Development 124, 3535-3541 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV4923

MAP kinase in situ activation atlas during *Drosophila* embryogenesis

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SUMMARY

Receptor tyrosine kinases (RTKs) and the signaling cascades that they trigger play central roles in diverse developmental processes. We describe the capacity to follow the active state of these signaling pathways in situ. This is achieved by monitoring, with a specific monoclonal antibody, the distribution of the active, dual phosphorylated form of MAP kinase (ERK). A dynamic pattern is observed during embryonic and larval phases of *Drosophila* development, which can be attributed, to a large extent, to the known RTKs. This specific detection has enabled us to determine the time of receptor activation, visualize gradients and boundaries of activation, and postulate the distribution of active ligands. Since the antibody was raised against the phosphorylated form of a conserved ERK peptide containing the TEY motif, this approach is applicable to a wide spectrum of multicellular organisms.

Key words: MAP kinase, ERK, receptor tyrosine kinase, *Drosophila*

INTRODUCTION

A wide range of processes involving intercellular communication is mediated by receptor tyrosine kinases (RTKs) and their signaling pathways. The growing list of processes regulated by these receptors across the phylogenetic tree is extremely broad, and includes induction of cell fates, guidance of cell and axon migration, and cell proliferation.

The diverse biological roles of RTKs are in contrast to the universal cytoplasmic signaling pathway that they activate. The receptors are activated by diffusible or membrane-anchored ligands and trigger, through adapter and GTP exchange factors, the activation of RAS. In turn, activated RAS induces a sequential activation of protein kinases, including RAF, MEK, ERK, RSK and, in some cases, also GSK3, collectively known as the MAPK signaling cascade (Seger and Krebs, 1995; Cobb and Goldsmith, 1995). This cascade may also be activated by non-RTK signaling pathways, such as those triggered by G-protein-coupled serpentine receptors (Dikic et al., 1996).

The cytoplasmic kinase cascade amplifies the signal elicited by the receptors. This amplification process is highly specific and tightly regulated. Thus, the activation of ERK is induced exclusively by the dual specificity kinase MEK, five minutes after receptor activation (Seger et al., 1992; Cobb and Goldsmith, 1995). In contrast to MEK, activated ERK has a wider range of substrates, both in the cytoplasm and in the nucleus. While the processes that lead to ERK activation are resolved, the down regulation events leading to ERK inactivation are less well understood, and appear to involve a battery of phosphatases including several that are ERK-specific (Sun et al., 1993).

ERK proteins are highly conserved in evolution, not only in multicellular organisms but also in single-celled organisms like yeast (Boulton et al., 1990; Levin and Errede, 1995). The nodal point they represent is exemplified by the fact that mutations in ERK genes give rise to phenotypes that are similar to those generated by loss of the RTKs that activate them. For example, ERK mutations in *Caenorhabditis elegans* lead to vulvaless phenotypes similar to *let*-23 EGF receptor mutations (Lackner et al., 1994), and in *Drosophila* to defects in photoreceptor differentiation in the eye, akin to EGF receptor and *sevenless* mutations (Biggs et al., 1994). These similar phenotypes also imply that there is no redundancy in signaling by ERK proteins. Indeed, in *Drosophila*, a single ERK gene has been identified and corresponds to the *rolled* locus (Biggs and Zipursky, 1992; Biggs et al., 1994). A complementary set of experiments showed that gain-of function *rolled* mutation activates pathways controlled by several RTKs (Brunner et al., 1994). Similar to ERK, a single *mek* gene was isolated in *Drosophila*, and shown to participate in several RTK pathways (Tsuda et al., 1993; Hsu and Perrimon, 1994).

Dissection of RTK signaling pathways has been achieved by isolation of genes and mutations, and their functions implied from the corresponding mutant phenotypes. While these analyses have provided a broad and comprehensive picture that could be compared between diverse organisms utilizing the same pathways, many issues remain open. The pattern and timing of RTK activation has so far been inferred indirectly from manipulations of temperature-sensitive alleles, dominant-negative constructs, mosaic clones and ligand expression patterns. Such analyses do not address, however, the extent of ligand diffusion and modulations that may occur downstream to the receptor, affecting the level and duration of activation. These are critical issues since in many cases, RTKs do not provide a simple on/off switch, and the final outcome is determined by the actual level of receptor activation. For example, it is believed that retinal axons projecting to the tectum are targeted by a finely tuned balance between opposing

expression gradients of the Mek4 receptor tyrosine kinase and ELF-1 ligand (Cheng et al., 1995; Nakamoto et al., 1996). The possibility of temporal overlap between different RTK pathways affecting the same tissue has been difficult to address. Finally, it is clear that there are novel RTK signaling pathways that have not been identified to date by genetic means or gene cloning, which may control central developmental programs.

