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Quantitative Variations in the Level of MAPK Activity Control Patterning of the Embryonic Termini in *Drosophila*

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We have examined the role in patterning of quantitative variations of MAPK activity in signaling from the *Drosophila* Torso (Tor) receptor tyrosine kinase (RTK). Activation of Tor at the embryonic termini leads to differential expression of the genes *tailless* and *huckebein*. We demonstrate, using a series of mutations in the signal transducers Corkscrew/SHP-2 and D-Raf, that quantitative variations in the magnitude of MAPK activity trigger both qualitatively and quantitatively distinct transcriptional responses. We also demonstrate that two chimeric receptors, Tor^{extracellular}-Egfr^{cytoplasmic} and Tor^{extracellular}-Sev^{cytoplasmic}, cannot fully functionally replace the wild-type Tor receptor, revealing that the precise activation of MAPK involves not only the number of activated RTK molecules but also the magnitude of the signal generated by the RTK cytoplasmic domain. Altogether, our results illustrate how a gradient of MAPK activity controls differential gene expression and, thus, the establishment of various cell fates. We discuss the roles of quantitative mechanisms in defining RTK specificity. © 1999 Academic Press

INTRODUCTION

One of the poorly understood aspects of receptor tyrosine kinase (RTK) signaling pathways is the basis of their specificities. An extremely wide array of developmental decisions are initiated by RTK activation which in turn regulates the activity of a signal transduction pathway commonly referred to as the "RTK signaling cassette" (see reviews by Egan and Weinberg, 1993; Perrimon, 1994; Dickson and Hafen, 1994). This cassette includes a series of "adapter" molecules, with and without catalytic functions, that upon association with the activated receptor, regulate the level of Ras-GTP in the cell. In turn, an increase in the level of Ras-GTP activates the Raf, MEK, and MAPK serine/threonine kinase cascade. The invariant nature of the RTK signaling cassette underscores the importance of determining the molecular mechanisms that underlie the generation of signaling specificity between RTKs. Three models have been put forward to explain this diversity (see Li and Perrimon, 1997, for review and references). The "qualitative model" proposes that the effects elicited by the activation of specific RTKs are specified by the array of transcription factors present in the cell. The "quantitative model" proposes that specificity arises from differences in the magnitude and/or duration of MAPK activation. In addition to these two models, the molecular basis of RTK specificity may reflect the unique abilities of these pathways to "cross-talk" with other signaling pathways.

The *Drosophila* Torso (Tor) or terminal system is a unique pathway to investigate the basis of RTK specificity (see reviews by Perrimon, 1993; Duffy and Perrimon, 1994). The *tor* mRNA is maternally provided and its translation, following fertilization, results in a uniform distribution of Tor receptors in the membrane of the early syncytial embryo (Casanova and Struhl, 1989; Sprenger *et al.*, 1989). At both embryonic termini, Tor receptors, likely activated by the Trunk protein (Casanova *et al.*, 1995), control the formation of terminal structures. The ligand activity is diffusible, located in the perivitelline space at each embryonic pole, and appears, unlike the Tor receptor, to be limiting in amount (Sprenger and Nusslein-Volhard, 1992; Casanova and Struhl, 1993). In this system, trapping of the ligand by Tor prevents its diffusion and ensures a spatially localized activation of the receptor. A number of studies

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have suggested that different levels of Tor or Ras activity specify distinct terminal structures (Casanova and Struhl, 1989; Hou *et al.*, 1995; Furiols *et al.*, 1996; Greenwood and Struhl, 1997). At the posterior pole, high levels of Tor activity specify the formation of the most posterior structures (e.g., the posterior midgut) while lower levels of Tor activity specify more anterior structures (e.g., the A8 denticle belt). Molecularly, this graded effect can be visualized by following the expression of *tailless* (*tll*) and *huckebein* (*hkb*) where, at the posterior end of the embryo, their expression is dependent solely on Tor activity (Weigel *et al.*, 1990). The domain of expression of *hkb* is smaller than that of *tll* (Pignoni *et al.*, 1990; Bronner and Jaeckle, 1991) presumably because higher levels of Tor activity are required to activate *hkb* expression than *tll* expression. *tll* and *hkb* encode transcription factors that regulate, at the posterior, the expression of a number of additional downstream target genes such as *hunchback* (*hb*) and *forkhead* (*fkh*) (see review in Perkins and Perrimon, 1991).

The molecular mechanism by which a gradient of activated Tor leads to differential gene activation, and thus the generation of different cell fates, is not well understood. In this paper we have tested if, by generating different gradients of signaling cassette activity, differential expression of *tll* and *hkb* can be obtained. To generate quantitative variations in the level of Tor signaling, we have used a series of mutations in the nonreceptor protein tyrosine phosphatase Corkscrew (Csw; aka SHP-2; Perkins *et al.*, 1992, 1996) and the serine/threonine kinase D-Raf (Ambrosio *et al.*, 1989; Melnick *et al.*, 1993), two components of the signaling cassette that positively transduce the signal received by Tor. We present evidence that by modulating the magnitude of the activity of the RTK signaling cassette, and thus, its more downstream component MAPK, both qualitatively and quantitatively distinct transcriptional responses can be triggered. Furthermore, to address whether the ability of Tor to deliver a precise quantitative signal is imprinted in the cytoplasmic domain of the RTK, we have examined the signaling activities of two chimeric RTKs which contain the Tor extracellular domain fused to the cytoplasmic signaling region of either the *Drosophila* epidermal growth factor (EGF) RTK (Egfr) or the *Drosophila* Sevenless (Sev) RTK. Previous analyses have shown that to signal both the EGF and the Sev RTKs requires the activities of Drk, Sos, Csw, Dos, Ksr, Gap1, Ras1, D-Raf, MEK, and MAPK (see review by Perrimon and Perkins, 1997, for references). Because these same signaling components are also required in Tor signaling (see review by Perrimon, 1993; Hou *et al.*, 1995), we expected that the chimeric receptors would be able to substitute for the wild-type Tor RTK. Interestingly, we found that these chimeric RTKs activate the RTK signaling cassette; however, they cannot fully functionally replace the wild-type Tor receptor. We conclude that a precise activation of MAPK by a specific RTK is determined by both the number of RTK molecules activated at the cell surface and the magnitude of the signal generated by the cytoplasmic domain of the activated RTK. Our results highlight the role of a quantitative mechanism in defining RTK specificity.

MATERIALS AND METHODS

Germline Mosaic Analyses

In this study a number of *D-raf* and *csw* mutations that are associated with different levels of activity were used. csw^{VA199} and $csw^{e13d.3}$ are null and near-null *csw* alleles, respectively, while csw^{6} and csw^{19-106} are associated with residual activity (Perkins *et al.*, 1992, 1996). $D\text{-}raf^{EA75}$ is a null allele, and both $D\text{-}raf^{C110}$ and $D\text{-}raf^{PB26}$ are weak alleles with residual activity (Melnick *et al.*, 1993). Germline clones of *csw* and/or *D-raf* mutations were generated using the following chromosomes: csw^{VA199} FRT^{101}, $csw^{e13d.3}$ FRT^{101}, csw^{6} FRT^{101}, csw^{19-106} FRT^{101}, $D\text{-}raf^{EA75}$ FRT^{101}, $D\text{-}raf^{C110}$ FRT^{101}, $D\text{-}raf^{PB26}$ FRT^{101}, csw^{VA199} $D\text{-}raf^{EA75}$ FRT^{101}, csw^{VA199} $D\text{-}raf^{PB26}$ FRT^{101}, csw^{6} $D\text{-}raf^{EA75}$ FRT^{101}, and csw^{6} $D\text{-}raf^{C110}$ FRT^{101}. Germline mosaics were generated using the "Flp-DFS" technique as previously described (Chou and Perrimon, 1992). Females carrying germline clones (GLCs) were crossed with wild-type males and egg collections were performed at 25°C.

