those conditions they are not limiting factors. The molecular analysis of *trk* is consistent with this observation since it suggests that the *trk* protein, as many growth factors, needs to be processed to be active (Casanova et al., 1995). Overexpression of unprocessed *trk* could lead to minor changes in the amount of active *trk* only relevant in situations or domains where the tor receptor is a limiting factor.

To investigate whether variations in the number of activated tor receptors at the cell surface might be responsible for distinct gene activation, we have generated situations in which the number of receptors or ligand molecules could be limiting. In particular, we have identified mutations that would give rise to low levels of either wild-type trk molecules secreted by the embryo or wild-type tor receptors at the cell surface, and have characterized their phenotypes. We used these mutations to demonstrate that different amounts of torso or trunk molecules correlate with expression of different zygotic genes, implicating changes in the number of activated torso molecules as one of the mechanisms defining differential gene expression.

MATERIALS AND METHODS

Stocks

trk¹, *trk⁴*, *trk⁵* and *tor^{HM}* are EMS mutations induced by Trudi Schüpbach (Lindsley and Zimm). *trk¹* results from a premature stop codon and behaves as a null allele (Casanova et al., 1995). *tor^{XR1}* is a small deficiency that deletes the *tor* gene (Sprenger et al., 1989). Df(2L)J77 is a deficiency spanning from 31C to 31F (T. Schüpbach, personal communication) that uncovers the *trk* gene. TR-11 is a third chromosome carrying a 6 kb genomic fragment that fully rescues the loss-of-function mutations in the *trk* gene. Egg laying took place at 25°C.

RNA analysis

Poly(A)⁺ RNA was obtained from embryos with the PolyAtract System (Promega), run in a 1.2% agarose gel containing formaldehyde and transferred to Hybond N (Amersham). Hybridization was performed with a *trk*-specific riboprobe derived from the antisense strand of the pR3 *trk* cDNA at 60°C in 50% formamide.

Sequencing

Genomic DNAs from *trk⁴*, *trk⁵* and *tor^{HM}* alleles, as well as genomic DNA from the isogenic stocks in which the mutations were induced, were PCR-amplified and sequenced. Two independent PCR products were sequenced in all the coding regions for each mutant strain and compared to the parental wild-type DNA in which the mutations were induced. For *trk⁴*, the only change that resulted in an amino acid substitution affected the codon for amino acid 18, which was changed from GGG (glycine) to AGG (arginine). No change was observed between the *trk⁵* DNA and the parental wild-type DNA. In the *tor^{HM}* allele, the only mutation that resulted in an amino acid change affected the codon for amino acid 11, which was changed from ATC (isoleucine) to AAC (asparagine).

Plasmid constructions

pBtor

pBtor has been described by Sprenger and Nüsslein-Volhard (1992).

pBHM

The mutation detected in the *tor^{HM}* allele was introduced into the pBtor cDNA by PCR. Using the oligos FS41 TTCCATGGT-TATTTTCTATGCGAAGTACGCATTTAACTT and FS2 CCAATTGCATTTTGTCTCCTGCC and pBtor as a template, a 1750 bp fragment was amplified, cut with *NcoI* and *BstXI*, and the resulting 528 bp fragment exchanged with the corresponding fragment of pBtor, yielding pBHM. In this vector the PCR-derived fragment was sequenced to exclude PCR-induced base changes.

TR11

A *BamHI-EcoRI* genomic fragment of around 6 kb from the *trk* region was inserted into the P(ry⁺) transformation vector C20. This genomic

fragment fully rescues the phenotype associated with the *trk* mutations.

In situ hybridization

ill and *hkb* probes were generated from cDNA clones provided by J. Lengyel and H. Jäckle, respectively. Whole-mount in situ hybridizations were done following the method of Tautz and Pfeifle (1989) with minor modifications.

Western analysis

Protein extracts were separated by SDS-PAGE, transferred to Immobilon membrane, and probed with anti-torso antibodies as described (Sprenger and Nüsslein-Volhard, 1992). Each lane contains material from approximately 20 eggs.