In order to resolve the issues raised above, it is essential to follow the activation of RTK pathways in situ at the time of activation. RTK signaling pathways may be tackled in this way because the active intermediates in the kinase signaling cascade are covalently modified by phosphorylation and can thus be distinguished from the non-active forms. We decided to follow specifically the activated form of ERK, since ERK represents one of the terminal steps in the signaling cascade and should, therefore, provide a maximal level of signal amplification. The active form of ERK is generated by phosphorylation on both threonine and tyrosine that lie adjacent to each other, in the unique TEY sequence (termed 'activation loop') (Payne et al., 1991). Other members of the MAP kinase family (e.g. Jun kinase) have a different residue between the threonine and tyrosine, suggesting that it should be possible to identify specifically the active form of ERK with appropriate antibodies. The 'activation loop' of ERK is highly conserved, so that the same antibody may be used to detect this kinase in different species.

We describe the utilization of a monoclonal antibody raised against a peptide containing the double phosphorylated form of ERK (diphospho-ERK). Immunohistochemical staining demonstrates that the antibody specifically detects the activated form of ERK (Gabay et al., 1997). In the *Drosophila* embryo, a very dynamic staining pattern is observed, providing an atlas of activated ERK during development. This pattern can be largely attributed to the RTKs that have previously been identified including Torso, the EGF receptor (DER) and the two FGF receptors, Heartless and Breathless. The pattern of DER-induced diphospho-ERK during embryonic and larval development is dynamic, in accordance with the pleiotropic roles of DER (Gabay et al., 1997).

The ability to follow the activation pattern, rather than its consequences, provides novel insights into the precise time, place and level of signaling of these RTKs. These observations reveal the distribution of the active form of the respective ligands. This is especially informative in the case of receptors for which the ligand has not been identified, e.g. Heartless. Novel patterns of diphospho-ERK suggest the involvement of additional receptor-triggered cascades during embryogenesis. In view of the structural conservation of ERK, the capacity to follow signaling pathways in situ during development should be applicable to a wide range of species.

MATERIALS AND METHODS

Antibodies

The monoclonal activated MAPK (dp-ERK) antibody was kindly provided by Sigma. It was raised against the 11 amino acid peptide HTGFLT(Pi)EY(Pi)VAT corresponding to the phosphorylated form of the ERK-activation loop (Yung et al., 1997). Additional antibodies used include rabbit anti-β-Gal antibody (Cappel), rabbit anti-Twist antibody (obtained from S. Roth), rat anti-Trachealess antibody, guinea pig anti-Stripe antibody (obtained from T. Volk), and rabbit anti-Myosin antibody (obtained from P. Fisher). Secondary antibodies include HRP-conjugated goat anti-mouse IgG, and FITC or LRSC-conjugated goat anti-mouse IgG+IgM or anti-rabbit, guinea pig or rat IgG (Jackson labs).

Antibody staining

Special care was taken to fix only fresh embryos in 8% formaldehyde (fresh) and keep them in 100% methanol at −20°C. All washes were done with PBS, 0.1% Tween 20. For DAB staining involving two primary antibodies (to identify mutant embryos by absence of a balancer chromosome carrying *lac*Z), the dp-ERK antibody staining was processed first to ensure efficient staining. For fluorescent double stainings, the second primary antibody was added only after completion of washes of the dp-ERK secondary antibody, to avoid any cross reactivity. All other aspects of staining were standard. For co-staining with Hoechst dye (No. 33258, 1 μg/ml), embryos were incubated with dye for 5 minutes after completion of DAB staining, washed and mounted in aqueous mountant. The dye was visualized by UV fluorescence.

Fluorescent staining was monitored with a Biorad 1024 confocal microscope. Timing of stages during embryogenesis is calculated at 25°C, according to Campos-Ortega and Hartenstein (1985).

Fly strains

The following mutant lines were used: $Torso^{4021}$, $Torso^{Y9}$, tsl^{691}, $rho^{\Delta 38}$, $htl^{\Delta 30}$ and btl^{LG19}. For induction of ectopic expression the Gal4-expressing lines included *69B* (provided by A. Brand) and *UAS-bnl B4-2* (provided by M. Krasnow).