The maternal effect phenotypes associated with *csw* and *D-raf* mutations are paternally rescuable to some extent (Perkins *et al.*, 1992, 1996; Melnick *et al.*, 1993). This paternal rescue is detectable after gastrulation and does not affect *tll, hkb,* and *hb* gene expression at the blastoderm stage.

Construction of P[tor-Egfr] and P[tor-sev]

To remove the intracellular domain of *tor,* the 11.5-kb *Eco*RI–*Eco*RI genomic *tor* DNA fragment, which was shown previously to fully rescue the *tor* null phenotype (Casanova and Struhl, 1989; Cleghon *et al.*, 1996), was digested with *Xmn*I and *Ase*I. The intracellular domain of *Egfr* (a gift from A. Michelson) was amplified by PCR using oligonucleotides containing *Xmn*I (for the 5′ end) and *Ase*I (for the 3′ end) sites. The intracellular domain of *Sev* (a gift from G. Rubin) was amplified by PCR using oligonucleotides containing *Sna*BI (for the 5′ end) and *Nde*I (for the 3′ end) sites, both of which have compatible ends with *Xmn*I and *Ase*I, respectively. After verification of the PCR-generated fragments by sequencing, the intracellular Egfr and Sev fragments were cloned onto the genomic *tor* DNA containing the entire extracellular and transmembrane domains; the precise fusion junctions occur three amino acids after the *tor* transmembrane domain.

These chimeric constructs were then cloned into CaSpeR4, which contains the marker mini-white (Thummel *et al.*, 1988). P-element-mediated transformation was performed according to Spradling (1986) following injection into the delta 2-3 transposase strain (Robertson *et al.*, 1988). Several stably transformed lines were obtained and subsequently used to generate flies that carry various copies of the *P[tor-Egfr]* or *P[tor-sev]* insertions in a tor^{XR1} background. tor^{XR1} is a deletion which entirely eliminates production of *tor* RNA and protein (Sprenger *et al.*, 1989, 1993). Similar results were obtained with multiple independent transformed lines (data not shown).

Embryonic Stainings

In situ hybridizations on whole-mount embryos using *tll* digoxygenin-labeled probes were performed according to Tautz and

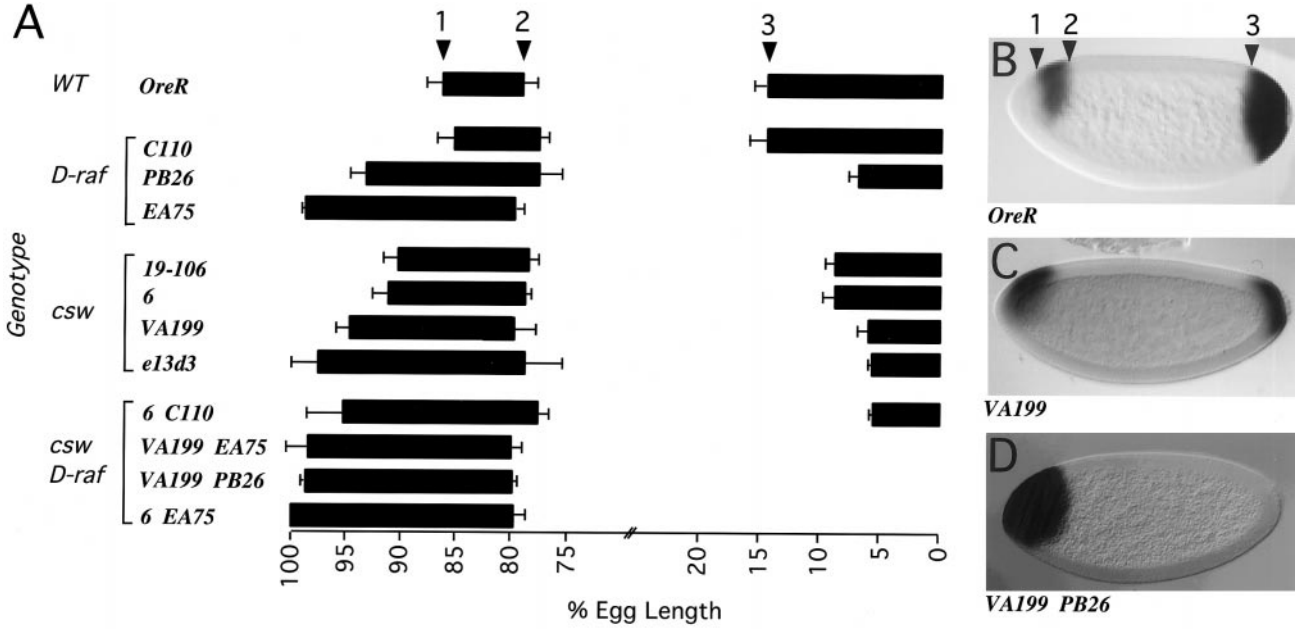

FIG. 1. The levels of *csw* and *D-raf* gene activities determine the domains of *tll* expression. The expression patterns of *tll* RNA in wild-type, *csw*, *D-raf*, and *csw D-raf* embryos, at the blastoderm stage, are presented both graphically (A) and in mutant embryos (B, C, and D). The limits of *tll* expression are denoted by numbered arrowheads, with 1 and 2 corresponding to the anterior and posterior limits of the anterior *tll* domain, respectively, and 3 corresponding to the anterior limit of the posterior domain of *tll* expression. In wild type, *tll* is expressed both anteriorly and posteriorly (A, B). With the exception of $D\text{-}raf^{C110}$, which is indistinguishable from wild type, in all single mutants for both *csw* (csw^{VA199}, $csw^{e13d.3}$, csw^{6}, csw^{19-106}) and *D-raf* ($D\text{-}raf^{EA75}$, $D\text{-}raf^{PB26}$) posterior *tll* expression is reduced and anterior *tll* expression is expanded (A, C). In two *csw D-raf* double-mutant combinations (csw^{6} $D\text{-}raf^{C110}$, csw^{VA199} $D\text{-}raf^{PB26}$) *tll* expression is reduced to a greater degree than either single mutant alone (A, D) In the remaining two *csw D-raf* double-mutant combinations (csw^{6} $D\text{-}raf^{EA75}$, csw^{VA199} $D\text{-}raf^{EA75}$) *tll* expression is indistinguishable from $D\text{-}raf^{EA75}$ alone. Of the various mutations tested, embryos mutant for either csw^{VA199} or $D\text{-}raf^{EA75}$ are completely devoid of Csw and D-raf activities, respectively. Note that 0% egg length corresponds to the posterior pole (right) and 100% egg length to the anterior pole (left); dorsal is up.

Pfeifle (1989). DNA probes were prepared by PCR labeling (N. Patel, personal communication) from plasmids containing the *tll* cDNA (Pignoni *et al.*, 1990, 1992).

The Hb (a gift from C. Desplan and E. Vimmer) and Hkb (a gift from C. Doe; McDonald and Doe, 1997) antibodies were used at 1:2000 and 1:150 dilutions, respectively. The stainings were visualized using the Vectastain ABC Kit (Vector Laboratories).

The monoclonal antibody against activated MAPK (dp-ERK; a gift from L. Gabay and B. Shilo) was used at a 1:500 dilution. Fixation of embryos was performed as described in Gabay *et al.* (1997a,b). The HRP detection utilized a biotinylated anti-mouse antibody at 1:2000 dilution followed by the Vectastain ABC Kit plus the TSA-Indirect (NEN), the latter to ensure efficient staining. The TSA-Indirect amplification system was essential in the detection of anti-dp-ERK staining in *csw* mutant and *tor-sev* embryos.