RESULTS

Different amounts of the trk product can trigger different embryonic responses

trk⁵ is a hypomorphic mutant allele. Embryos from *trk⁵* females display a gradation of phenotypes ranging from *trk⁻* null phenotypes to almost wild-type phenotypes. It is worth noting that those embryos with partial terminal development always lack the most terminal structures, those that are thought to require higher levels of terminal signaling. We have sequenced the DNA of the *trk* coding region from this mutant and have not found any change when compared to the wild-

Table 1. Dose-dependance of *trk⁵* and *tor^{HM}* phenotypes

Maternal genotype	Size of terminal structure (% of embryos)			n
	A7 segment	A8 segment	More terminal	
1 copy of <i>trk⁵</i>	22	76	1	143
2 copies of <i>trk⁵</i>	12	37	51	65
1 copy of <i>tor^{HM}</i>	76	24	0	41
2 copies of <i>tor^{HM}</i>	46	41	14	37

tor^{HM} phenotypes are dependent on the dose of trk

Maternal genotype	Copies of <i>tor^{HM}</i>	Copies of <i>trk⁺</i>	Percentage of embryos whose most posterior structures belong to			n
			A7 segment	A8 segment	More terminal	
1	1	98	2	0	48	
1	2	76	24	0	41	
1	3	61	39	0	41	
2	1	42	53	6	36	
2	2	46	41	14	37	
2	3	9	14	77	37	

Cuticles from embryos derived from females of the appropriate phenotypes were examined and classified according to whether their more terminal structures belonged to the seventh abdominal segment (A7), to the eighth abdominal segment (A8), or were of more terminal origin such as the anal pads and tuft. n, number of embryos analysed for every genotype. Egg laying took place at 25°C.

The actual genotypes for each combination are as follows:

1 copy of *trk⁵*; *trk⁵*/Df Jder 77 (similar results were obtained for *trk⁵/trk¹*).

2 copies of *trk⁵*; *trk⁵* homozygous.

1 copy of *tor^{HM}* and 1 copy of *trk⁺*; *trk¹tor^{HM}/tor^{XR1}*.

1 copy of *tor^{HM}* and 2 copies of *trk⁺*; *trk¹tor^{HM}*; TR11.

1 copy of *tor^{HM}* and 3 copies of *trk⁺*; *tor^{HM}/tor^{XR1}*; TR11.

2 copies of *tor^{HM}* and 1 copy of *trk⁺*; *trk¹tor^{HM}*; TR11.

2 copies of *tor^{HM}* and 2 copies of *trk⁺*; *trk¹tor^{HM}/tor^{HM}*; TR11.

2 copies of *tor^{HM}* and 3 copies of *trk⁺*; *tor^{HM}*; TR11.

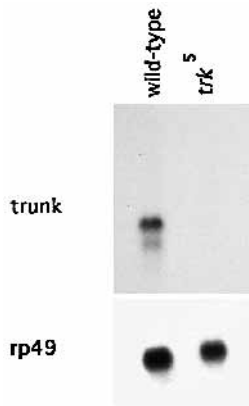


Fig. 1. Analysis of *trk* message from the *trk*⁵ mutants. Northern blot of poly(A)⁺ RNA from wild-type and *trk*⁵ mutant embryos from 0 to 3 hours after fertilisation hybridised with a labelled riboprobe derived from the antisense strand of *trk* cDNA. In *trk*⁵ embryos the *trk* transcripts (Casanova et al., 1995) are barely detectable. The blot was also probed for *rp49* transcripts to confirm the integrity of each RNA sample.

type gene. However, a northern blot reveals that the *trk* message is hardly detectable in embryos from *trk*⁵ females (Fig. 1), which suggests that a reduced amount of otherwise wild-type product is causing the mutant phenotype associated with the *trk*⁵ allele. Interestingly, the range of terminal deletions is dependent on the number of copies of the *trk*⁵ allele: embryos from females carrying two copies of the *trk*⁵ allele tend to have more terminal structures than those from females with just one copy of the *trk*⁵ allele (Table 1; see table legend for exact phenotypes). Hence, we can correlate the amount of *trk* product with the appearance of different terminal structures and therefore with the level of tor signaling. Similar results were obtained with *trk*⁴, another hypomorphic mutant allele with a single amino acid substitution of a glycine for arginine at position 18 in the putative signal sequence (data not shown).