RESULTS

Torso-dependent ERK activation

Torso represents the initial RTK pathway that functions during embryogenesis and is responsible for inducing terminal cell fates (Sprenger et al., 1989; Perrimon, 1993). Activation of Torso by ligands during embryogenesis is spatially restricted by the Torso-like protein, deposited by the follicle cells at the anterior and posterior ends of the egg chamber (Savant-Bhonsale and Montell, 1993). A transient and graded activation of ERK is observed in both poles of the embryo (Fig. 1A,B). The Torso-dependent dp-ERK pattern is restricted to embryonic stage 4. Following the cycles of nuclear division, by co-staining with Hoechst dye, allowed a more accurate timing. The staining first appears at cycle 12, 2 hours and 15 minutes after egg lay (AEL). It peaks at cycle 13/14, 30 minutes later, and is diminished shortly afterwards, by the time cellularization is half way. Staining is not observed in the pole cells. As expected, dp-ERK is diminished in embryos derived from homozygous *torso-like* mutant females and expands uniformly in embryos derived from *Torso*-dominant females (Fig. 1C,D). It is interesting to note that, in the latter embryos, prominent dp-ERK staining is detected already at cycle 12. This may result from the constitutive activity of Torso, which is manifested prior to the normal activation by ligand.

The onset and termination of Torso activation were indirectly inferred from elegant rescue and dominant-negative experiments (Sprenger and Nusslein-Volhard, 1992). It was argued that the initiation of Torso activation should be highly regulated. Since the source of the ligand is emanating from the two poles, an excess of receptor molecules on the cell surface is essential to trap the released ligand and facilitate a graded profile of activation. The receptor molecules are derived by

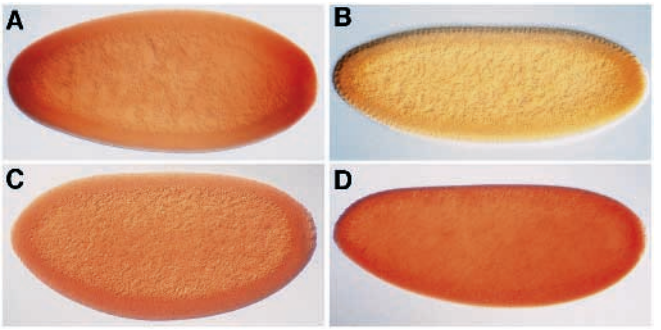

Fig. 1. Torso-dependent ERK activation. **(**A) In *wt* embryos prominent dp-ERK staining at both poles is detected at cycle 13/14. (B) During cellularization, after completion of cycle 14, dp-ERK diminishes. (C) In embryos derived from homozygous tsl^{691} females, no dp-ERK staining is detected at cycle 13/14. (D) In embryos derived from $Torso^{4021}$/ $Torso^{Y9}$ females, ubiquitous dp-ERK staining is detected already at cycle 12. This may result from the constitutive activity of Torso which is manifested prior to the normal activation by ligand.

translation of maternal *torso* RNA that is ubiquitously deposited in the egg. Indeed, we observed that ERK activation initiates abruptly at ~2 hours AEL. The rapid termination of signaling, 30 minutes after its onset, implies that the amount of Torso ligand or other activating components is limited, allowing only a restricted burst of receptor activation. Graded distribution of dp-ERK is observed in the two poles, in accordance with the capacity of Torso activation to induce nested sets of genes in different parts of the embryo, depending upon the level of activation (Furriols et al., 1996). This graded profile is generated prior to cellularization in the syncytial embryo, at stage 4. To maintain this pattern, restricted diffusion of dp-ERK or rapid dephosphorylation are required.

Heartless-dependent activation

During normal development, the invaginated mesoderm is initially clustered. It then spreads dorsally over the ectodermal surface during stages 7-8, to generate a single mesodermal layer, encompassing up to 30 cell rows. Heartless (Htl) encodes an FGF receptor that is expressed in the mesoderm, from gastrulation to the completion of mesoderm differentiation (Shishido et al., 1993). *heartless* mutations lead to incomplete mesodermal spreading over the ectoderm and, consequently, to defects in mesoderm patterning by ectodermal cues (Beiman et al., 1996; Gisselbrecht et al., 1996).

Following gastrulation, dp-ERK is detected in the mesoderm 3 hours and 15 minutes AEL, at stage 8. It is not displayed, however, by all mesodermal cells: during the process of spreading, dp-ERK is restricted to the dorsalmost 3-4 cell rows on each side of the embryo (Fig. 2A,B). Within these rows, staining is stronger in the dorsal ones, where its nuclear localization is also more prominent. As spreading advances, the pattern becomes more restricted and is confined only to the dorsalmost 1-2 cell rows (Fig. 2C,D), until it diminishes by 4 hours AEL. This mesodermal staining is Heartless-dependent, as it is completely absent in *heartless* null mutant embryos (Fig. 2E).