For visualization, embryos were dehydrated through an ethanol series, mounted in Euparal (Carolina Biological Supply) or mounted directly in 80% glycerol, and photographed under Nomarski optics with a Zeiss Axiophot microscope. When the expression domains of the different markers were measured, an average of 30 late syncytial blastoderm-staged embryos were scored in each experiment. The domains of expression were determined as the percentage of egg length (EL), 0% EL corresponding to the posterior embryonic pole and 100% EL to the anterior pole.

Embryonic cuticles were prepared according to van der Meer (1977) and visualized using dark-field optics.

RESULTS

Quantitative Variations in the Level of Activity of Csw and D-Raf Triggers Qualitatively Distinct Transcriptional and Morphological Responses

To determine whether the quantitative level of activation of the RTK signaling cassette determines the domains of activation of downstream target genes, we have carefully examined the domains of expression of both *tll* and *hb* using a number of *D-raf* and *csw* mutations, as well as double-mutant combinations. *tll* is one of the first zygotic genes to be regulated directly by Tor activity. At the posterior ends of wild-type embryos at the syncytial blastoderm stage, *tll* is expressed from 0 to 20% EL and resolves by the cellular blastoderm stage to between 0 and 15% EL (Pignoni *et al.*, 1990; Figs. 1A and 5A). As a second marker for terminal activity, we followed the expression of *hb*, which posteriorly, at blastoderm stages, begins as a cap that

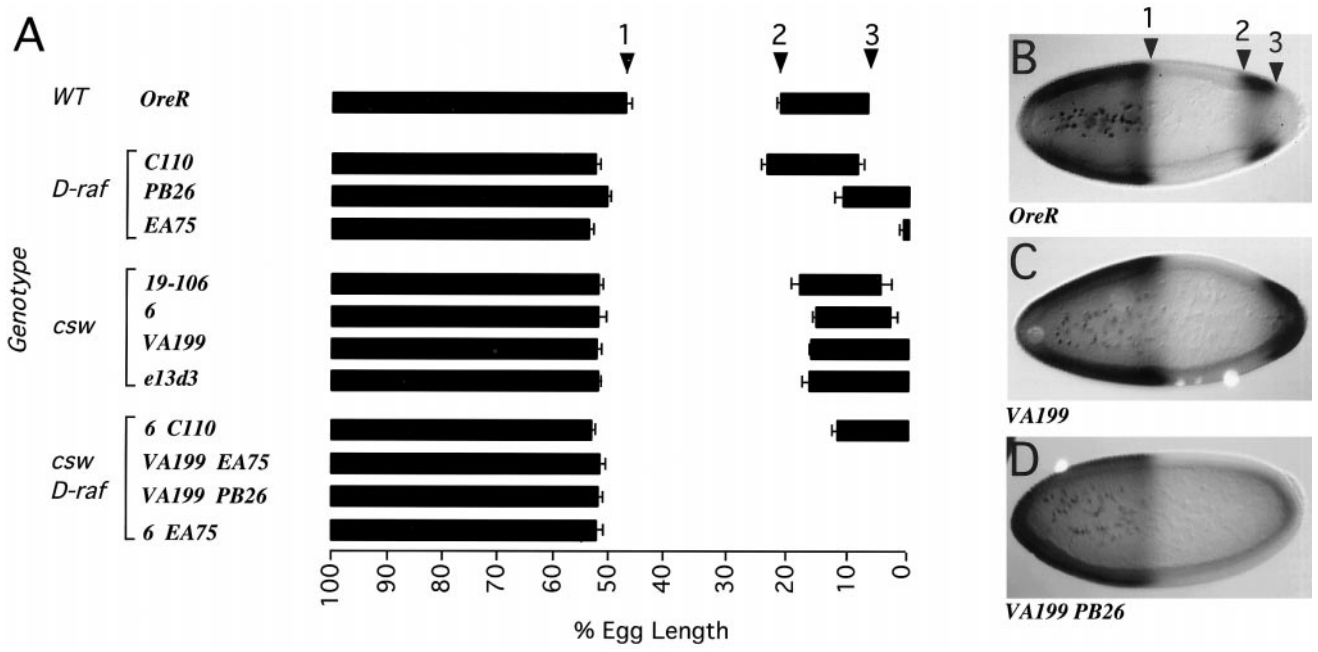

FIG. 2. The levels of *csw* and *D-raf* gene activities determine the domains of Hb expression. The expression patterns of Hb protein in wild type, *csw*, *D-raf*, and *csw D-raf* embryos, at the blastoderm stage, are presented both graphically (A) and in mutant embryos (B, C, and D). The limits of Hb expression are denoted by numbered arrowheads, with 1 corresponding to the posterior limit of the anterior Hb domain and 2 and 3 corresponding to the anterior and posterior limits of the posterior domain of Hb expression, respectively. In wild type, Hb is expressed both anteriorly and posteriorly (A, B). With the exception of $D\text{-}raf^{C110}$, which is indistinguishable from wild type, in all single mutants for both *csw* (csw^{VA199}, $csw^{e13d.3}$, csw^{6}, csw^{19-106}) and *D-raf* ($D\text{-}raf^{EA75}$, $D\text{-}raf^{PB26}$) posterior Hb expression is shifted posteriorly and reduced (A, C). In all *csw D-raf* double-mutant combinations (csw^{6} $D\text{-}raf^{C110}$, csw^{VA199} $D\text{-}raf^{PB26}$, csw^{6} $D\text{-}raf^{EA75}$, csw^{VA199} $D\text{-}raf^{EA75}$) Hb expression is shifted and reduced to a greater degree than either single mutant alone (A, D). In all cases the posterior limit of the anterior Hb expression domain remains near to that observed in wild type (A).

subsequently is repressed by Hkb at the posterior pole to resolve into a single stripe from 10 to 20% EL (Tautz *et al.*, 1987; Casanova, 1990; Figs. 2B and 5C). Previous studies have shown that *hb* expression is under the control of the terminal system since in *tor* mutant embryos, or *tll hkb* doubly mutant embryos, posterior *hb* expression is absent (Tautz *et al.*, 1987; Casanova, 1990).

Previous analyses have demonstrated that the Tor signal, in its entirety, is mediated through D-Raf; i.e., D-raf is both necessary and sufficient for Tor signaling. First, in embryos containing complete loss-of-function mutations of either *D-raf* (Fig. 1A) or *tor*, posterior *tll* expression is completely eliminated (see review by Duffy and Perrimon, 1994). Second, the effect of gain-of-function *tor* mutations that lead to an expansion of both *tll* expression and posterior terminal structures is suppressed by complete removal of *D-raf* activity (Ambrosio *et al.*, 1989). Third, expression of an activated form of either *Drosophila* or mammalian Raf in *tor* mutant embryos is sufficient to rescue posterior *tll* expression as well as to promote the development of terminal structures (Casanova *et al.*, 1994; A. Brand, X. Lu, and N. Perrimon, unpublished data). Finally, consistent with biochemical data demonstrating that MAPK activity is regulated by MEK, which itself is activated by Raf, activation of MAPK, as revealed with the dp-ERK antibody, is completely absent in embryos mutant for either *tor* or *D-raf* (Gabay *et al.*, 1997a,b; this study, see below).