To extend this correlation to differential gene activation we have examined the activity of *tll* and *hkb* in those embryos by in situ hybridization. As mentioned before, tor signaling is solely responsible for activating the expression of the genes *tll* and *hkb* at the posterior end of the embryo (Weigel et al., 1990). Accordingly neither *tll* nor *hkb* expression is detected at the posterior pole of *trk*⁻ embryos (Fig. 2C,D), as it occurs in *tor*⁻ embryos (Brönner and Jäckle, 1991). We have examined independently *hkb* and *tll* expression in embryos from *trk*⁵/*trk*⁻ and *trk*⁵ homozygous females. Embryos from mothers of the same mutant genotype (either *trk*⁵/*trk*⁻ or *trk*⁵ homozygous) exhibit some variability in their pattern of *tll* and *hkb* expression, in agreement with the variability observed in the cuticle structures that they develop (see Table 1). However, while *tll* and *hkb* expression is detected in some embryos from *trk*⁵ homozygous females (Fig. 2G,H), only *tll* expression but not *hkb* expression is detected in some embryos from *trk*⁵/*trk*⁻ females (Fig. 2E,F). It is worth noting that the domain of *tll* expression is often reduced in those embryos, suggesting that fewer tor receptors have been activated by the ligand diffusing from the poles. Thus, the amount of *trk* product from one copy of *trk*⁵ can trigger tor

signaling to the levels required to activate *tll* but not *hkb*. Conversely, the amount of *trk* product from two copies of *trk*⁵ can trigger tor signaling to the levels required to activate both *tll* and *hkb*.

Different numbers of tor receptors activated at the cell surface can originate diverse terminal structures

tor^{HM} is a temperature-sensitive allele in which no terminal structures are present when embryos are raised at high temperatures (27°C). At 18°C, on the other hand, terminal structures are present (Sprenger, 1991). *tor*^{HM} encodes a protein with a single amino acid substitution of an asparagine for isoleucine at position 11 in the signal sequence. This change results in a significant alteration of the hydrophobicity of the leader peptide. In western blots the majority of *tor*^{HM} migrates differently from the mature wild-type protein that is glycosylated (F. S., unpublished data) and with an electrophoretic mobility that compares to the non-glycosylated form (Fig. 3) (for details see legend to Fig. 3). When embryos are developed at lower temperatures, in which terminal structures are present, small amounts of *tor*^{HM} protein can be