While the essential role of Heartless in mesodermal spreading has been previously demonstrated, it was not possible to determine whether the activated receptor actually plays an instructive role in guiding migration, or a more per-

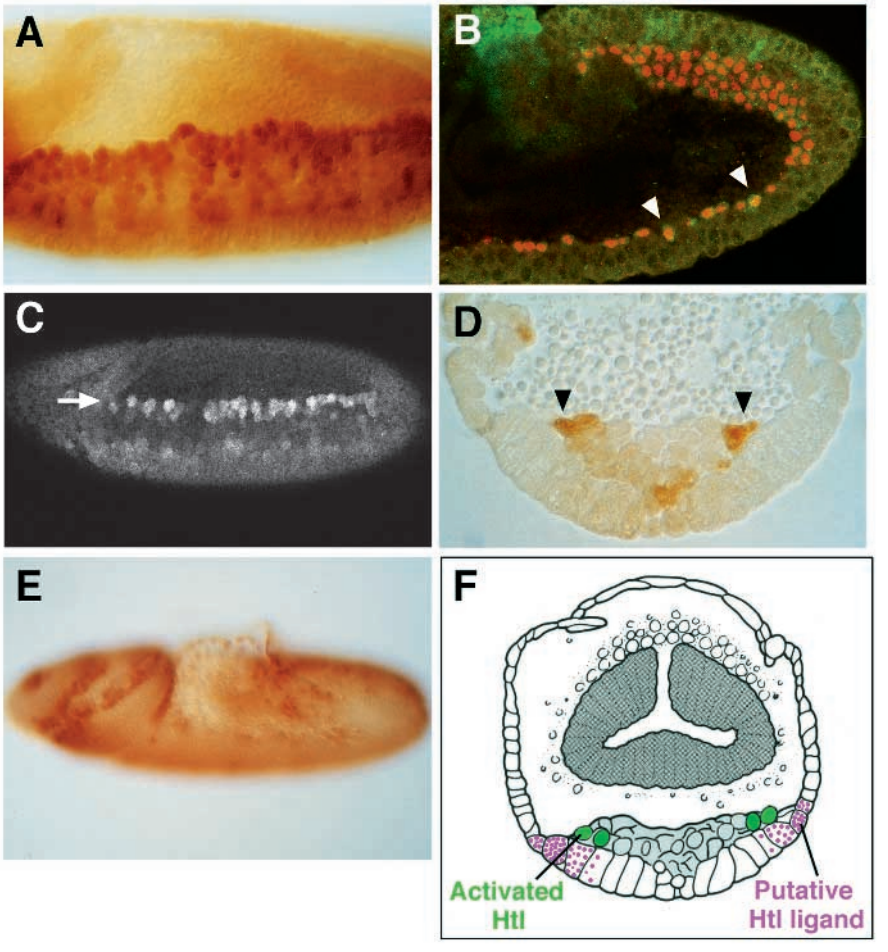

Fig. 2. Heartless-dependent ERK activation. Htl-dependent dp-ERK pattern is restricted to the mesodermal cells at stage 8. (A) Initially, several rows display staining, with a higher intensity in the more dorsal rows. The pattern becomes more restricted as migration proceeds. (B) Double staining with anti-Twist (red) and dp-ERK antibody (green). The mesodermal cells that display dp-ERK are marked by arrowheads. (C) Only the dorsalmost row (arrow) is labeled by dp-ERK antibodies. This embryo is mutant for the *rho* gene, indicating that the DER pathway does not contribute to mesodermal dp-ERK staining at this phase. (D) Section showing preferential dp-ERK staining in the dorsalmost mesodermal cells (arrowheads). (E) In a $htl^{\Delta 30}$ null mutant, mesodermal staining of dp-ERK is abolished, while other aspects (e.g. in the head furrows) are normal. (F) A model showing the dorsal mesodermal cells displaying dp-ERK, and a putative Htl ligand expressed in a subset of ectodermal cells, in a dynamic pattern progressing dorsally, thus attracting the migration of the mesoderm.

missive one. The ligand for Heartless has not been identified to date. Thus, the pattern of dp-ERK in the mesoderm is extremely informative in terms of charting the expected expression pattern of the putative Heartless ligand(s) and indicates the instructive role of receptor activation in migration.

Heartless activation is transient and highly dynamic in the migrating mesodermal cells. At any given time point, only a subset of mesodermal cells display dp-ERK, indicating that the active form of the ligand is not produced by all mesodermal or ectodermal cells. Activation is observed in more dorsal mesodermal cell rows, but not in the cells that have already contacted the ectoderm in the ventral rows. The ligand source is thus likely to have a dynamic expression pattern on the ectoderm, that is progressing dorsally to attract the migrating mesodermal cells. These results also suggest that the ligand has a limited diffusion range and activates only the mesodermal cells closest to the ligand source. The similarities to the activation profile of another FGF receptor, Breathless, will be presented below.