Unlike D-raf, Csw activity is only partially required for transducing the Tor signal (Perkins *et al.*, 1992). Further, a number of experiments support a model that during Tor signaling Csw functions downstream of Torso and upstream of Ras1, D-raf, and MAPK (Perkins *et al.*, 1992, 1996; Lu *et al.*, 1993; Cleghon *et al.*, 1996, 1998). First, the effect of gain-of-function *tor* mutations that lead to an expansion of expression of both *hkb* as well as posterior terminal structures is suppressed by removal of *csw* activity (Perkins *et al.*, 1992, Perkins *et al.*, 1996). Second, activated Ras is able to rescue *hkb* expression in *csw* mutant embryos (Lu *et al.*, 1993). Third, activation of MAPK, as revealed with the dp-ERK antibody, is reduced at posterior termini of *csw* mutant embryos (this study, see below). Fourth, we have found that the zygotic lethality associated with *csw* mutations can be rescued by the activated MAPK allele *Sevenmaker* (Brunner *et al.*, 1994). These *csw/Y; Sem/+* males are viable and fertile and can be used to generate *csw/csw; Sem/+* females (L. A. Perkins, unpublished data). Larvae

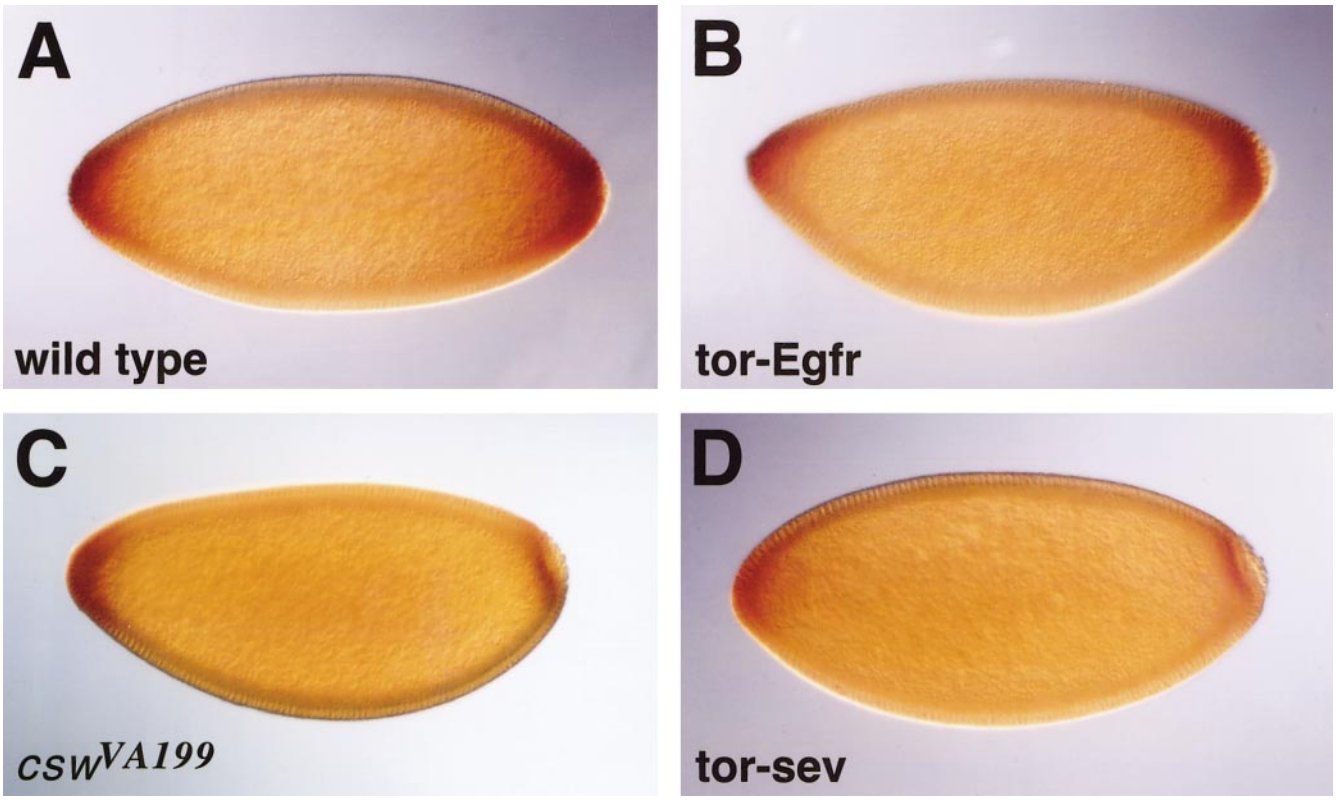

FIG. 3. The magnitude of MAPK activity directly reflects the magnitude of Tor signaling. At the blastoderm stage the expression pattern of activated MAPK, detected by dp-Erk (see Materials and Methods), is revealed in wild-type (A), tor-Egfr (B), csw^{VA199} (C), and tor-Sev (D) embryos. All embryos are oriented with anterior to the left and dorsal up.

with no terminal defects can be generated from such females, which is consistent with the model that Csw regulates the level of MAPK activity in the Tor pathway. Finally, Cleghon *et al.* (1996, 1998) have shown that Csw associates with pY630 of Tor. This association results in phosphorylation of a conserved tyrosine residue (Y666) in the C-terminal tail of Csw, which then serves as a binding site for Drk/Grb2, a known regulator of Ras activity. The physical association between the activated Tor RTK and Csw also allows Csw to specifically dephosphorylate the negative pY918 site of Tor, which binds RasGAP, thus effectively lowering the local concentration of RasGAP and thereby potentiating a positive signal.

For the reasons detailed above, in this study we have used mutations in both *D-raf* and *csw* as a means to regulate the level of activated MAPK activity in embryos. Specifically, we have characterized the effect of two hypomorphic *D-raf* alleles ($D\text{-}raf^{C110}$ and $D\text{-}raf^{PB26}$; Melnick *et al.*, 1993; Hou *et al.*, 1995) on terminal development. By examining the domains of expression of both *tll* RNA and Hb protein expression in embryos derived from GLCs, we could not detect any obvious defects in terminal development in the presence of the $D\text{-}raf^{C110}$ mutation (Figs. 1A and 2A). However, in embryos derived from $D\text{-}raf^{PB26}$ GLCs there is a pronounced reduction in terminal activity as demonstrated by a reduction in posterior *tll* expression (Fig. 1A) and a shift in the posterior domain of Hb (Fig. 2A).

Complete removal of Csw activity from the germ line (csw^{VA199} and $csw^{e13d.3}$) reveals results similar to $D\text{-}raf^{PB26}$ (Figs. 1A and 1B), and the Hb domain is shifted posteriorly (Figs. 2A and 2B). Characterization of two weak alleles of Csw (csw^{6} and csw^{19-106}) reveals similar, yet less severe reduction of *tll* expression (Fig. 1A) and shift of the Hb domain (Fig. 2A). Collectively, the data presented above, from analyses of single mutations in either *csw* or *D-raf*, suggest that a distinct transcriptional response is elicited with each mutant activity.

To determine whether a reduction in both *D-raf* and *csw* gene activities could act synergistically, we analyzed the expression of *tll* and Hb in double-mutant combinations. In each double-mutant combination tested, posterior expression of both *tll* and Hb were severely reduced compared to either single mutant alone (Figs. 1A and 2A). For example, in GLC-derived embryos doubly mutant for the hypomorphic alleles csw^{6} and $D\text{-}raf^{C110}$, *tll* expression is reduced to a greater extent than for either single mutant alone (Fig. 1A). Furthermore, in the same genetic background, Hb protein, which is expressed as a stripe of posterior expression in each

single mutant, is shifted posteriorly, where it appears as a "cap" in the csw^{6} $D\text{-}raf^{C110}$ mutant embryos. Similarly, while both *tll* and Hb are entirely deleted from GLC-derived embryos doubly mutant for csw^{VA199} and $D\text{-}raf^{PB26}$ (Figs. 1A, 1D, 2A, and 2D), both of these markers are present, though reduced and mispositioned, in each single mutant alone (Figs. 1 and 2). These analyses of GLC-derived doubly mutant embryos suggest that the magnitude of the transcriptional response is a cumulative event determined by the relative activities of the upstream signal transducers.