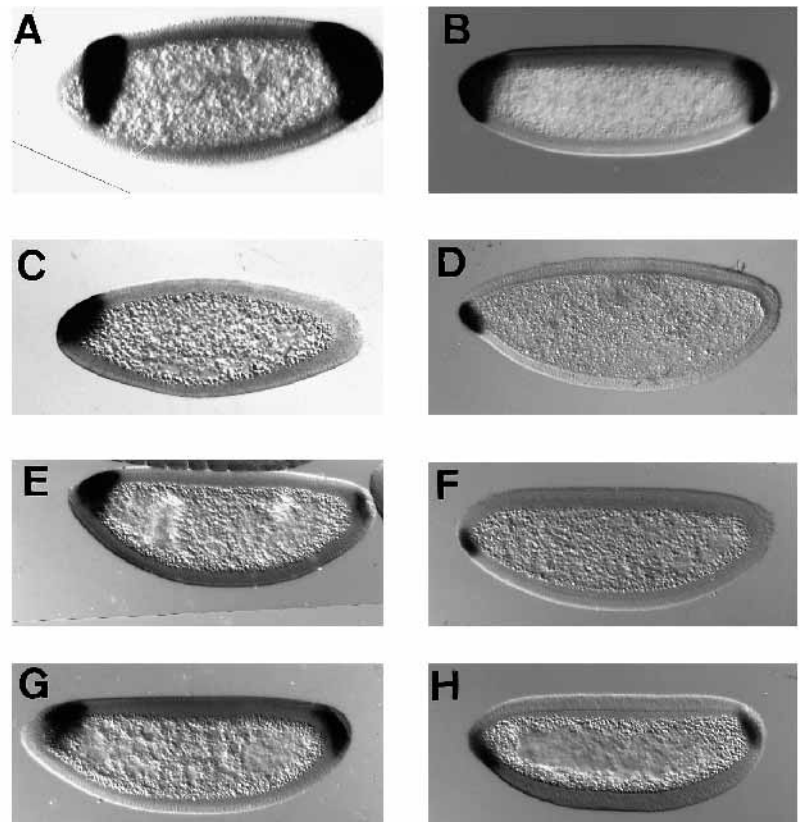


Fig. 2. *tll* and *hkb* expression in *trk* mutant embryos. *tll* (A,C,E,G) and *hkb* (B,D,F,H) expression, as monitored by in situ hybridisation. (A,B) At the blastoderm stage, *tll* and *hkb* are expressed at both poles of the embryo. (C,D) In embryos from *trk*¹ mothers, neither *tll* nor *hkb* expression is detected in the posterior pole; expression remains in the anterior domain, since at the anterior end the terminal signal acts in conjunction with the anterior system. (E,F) Embryos from females with one copy of the *trk*⁵ allele (*trk*⁵/*trk*¹). *tll* expression is detected in some of those embryos; however, *hkb* expression is not detected. (G,H) Embryos from females with two copies of the *trk*⁵ allele (*trk*⁵ homozygous); both *tll* and *hkb* expression can be detected among those embryos.

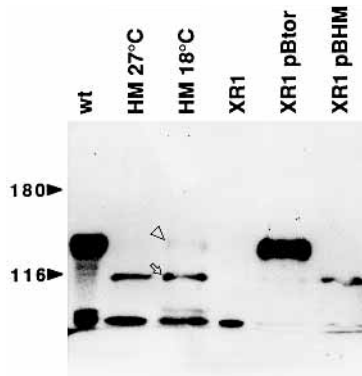


Fig. 3. Torso expression in *tor*^{HM} mutant eggs. wt, 0-3 hour wild-type eggs (22°C). HM 27°C, 0-3 hour eggs from *tor*^{HM} homozygous mothers developed at 27°C. HM 18°C, 0-4 hour eggs from *tor*^{HM} homozygous mothers developed at 18°C. XR1, 0-3 hour eggs (22°C) from *tor*^{XR1} homozygous mothers that lack torso protein. XR1 pBtor, 0-1 hour eggs from *tor*^{XR1} homozygous mothers were injected with 1 mg/ml pBtor mRNA, encoding wild-type torso protein, and incubated for 2 hours at 22°C. XR1 pBHM, 0-1 hour eggs from *tor*^{XR1} homozygous mothers were injected with 1 mg/ml pBHM mRNA, encoding the *tor*^{HM} protein, and incubated for 2 hours at 22°C. Most of the torso protein of embryos from *tor*^{HM} homozygous mothers migrates with faster electrophoretic mobility than wild-type torso. The approximate size of this *tor*^{HM} protein (arrow) compares to the non-glycosylated form of torso. However, at 18°C, a little bit of wild-type sized torso can be detected (arrowhead). To show that the change found in the sequence of *tor*^{HM} is solely responsible for the *tor*^{HM} phenotype, the mutation was introduced into the pBtor cDNA, the resulting cDNA (pBHM) transcribed in vitro and the RNA injected into embryos that lack endogenous torso. In these embryos, the *tor*^{HM} produced from the injected RNA migrated with the same electrophoretic mobility as torso from embryos mutant for *tor*^{HM}. In addition, only a little induction of terminal structures was observed, similar to *tor*^{HM} mutant embryos developed at 22°C (not shown).

detected that migrate with the same electrophoretic mobility as wild-type torso. However, the majority of the *tor*^{HM} protein still migrates like non-glycosylated wild-type torso molecules. This suggests that the first amino acids of the *tor*^{HM} molecule are a very poor leader peptide. The majority of protein does therefore not get glycosylated and does not get incorporated into the cytoplasmic membrane. Only at lower temperatures is a small fraction of *tor*^{HM} apparently glycosylated and presumably reaches the cytoplasmic membrane.