Breathless-dependent activation

Breathless (Btl) is an FGF receptor that is expressed in tracheal pit cells, and is essential for all aspects of the elaborate and stereotyped process of tracheal cell migration and extension (Glazer and Shilo, 1991; Klämbt et al., 1992; Reichman-Fried and Shilo, 1995). The specific function of Breathless was recently identified by isolation of its ligand, Branchless. This FGF-like molecule is expressed on the ectodermal and mesodermal cells that prefigure the migration pattern of the tracheal cells. After formation of the tracheal branch primordia, restricted activation by Branchless is also responsible for the induction of different tracheal cell fates within the migrating tips (Sutherland et al., 1996).

Initially, at stage 10, dp-ERK is detected in the tracheal placodes following activation of DER (Gabay et al., 1997; Wappner et al., 1997). At stage 11, a fairly broad dp-ERK pattern is observed in the tracheal pits (Fig. 3E,F). It is not diminished in *spitz* or *rho* mutant embryos, indicating that the pattern is not induced by the DER pathway. The dp-ERK staining at this stage was absent in *btl* mutant embryos, thus representing a second, Btl-dependent, wave of RTK activation in the trachea. As migration of the tracheal branch tips begins, the pattern of dp-ERK refines and can be observed within each branch only in the migrating tip cells (Fig. 3A-C). This pattern is also Breathless dependent; ubiquitous expression of Branchless gives rise to the appearance of dp-ERK in all tracheal cells (Fig. 3D). The corollaries between localized Heartless and Breathless FGF receptor activation, which in turn guide cell migration in the respective tissues, are striking. The intracellular molecular mechanisms responsible for these directed migrations and the possible roles of ERK in these processes remain to be elucidated.

Novel dp-ERK profiles

Most aspects of the dp-ERK pattern could be accounted for by known *Drosophila* RTK pathways. Several of the patterns revealed are novel however, with respect to the receptor they are triggered by. These patterns may be induced by unknown RTKs, or by other pathways (e.g. G protein-coupled receptors) which may activate ERK.

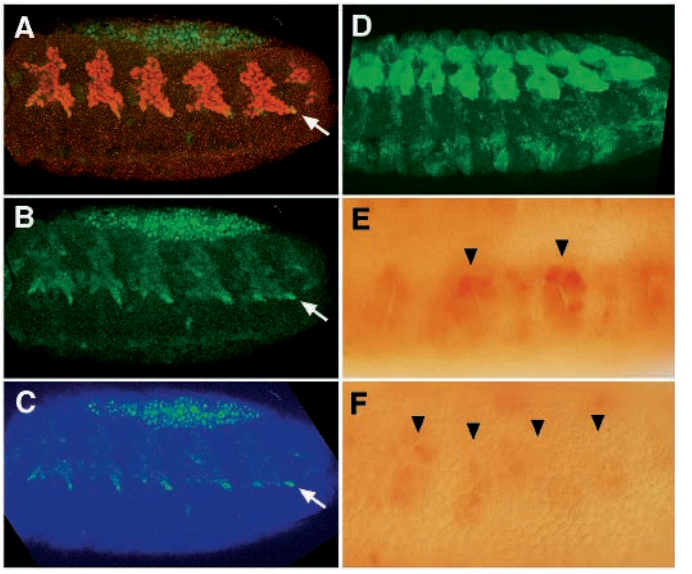

Fig. 3. Breathless-dependent ERK activation. (A) Stage 12 embryo stained with anti-Trachealess (red) and dp-ERK antibody (green). dp-ERK is specifically detected in the tips of the migrating lateral tracheal branches (arrow). The prominent expression in the posterior lateral branch corresponds to the high expression of Branchless at stage 12 in the vicinity of this tip (Sutherland et al., 1996). (B) dp-ERK antibody. Note: green staining in the yolk represents auto fluorescence. (C) False colors of dp-ERK antibody, displaying the highest intensity of staining in the migrating tips. (D) Stage 12 embryo following ubiquitous induction of the Breathless ligand, Branchless, by *69B-Gal4*. Since Breathless is expressed in all tracheal cells, dp-ERK is detected in the same cells. (E) Stage 11 *rho* mutant embryo. dp-ERK staining in the tracheal pits (arrowheads) is clearly detected, indicating that the tracheal staining is not DER-dependent at this phase. (F) *breathless* mutant embryo at the same stage, showing no dp-ERK staining in the tracheal pits (arrowheads).