To demonstrate that the gradients of activity observed in various *csw* and *D-raf* mutant backgrounds reflect a gradient of MAPK activity, we used an antibody that detects only the activated form of MAPK, dp-ERK (Gabay *et al.*, 1997a,b). As previously reported, at the blastoderm stage dp-ERK is localized in two terminal caps reflecting the activation of MAPK by the Tor pathway (Gabay *et al.*, 1997b; Fig. 3A). While in the absence of *D-raf* activity no staining was observed at the posterior pole (data not shown), in *csw* null mutant embryos, where the *tll* and Hb expression domains are present though mispositioned (see above), reduced levels of dp-ERK reactivity were observed (Fig. 3C). This latter result differs from that of Gabay *et al.* (1997a), who reported no dp-ERK reactivity in *csw* mutant embryos. This discrepancy likely reflects the enhanced detection system utilized in our analysis (see Materials and Methods).

Differential Gene Expression Correlates with the Generation of Different Cell Responses

In the experiments described above, the transcriptional response or gene expression of *tll* and Hb was monitored as readouts for terminal activity. When development is allowed to proceed, none (with the exception of $D\text{-}raf^{C110}$) of the *csw* and *D-raf* singly and doubly mutant embryos derived from GLCs hatch into larvae; i.e., they are 100% embryonic lethal. When the cuticles of the dead embryos are examined it is apparent that the different transcriptional responses observed above correlate nicely with the generation of different cell responses. For example, the cuticle of a $D\text{-}raf^{C110}$ mutant embryo (Fig. 4A) is indistinguishable from wild type in that all pattern elements posterior to abdominal segment 7 are present and appear normal. When terminal activity is progressively removed (Figs. 4B, 4C, and 4D), there is a corresponding progressive malformation and eventual loss of terminal cuticular structures. The first terminal cuticular elements malformed/lost require the highest terminal activation (e.g., the anal tuft and posterior spiracles visualized by the presence of Filzkorper material; Fig. 4B). The next elements malformed/lost require intermediate levels of the terminal signal (e.g., the abdominal 8 (A8) denticle belt and the posterior spiracles; Fig. 4C). Finally, the last elements malformed/lost require the lowest levels of terminal activity (e.g., posterior A7; Fig. 4D). Collectively, these results reveal that a precise transcriptional response translates into a specific cell identify.

In summary, the results presented above support the model whereby Tor triggers a gradient of signaling activity, mediated by both Csw and D-raf, which modulates the level of MAPK activity that in turn determines a precise transcriptional response. It is the precise transcriptional response, the magnitude of which is a cumulative event determined by the relative activities of the upstream signal transducers, that ultimately generates the identity of each cell under the control of the Tor signaling pathway.

The Kinase Domain of an RTK Determines the Precise Level of Activation of the MAPK Pathway

What are the parameters which are critical for the precise activation of MAPK and the domains of expression of downstream target genes? Previous analyses have shown that the domains of Tor activation are defined by the number of receptors which become activated by a ligand which is limited in amount (Sprenger and Nusslein-Volhard, 1992; Casanova and Struhl, 1993). However, we wanted to test whether, in addition, the ability of Tor to trigger the appropriate magnitude of activation of the signaling cassette is imprinted in the RTK cytoplasmic domain. To test this hypothesis, we constructed two different chimeric RTKs, $Tor^{extracellular}$-$Egfr^{cytoplasmic}$ and $Tor^{extracellular}$-$Sev^{cytoplasmic}$. To ensure that these receptors are activated only in the appropriate Tor domains, each chimera encoded the extracellular and transmembrane domains of Tor. These receptors, *P[tor-Egfr]* or *P[tor-sev]*, stably transformed into tor^{XR1} animals were expressed under the control of the *tor* promoter to express them at levels comparable to wild-type Tor. Finally, their signaling activities were assessed by examining *tll*, Hkb, and Hb expression in progeny from tor^{XR1} homozygous females carrying various copies of either *P[tor-Egfr]* or *P[tor-sev]*.

The tor-Egfr chimera. When a single copy of *P[tor-Egfr]* is introduced into tor^{XR1}-homozygous females, embryos from these females express *tll* between 0 and 11% EL (Fig. 5D), in contrast to the wild-type situation in which *tll* is expressed from 0 to 15% EL. This domain of expression can be increased to 0–11.5% when a second copy of *P[tor-Egfr]* is introduced, but no further increase is observed by the addition of up to four copies of *P[tor-Egfr]*. These results contrast with those previously reported in which a single copy of *P[tor]* is sufficient to provide wild-type expression of *tll* in the 0–15% EL domain (Casanova and Struhl, 1989; Cleghon *et al.*, 1996).

Similar results are obtained when the posterior expression domain of Hkb is used as a measure of the signaling ability of the chimeric RTK. In wild-type embryos, Hkb is expressed as two polar caps, the posterior domain extending from 0 to 8.3% EL (Fig. 5B). In embryos derived from females carrying two or more copies of *P[tor-Egfr]*, this posterior domain extends no further than 0–4.5% EL (Fig. 5E).

A third measurement of the signaling ability of the chimeric RTK is the posterior expression domain of Hb. In