The *tor*^{HM} mutant phenotype, similar to the *trk*⁵ mutation, is dose-sensitive (Table 1). Interestingly, the *tor*^{HM} phenotype is very sensitive to the dose of the *trk* gene. We have analyzed the phenotype of embryos from females at 25°C carrying either one or two copies of the *tor*^{HM} gene and varying doses of the *trk* gene (Table 1). For a given number of tor receptors, an increase in the doses of *trk* results in the appearance of more terminal structures, indicating that a higher level of terminal signaling is being generated. Under these experimental conditions, the sole variation lies in the number of tor or trk molecules, implying that it is the different number of activated tor receptors that dictates the expression of the distinct genes responsible for the determination of diverse terminal structures.

DISCUSSION

The torso signal transduction and generation of pattern

Activation of the torso pathway is responsible for specifying the terminal portions of the embryo, which include a variety of structures that appear in an organized pattern. The generation of this pattern involves the activation of at least two different genes along the terminal region. An open question is how activation of the torso receptor can lead to differential gene expression. By using mutations coding for constitutive torso receptors it has been possible to show that the level of active tor protein can be causally related to the specification of particular portions of the terminal pattern (Casanova and Struhl, 1989). However, it has been pointed out that these results should be interpreted with caution, as mutant proteins with altered activity may represent unique situations that a wild-type form of the tor protein may not be capable of achieving (Duffy and Perrimon, 1994). Here we report the identification of mutations that either reduce the amount of trk molecules or that lower the amount of tor receptors at the cell surface. These mutations allow us to show that variation in the number of activated torso receptors can be correlated with differential expression of the distinct genes responsible for the determination of diverse terminal structures. Probably other factors are also involved in translating the tor signal into the proper pattern. For instance, diffusion of intracellular phosphorylated substrates could also generate an instructive gradient specifying different pattern elements. Moreover, many experiments suggest that uniform levels of ubiquitous tor activity (Casanova and Struhl, 1989) or uniform levels of signal transducers (Casanova et al., 1994) can generate organized pattern. These observations suggest that the terminal signal can also be modulated by its interaction with other molecules present in the embryo (e.g. gap gene products) to give rise to differential gene expression. In wild-type development all these mechanisms might cooperate to translate the terminal signal into distinct domains of gene expression responsible for organization of the terminal pattern.

Variation in the number of activated receptors at the cell surface: a general mechanism to elicit distinct responses from cells?

In this report, we have shown that by modulating the amount of receptor and activating molecules we can elicit different responses in cells, in terms of their gene activation. Recently, results have been reported showing that stimulation of over-expressed EGF receptor can mimic the cellular response to activation of the NGF receptor (Traverse et al., 1994). Conversely, reduction in the amount of the NGF receptor can mimic the cellular response to EGF receptor activation (Schlessinger and Bar-Sagi, 1995). This behavior has been interpreted as a quantitative difference in ERK activation that is translated into a qualitative difference in transcription factor activation, but a limitation of this model is that it is based wholly on 'in vitro' experiments with PC12 cells (Marshall, 1995). In addition, many other transducing signaling pathways operating in development have been shown to generate graded responses. This is the case for the dorsoventral patterning in *Drosophila* (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989; Chasan and Anderson, 1993), embryonic ventral

ectoderm determination also in *Drosophila* (Schweitzer et al., 1995) and for patterning of vulval fates in *Caenorhabditis elegans* (Sternberg and Horvitz, 1986; Hill and Sternberg, 1993). Here we document the case of the terminal system where activation of a unique transduction pathway can elicit at least two responses in terms of gene activation and we show this by altering the number of activated receptors at the cell surface. We would like to suggest that variation in the number of activated receptors, by changes either in ligand concentration or in the amount of receptor present at the cell surface, could represent a general mechanism to elicit distinct responses from cells.

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