Prominent dp-ERK staining is observed in the visceral mesoderm at stage 11. It is first seen as segmental patches, before fusion of the visceral arches from each segment, and is subsequently observed as a continuous waved line (Fig. 4A,B). The uniform pattern of dp-ERK in the visceral mesoderm suggests that it may not be involved in the directed migration of these cells, but possibly in their differentiation. Heartless was shown to be essential for differentiation of several mesodermal lineages, including the visceral mesoderm, following the initial spreading of the mesoderm (Beiman et al., 1996). However, the dp-ERK pattern in the visceral mesoderm is not Heartless dependent; In *heartless* mutant embryos small clusters of visceral mesoderm remain and these residual patches display dp-ERK at a normal intensity (not shown).

Finally, staining is observed at stage 15 in several muscle-attachment cells on the ectoderm and in the ventral-transverse (VT1) muscles (Fig. 4C,D). Expression of DER in the muscle-attachment cells (Zak et al., 1990) may be correlated to this pattern. Another RTK, Derailed, is expressed in the LT1-3 muscles and in a cluster of epidermal cells surrounding their attachment sites (Callahan et al., 1996) and is therefore not a candidate for inducing this pattern.

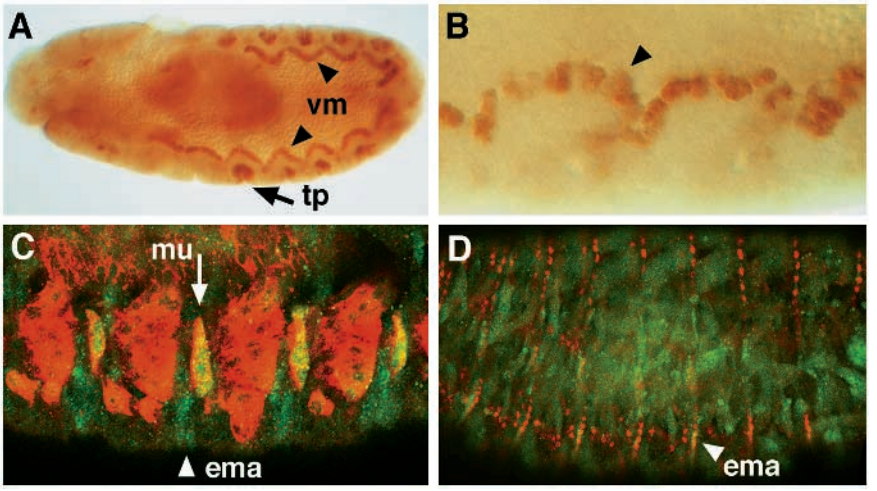

Fig. 4. Novel patterns of dp-ERK in embryogenesis. (A,B) At stage 11 dp-ERK is detected in the visceral mesoderm (vm), prior to and after the generation of a continuous visceral stripe (arrowheads). Btl-dependent staining in the tracheal pits (tp) is observed under the visceral arches. (C) At stage 15 dp-ERK is detected in several muscles (mu) and ectodermal muscle-attachment cells (ema) (red, anti-myosin; green, dp-ERK antibody). (D) dp-ERK muscle attachment staining (green) can also be visualized by double staining with anti-Stripe (red).

DISCUSSION

This work describes the active state of RTK signaling pathways in situ. An atlas of the spatial and temporal distribution of the activated form of ERK during embryogenesis is presented in Fig. 5, and marked according to the RTKs that trigger ERK at each phase. We could account for all known functions of RTKs by the dp-ERK staining pattern, except for the role of the DER pathway in cell proliferation, anterior to the morphogenetic furrow of the eye imaginal disc. However, it is possible that there are cases in which low levels of RTK activation elicit a biological response, but induce only minute levels of dp-ERK which are undetectable by the antibody. Highlighted below are the general conclusions and their application to other developmental systems, in view of what was learned by this approach in *Drosophila*.

Timing and levels of RTK activation

The timing of RTK activation could only be deduced previously from temperature-sensitive mutations or dominant-negative constructs. The level of activation, its precise boundaries and the existence of gradients could only be inferred from resulting patterns of gene expression and mosaic clones. By following dp-ERK, it is now possible to address these issues directly.