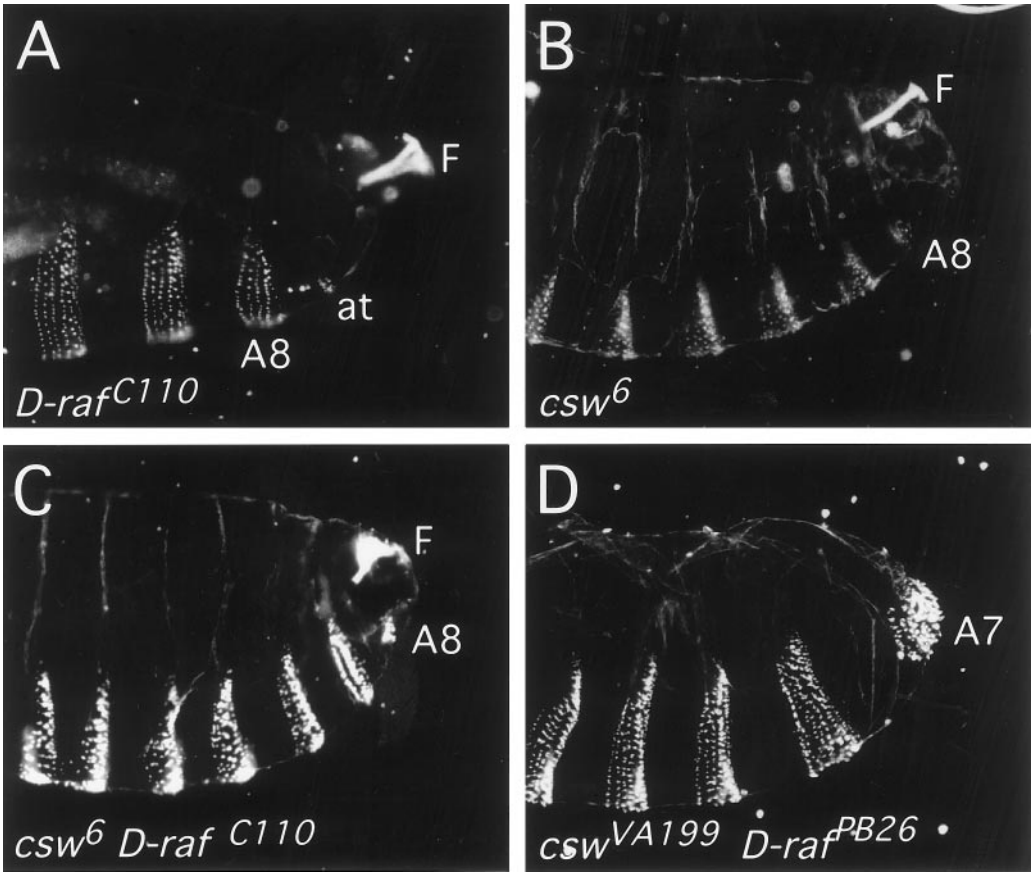

FIG. 4. The extent to which posterior pattern elements are formed reflects directly the magnitude of the Tor signal. Shown are dark-field photographs of cuticular preparations of the posterior ends of paternally rescued embryos derived from females bearing GLCs of genotypes *D-raf*^{C110} (A), *csw*^{6} (B), *csw*^{6} *D-raf*^{C110} (C), and *csw*^{VA199} *D-raf*^{PB26} (D). Note that the cuticle of *D-raf*^{C110} is indistinguishable from wild type in that all posterior pattern elements are present. As the magnitude of the Tor signal is progressively reduced by mutation (B > C > D; relative Tor signaling activities are also shown in Figs. 1 and 2), so is the extent to which posterior cuticular structures are present. All cuticles are oriented with anterior to the left and dorsal up. Abbreviations: A7, A8, abdominal denticles belts 7 and 8, respectively; at, anal tuft; F, Filzkorper material.

embryos derived from females carrying two or more copies of *P[tor-Egfr],* Hb does not retract from the posterior pole, but rather remains as a terminal cap (Fig. 5F). Further, the anterior border of this posterior Hb domain is shifted posteriorly. This altered pattern of Hb expression is like that observed in *csw*^{VA199} mutant embryos (compare Figs. 2C and 5F), which presumably is due to the absence of, or only weak, repression by Hkb (in *csw*^{VA199} mutant embryos the posterior *hkb* domain is entirely deleted or only a few cells at the posterior pole are occasionally Hkb positive; Perkins *et al.*, 1996; this report).

Collectively, examination of the domains of expression of *tll*, Hkb, and Hb, used as readouts of the magnitude of receptor signaling, demonstrate that in the presence of the Tor-Egfr chimeric receptor the magnitude of the Tor signal is decreased. These results are consistent with the reduced levels of dp-ERK reactivity that we observe in embryos derived from females carrying two copies of *P[tor-Egfr]* (Fig. 3B). In these experiments the components that regulate the activity of the Tor-Egfr chimera, i.e., the Tor ligand and the Tor extracellular region, are the same as those that activate wild-type Tor, suggesting that the cytoplasmic domain of the Egfr is less efficient than the Tor cytoplasmic domain at transducing the signal. In addition, the fact that we do not observe changes toward the wild-type condition of the *tll*, Hkb, or Hb domains when more than two copies of *P[tor-Egfr]* are added is consistent with the fact that the ligand for Tor is present in a limiting amount (see also Sprenger and Nusslein-Volhard, 1992; Casanova and Struhl, 1993).

The tor-sev chimera. In experiments similar to those described above, when a single copy of the *P[tor-sev]* chimera is introduced into *tor*^{XR1}-homozygous females, approximately 15% of the resulting embryos express *tll* in a posterior domain composed of a few cells and not extending

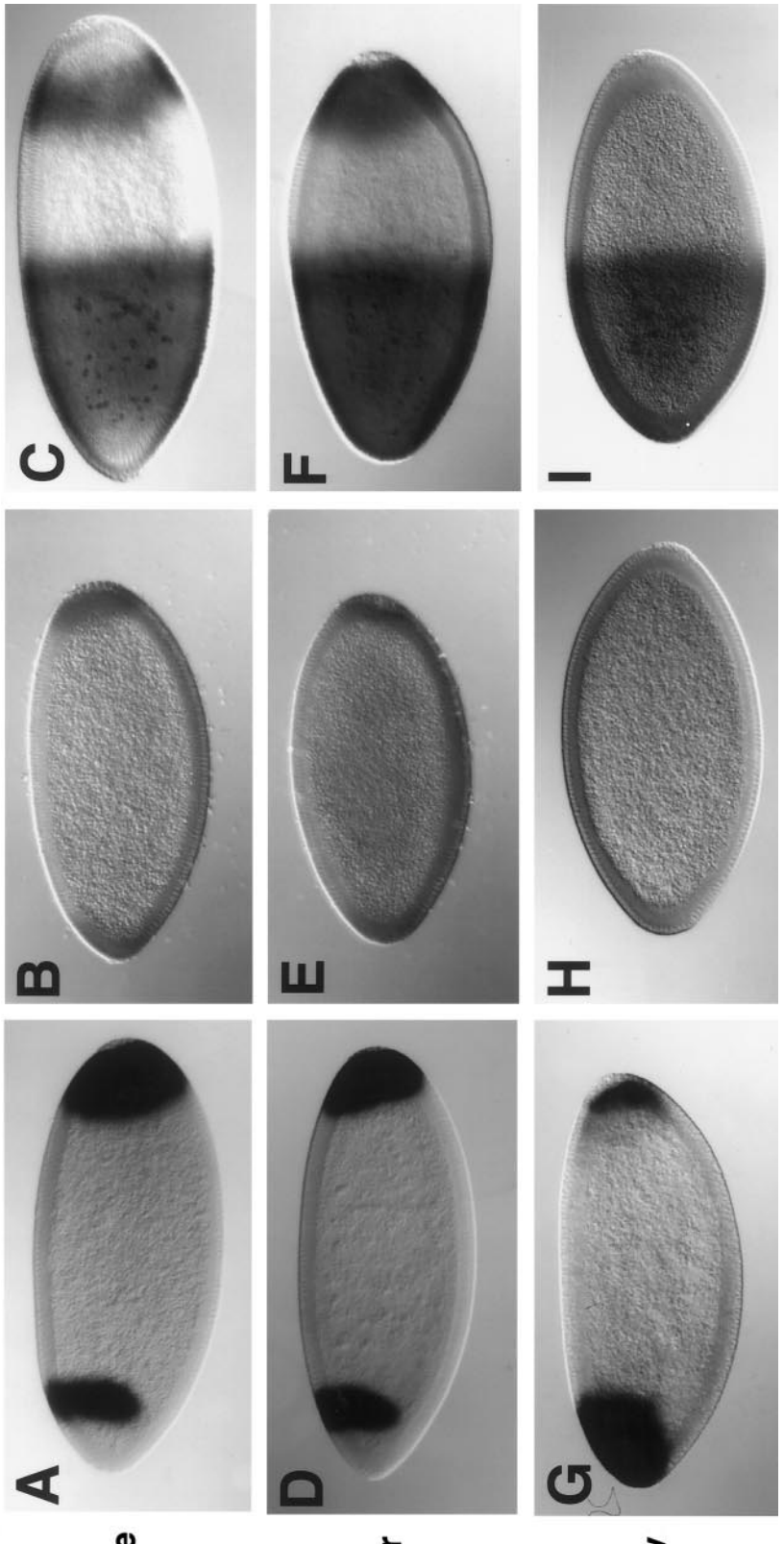

FIG. 5. The cytoplasmic domains of the EGF and Sev RTKs are less efficient than the Tor cytoplasmic domain at transducing the Tor signal. Shown are blastoderm-stage embryos that reveal the expression patterns of *tll* RNA (A, D, and G), Hkb protein (B, E, and H), or Hb protein (C, F, and I). Compared to wild type (A, B, C), the expression patterns of *tll* + Hkb, and Hb are significantly reduced in embryos derived from tor^{XR1} mutant mothers carrying either four copies of *P[tor-Egfr]* (D, E, F) or two copies of *P[tor-sev]* (G, H, I). Note that these terminal-specific markers are reduced to a greater extent in embryos derived from *P[tor-sev]* females than in the case of *P[tor-Egfr]*, reflecting the efficiency to which these chimeric RTKs transduce the Tor signal. All embryos are oriented with anterior to the left.