The ligand for the *Drosophila* RTK Heartless has not been identified to date. Consequently, the precise role of this receptor in cell migration could not be defined. Heartless-

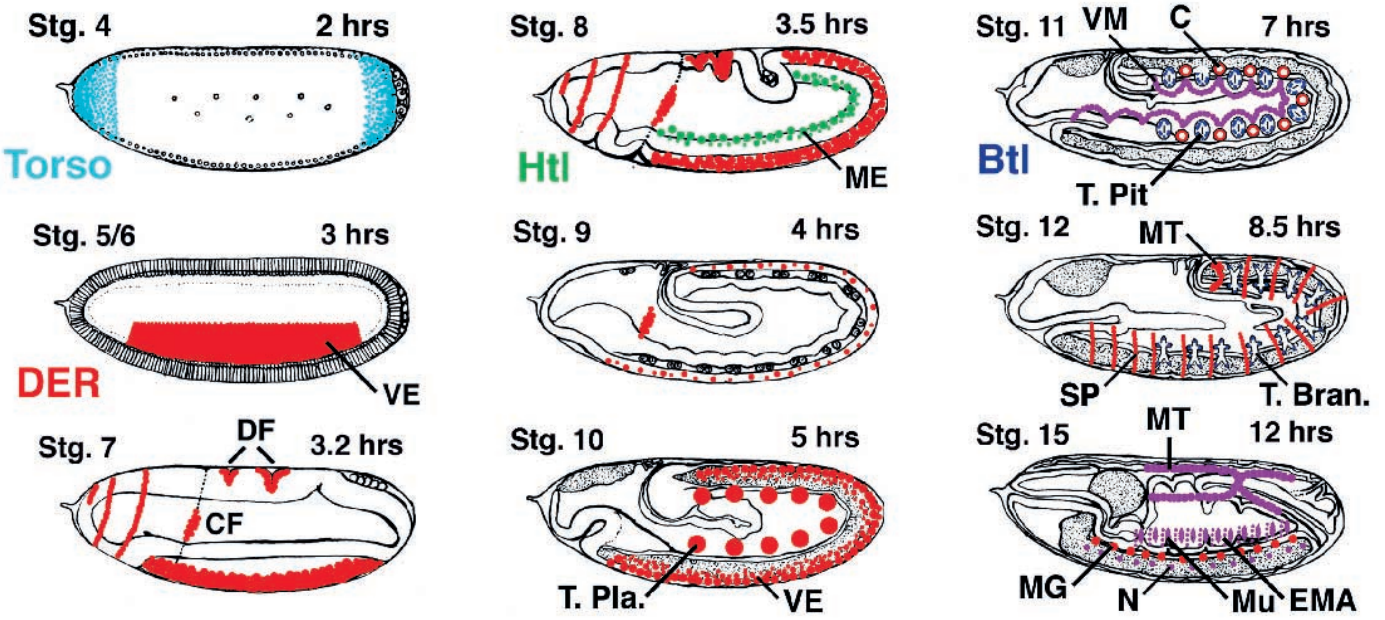

Fig. 5. Atlas of dp-ERK during embryogenesis. The distribution of dp-ERK during embryogenesis is presented schematically. The RTKs inducing each pattern are color coded (Torso, light blue; DER, red; Heartless, green; Breathless, dark blue; Unidentified receptors, purple). DER-induced patterns disappear in mutants for the DER pathway, including *DER/flb*, *spitz*, *Star* and *rhomboid* (Gabay et al., 1997). VE, ventral ectoderm; CF, cephalic furrow; DF, dorsal anterior and posterior folds; M, mesoderm; T. Pla., tracheal placodes; T. pit, tracheal pits; VM, visceral mesoderm; C, chordotonal organs; MT, Malpighian tubules; SP, segmental pattern; T. Bran., tracheal branches; MG, midline glial cells; EMA, ectodermal muscle-attachment cells; MU, muscles; N, unidentified neurons.

dependent dp-ERK pattern is detected initially in several rows of mesodermal cells, but always in the more dorsal ones, with a decreasing intensity in the ventral rows. As mesoderm spreading proceeds, the activation is confined to the dorsalmost row, until it totally declines. The restriction of dp-ERK to the mesodermal rows that are undergoing active migration and spreading over the ectoderm, strongly suggests that the Heartless ligand is playing an instructive role in guiding migration. The ligand may be expressed or presented in a dynamic pattern on the ectoderm, such that only the more dorsal mesodermal cells will be exposed to it at any given point in time.

For RTK pathways in which the ligand is known, its expression profile does not necessarily indicate when and where an active form of the ligand is presented to the receptor. The pattern of dp-ERK allows us to follow the extent of ligand diffusion, which may be restricted via trapping by extracellular matrix or by an excess of receptor molecules presented on the surface of the cells that the ligand encounters. In addition, the duration of activation allows assessment of the time window in which ligand is presented and of the activity of intracellular pathways dephosphorylating ERK.