beyond 0–3% EL. With two copies of this chimera, approximately 30% of the embryos express *tll* from 0 to 4% EL (Fig. 5G). At the anterior termini, *tll* expression is expanded toward the anterior pole (Fig. 5G). Likewise, in embryos derived from females carrying two copies of *P[tor-sev],* Hkb expression is completely deleted both anteriorly and posteriorly (Fig. 5H). Further, in embryos derived from females carrying two copies of *P[tor-sev],* posterior Hb expression is completely deleted (Fig. 5I). Note that in embryos derived from either Tor-Egfr or Tor-Sev females, the posterior position of the anterior domain of Hb expression is similar to that of wild type and remains at approximately 50% EL (compare Figs. 5F and 5I with Fig. 5C).

Altogether these results suggest that the cytoplasmic domain of the Sev RTK is much less efficient than either the Tor or the Egfr cytoplasmic domains at transducing the signal. This result is also apparent when embryos derived from *P[tor-sev]* females are stained with dp-ERK antibody, as the staining is much weaker than in the presence of *P[tor-Egfr]* (Fig. 3D compared to Fig. 3B). In conclusion, our data indicate that, to correctly specify cell fates at each embryonic termini, not only do the correct number of receptors need to become activated, but in addition another important parameter resides in the magnitude of the signal triggered by each activated receptor.

DISCUSSION

Graded Activation of the RTK Signaling Cassette by Tor

In this article we have used mutations in both *D-raf* and *csw* as a means to regulate the level of MAPK activity in embryos. Using this experimental design, we demonstrate that a precise activation of MAPK is critical to establish the appropriate domains of expression of downstream target genes such as *tll* and *hkb* at the posterior pole. The interpretation of our results relies, in part, on the model that both Csw and D-Raf regulate the level of MAPK activity to define the domains of downstream target genes. As discussed under Results we believe that this assumption in the Tor pathway is valid as there is no evidence in this system for MAPK-independent pathways that regulate either *tll* or *hkb* expression.

At the point of the receptor there are at least two different levels of control. First, as shown by Casanova and Struhl (1989), and later by Furriols *et al.* (1996), variations in the number of activated Tor molecules correlate with both differential gene expression of downstream target genes and the generation of different cell responses or fates. In this paper it is shown that the strength or magnitude of the signal emanating from activated Tor receptors, the number of which is controlled by a ligand which is limiting in amount, is imprinted in the cytoplasmic domain of the RTK itself. This latter level of control is apparent from our observations that chimeric receptors, either $Tor^{extracellular}$-$Egfr^{cytoplasmic}$ or $Tor^{extracellular}$-$Sev^{cytoplasmic}$, cannot fully functionally replace the wild-type Tor receptor. It should be emphasized that the interpretation of these results relies on the assumption that the chimeric receptor molecules are able to achieve efficient transphosphorylation following dimerization. We believe that the possibility that dimerization of the chimeric receptors does not position the catalytic domains of the receptors in the right orientation to achieve efficient transphosphorylation is unlikely. First, results obtained following experimentation with numerous RTK chimeras have detected no problems in transphosphorylation. For example, chimeras between the extracellular domain of the EGFR and the tyrosine kinase domains of the Axl (McCloskey *et al.*, 1994), LTK (Ueno *et al.*, 1995), PDGFR (Seedorf *et al.*, 1991), and JAK2 (Nakamura *et al.*, 1996) tyrosine kinases have been generated and found to transphosphorylate extremely well. Additional examples include chimeras between the insulin receptor extracellular and the EGFR cytoplasmic domains (Riedel *et al.*, 1986) and the Trk extracellular domain fused to the insulin receptor cytoplasmic domain (Isakoff *et al.*, 1996). Second, structural studies suggest that the cytoplasmic domains of RTKs are probably able to rotate freely relative to the transmembrane domain (S. Hubbard, personal communication; D. Stern. personal communication). However, Burke and Stern (1998) have reported that structural constraints do exist between the transmembrane domain of the Neu RTK and the extracellular domain. This is not a problem in this study since in both of the chimeras utilized the transmembrane domain of Tor, along with its native extracellular domain, have been conserved; i.e., the fusion occurs within the cytoplasmic domain three amino acids downstream of the transmembrane domain.

Our finding that the strength or magnitude of the signal emanating from activated Tor receptors is imprinted in the cytoplasmic domain of the RTK is consistent with previous studies with a cytoplasmic Tor mutation, Y630F (Cleghon *et al.*, 1996). The Y630F mutation on Tor specifically eliminates the interaction between Tor and Csw following receptor activation and results in a decrease in the posterior domains of expression of both *tll* and *hkb*. In both cases, the Tor Y630F mutation and the chimeric RTKs, the results are the same; i.e., the magnitude of the Tor signal is decreased, as is MAPK activation, the domains of downstream target genes are altered, and ultimately fewer terminal cell fates are specified. Our analysis agrees with the recent study by Greenwood and Struhl (1997) who showed that different levels of Ras activity specify distinct terminal structures.

One possible way to generate differing magnitudes of activity from the cytoplasmic domains of specific RTKs might be dependent on the specific affinities of the downstream signal transducers to the receptor. Csw binds through one of its SH2 domains to only one phosphotyrosine on Tor. Perhaps a higher or lower affinity of Csw to this site, or addition of another site that would also engage the second SH2 domain of Csw, would increase or decrease the signal output. Alternatively, differing magnitudes of activity from the cytoplasmic domains of specific RTKs

might be dependent on the constellations of downstream transducers recruited to the receptor following activation. For example, although Drk/Grb2 has been shown to bind Sev at a single phosphotyrosine residue (Raabe *et al.*, 1995), it does not associate directly with Tor (Cleghon *et al.*, 1996). Similarly, although Csw association with Tor is mediated by a phosphotyrosyl residue and dependent upon receptor activation, the association between Csw and Sev, observed biochemically following immunoprecipitation, is not dependent upon the presence of a phosphotyrosine (Allard *et al.*, 1996; Herbst *et al.*, 1996; see Perrimon and Perkins, 1997, for discussion). Clearly, although the players may be conserved, the molecular mechanisms by which RTKs propagate their specific signals vary with each receptor under consideration and these differences can easily be interrupted to alter the magnitude of the RTK signal.

Rather remarkably, the presence of a graded activity of Tor signaling, which can be visualized by modulating the levels of activity of at least two of the signal transducers (D-raf and Csw), suggests that the signaling cassette has built-in mechanisms to provide a precise quantitative output (see also Greenwood and Struhl, 1997). Further, this precise quantitative output can be interpreted at the level of the promoters of the downstream genes, *tll*, and *hkb* in the case of Tor. It has been hypothesized that the main function of the Raf-MEK-MAP kinase cascade, functioning downstream of Ras, is to amplify the RTK-initiated signal. However, since a number of studies have illustrated the existence of parallel signaling pathways emanating from an RTK (Hou *et al.*, 1995; Raabe *et al.*, 1995; Herbst *et al.*, 1986), an alternative hypothesis is that the Raf–MEK–MAP kinase cascade is a multistep means to integrate and precisely refine the signals received from multiple upstream parallel pathways.

Conservation of the RTK Signaling Cassette

Each of the chimeric RTKs used in this paper, both $Tor^{extracellular}$-$Egfr^{cytoplasmic}$ and $Tor^{extracellular}$-$Sev^{cytoplasmic}$, are able to partially activate the downstream response gene *tll*, indicating that a shared set of signaling molecules is utilized by the Tor, Sev, and Egfr RTKs. These observations are consistent with the previous analyses of Reichman-Fried *et al.* (1994) in which it was shown that chimeric receptors composed of a constitutively active $Tor^{Dominant}$ extracellular domain fused to the cytoplasmic domain of DFGF-R2 (aka Heartless), Egfr, Tor, or Sev were all able to partially rescue migration defects associated with a *DFGF-R1* (aka *breathless*) mutation. Similarly, using two of the same chimeras as above, Freeman (1996) found that the $Tor^{Dominant}$-Egfr chimera behaves similarly to the $Tor^{Dominant}$-Sev chimera in causing overrecruitment of R7 photoreceptors in the developing ommatida. The final readouts in these two analyses consisted of describing the phenotypic effects of unregulated, constitutively active chimeric RTKs.

We have extended these previous studies not only by analyzing the phenotypic effects of chimeric RTKs (Figs. 3 and 5), but, in addition, we have conducted quantitative analyses by measuring the magnitudes of activation of downstream target genes in a regulated system in which the receptors are activated by the endogenously produced ligand. Significantly, our results, unlike the previous studies, can be used to assess the relative signaling capabilities of the cytoplasmic domains of various RTKs as well as to determine the extent to which a quantitative mechanism of RTK activation plays a role in the ultimate outcome of a given signaling pathway. Finally, the Tor chimeric system that we have developed provides a powerful assay to characterize the function of specific motifs located in the cytoplasmic domain of the RTK. For example, it will be of interest to determine whether all of the signaling activity of the $Tor^{extracellular}$-$Sev^{cytoplasmic}$ chimeric RTK in the activation of *tll* is mediated through the single Drk/Grb2 binding site which has been identified (Raabe *et al.*, 1995).

A Gradient of MAPK Activation Underlies the Quantitative Mechanism

Our finding that different levels of MAPK activity can lead to different cell responses/fates is relevant to a number of studies conducted in PC12 cells (for review see Li and Perrimon, 1997). Addition of FGF or NGF to PC12 cells results in prolonged MAPK activation and nuclear translocation, which culminates in cessation of cell division and neuron differentiation. Conversely, addition of EGF to PC12 cells results in transient MAPK activation, does not affect MAPK nuclear translocation, and culminates in cell proliferation. The receptors of these growth factors, which likely use the same signaling pathway, clearly have very different effects on MAPK activity.

Like the Tor system, in PC12 cells it is the number of RTK molecules which become activated that determines both the extent to which MAPK is activated and the cellular outcome. Insulin added to PC12 cells, where the endogenous insulin receptor numbers are low, only transiently stimulates MAPK activation and acts as a mitogen (Dahmer and Perlman, 1988). However, when the insulin receptor is overexpressed, MAPK undergoes prolonged activation and nuclear translocation and the cells undergo neuronal differentiation (Dikic *et al.*, 1994), the same as if they were treated with NGF. Similarly, while normal activation of the endogenous EGF receptor results in proliferation, overexpression of the EGF receptor results in sustained MAPK activation and nuclear translocation (Traverse *et al.*, 1994).

Interpretation of Graded Levels of MAPK Activity

How the differing magnitudes of activity from the cytoplasmic domains of RTKs are integrated at the level of the promoters of the downstream response genes, such as *tll* and *hkb* in the Tor pathway, is still not resolved. Presumably, in each individual cell there exists a mechanism built into the enhancer elements of the promoters of both *tll* and *hkb* that acts to read directly the magnitude of Tor signal-

ing. In the *tll* promoter, a Tor-response element that mediates the repression of *tll* has been identified, indicating that the Tor signal activates *tll* by a mechanism of derepression (Liaw *et al.*, 1995). A putative candidate for this repressor activity is encoded by the transcription factor NTF-1 (ELF-1), the product of the *grainyhead* gene. NTF-1 binds to the Tor-response element and can be directly phosphorylated by MAPK *in vitro*, and a decrease in NTF-1 activity has been shown to cause *tll* expansion in early embryos (Liaw *et al.*, 1995). Further, the transcriptional corepressor Groucho (Gro) is required for terminal patterning, because embryos that lack maternal Gro activity show an expansion of both *tll* and *hkb* expression domains toward the center of the embryo (Paroush *et al.*, 1997). Further characterization of how NTF-1 and/or Gro activities are regulated by activated MAPK should clarify how differing levels of phosphorylation translate into derepression of terminal target genes.

Gradients of Gene Activity and Other RTKs

As the results in this paper detail, to correctly specify the various cell fates at the posterior embryonic termini not only do the correct number of Tor receptors need to become activated, but in addition, another important parameter resides in the magnitude of the signal generated by the Tor cytoplasmic domain. The *Drosophila* EGF receptor, Egfr, is another clear example whereby the degree to which the receptor becomes activated determines the fates of responsive cells. During embryogenesis, the Egfr, activated by the TGFα homolog Spitz (Spi), is responsible for determination of ventral ectodermal cell fates (Raz and Shilo, 1993). Spi is active as a processed, secreted form which is produced in the ventral midline and thus presented to the ubiquitously expressed Egfr as a gradient; i.e., cells closest to the midline are exposed to higher concentrations of Spi than are cells located farther from the midline (Schweitzer *et al.*, 1995a). While complete loss of Egfr function is associated with complete loss of ventral ectodermal cell fates, graded, intermediate levels of activation of the Egfr signaling pathway result in the loss of discrete sets of cell fates (see reviews by Perrimon and Perkins, 1997; Schweitzer and Shilo, 1997). In tissue culture it can be shown that activation of the Egfr, by secreted Spi, induces MAPK activation in a dosage-sensitive fashion; i.e., the magnitude of MAPK activation is dependent upon the dosage of Spi used (Schweitzer *et al.*, 1995b). Together, and like those for the Tor pathway, these results support a quantitative model for Egfr signaling whereby different ventral ectoderm cell fates are generated by the graded activation of the Egfr which in turn controls the magnitude to which MAPK becomes activated.

These observations on the Egfr signaling pathway in the embryonic ectoderm are similar to those in the *Drosophila* ovary where, again, graded activation of the Egfr organizes a gradient of gene expression (see review by Li and Perrimon, 1997). Clearly, other developmental systems will have to be carefully examined to gain a fuller understanding of the extent to which a quantitative mechanism of RTK activation plays a role in the ultimate outcomes of other signaling pathways.

Concluding Remarks

Both qualitative and quantitative mechanisms contribute to the specificity of the signal generated by the Tor RTK. First, the qualitative mechanism implies that specific signal transducers and transcription factors (e.g., NTF-1) be available in the embryo in order for responding nuclei to have the potential of activating the terminal-specific genes *tll* and *hkb*. Second, the quantitative mechanism implies that the magnitude of activation of the RTK signaling pathway determines a precise transcriptional response. In the Tor pathway the quantitative mechanism is a finely tuned process that generates a graded signal. The magnitude of this signal is determined by two parameters: (1) a ligand limited in amount and (2) the cumulative effect of each activated RTK molecule whose cytoplasmic domain is imprinted with an intrinsic signaling capability. Ultimately, it is the precise transcriptional response within each cell, under the control of the Tor signaling pathway, that determines specific cell fates.

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