We have shown that the time window of Torso activation is confined to a period of only ~30 minutes, possibly because of the limited amount of active ligand released from the vitelline membrane. Graded activation is observed within the syncycial embryo, indicating a limited diffusion of dp-ERK. In addition, rapid dephosphorylation mechanisms may restrict the position of dp-ERK adjacent to its site of activation.

The profile of Breathless activation has several interesting implications with respect to tracheal migration and differentiation. A very limited diffusion range is identified for the ligand Branchless, which triggers Breathless at the phases of branch migration only in the tracheal cells at the tips, immediately adjacent to the ligand source. During migration, dp-ERK was detected only in 1-2 cells, forming the tip of each branch. Thus, we should consider the possibility that Breathless is not transmitting signals for guided migration to all tracheal cells, but only to the leading ones. Other mechanisms, which may involve cell adhesion molecules, could be responsible for the recruitment of additional cells into each migrating branch. The level of Breathless activation was shown to be crucial not only for cell migration, but also for determining distinct cell fates within the migrating branches (Sutherland et al., 1996; Lee et al., 1996). The restricted pattern of dp-ERK provides a visual illustration for the capacity of localized Branchless expression to activate Breathless predominantly in the tip cells, and consequently induce differentiation of terminal tracheal cells.

Hierarchy of signaling components

Genetic analyses allow the unbiased isolation of mutations giving rise to a similar phenotype that represent different elements in a given signaling pathway. In some instances, the structure of the encoded protein immediately reveals its position in the signaling hierarchy. However, in the case of novel proteins, it is not obvious. Genetic epistasis experiments can be carried out only when dominant and recessive mutations in different signaling elements of the same pathway can be combined. The ability to directly monitor the pattern of dp-ERK in the different mutant backgrounds, provides a rapid and powerful tool to place genes in the signaling cascade, upstream or downstream to ERK.

Moreover, since we have assigned most dp-ERK patterns to known RTKs, it is possible to determine which RTK pathways require the activity of a given signaling component. The *corkscrew* gene, encoding an SH2-containing tyrosine phosphatase (Perkins et al., 1992), is a case in point. Although *corkscrew* was originally identified on the basis of its capacity to give rise to an embryonic phenotype resembling that of *torso* (Perkins et al., 1992), subsequent work has suggested that it may be required for signaling by other RTKs as well (Perkins et al., 1996). By following the dp-ERK pattern in embryos lacking both maternal and zygotic contributions of *corkscrew*, we have shown that all aspects of dp-ERK are diminished (Gabay et al., 1997). Thus, Corkscrew is absolutely required for signaling by all RTKs and its activity converges into the signaling pathway upstream to ERK.

No temporal overlaps between different RTKs

Since different RTK pathways elicit divergent biological responses, it was not previously feasible to examine the possibility of temporal overlaps between RTK pathways. The fact that ERK is a common universal denominator of different RTK pathways allows us to address this issue. We have observed no temporal overlaps in tissues where more than one RTK is operating. For example, in the tracheal system the DER pathway is induced in the placodes at stage 10, while Breathless is activated only after DER activation has diminished, from stage 11 onwards. Similarly, in the visceral mesoderm, dp-ERK is induced by an unknown receptor, after Heartless activation has declined. The lack of temporal overlap may suggest that the timing of ERK activation is crucial. This argument is especially pertinent when a given RTK induces different cell fates in the same tissue, in subsequent cycles of activation.

The universal role of ERK in all RTK signaling pathways raises the issue of specificity, since the different RTK pathways regulate versatile biological processes. An important factor in the induction of distinct cellular responses may be the capacity of different RTKs to trigger diverse parallel signaling cascades, which may include phospholipases, PI_3 kinase, JAK/STAT or other unknown pathways. Another option is that the responding cells are not naive, but are undergoing a dynamic set of changes during development allowing them to respond differently to the same signaling pathway. Thus, ERK may trigger distinct responses during development, according to the repertoire of ERK targets that the cells express. Transient versus sustained activation of ERK was also suggested to be instrumental in determining different outcomes (Marshall, 1995). Finally, the cellular compartments in which the pathway is induced may determine the final consequences.

In conclusion, the ability to visualize in situ dp-ERK has allowed us to follow the pattern of RTK activation during *Drosophila* development. This methodology should be applicable to a wide range of multicellular organisms. It may also serve as the most direct approach to identify the involvement of unknown RTK pathways and, possibly, also of other receptor types, in a wide range of processes controlling cell growth, differentiation and migration.

We thank Y. Dolginov and D. Zharhary from Sigma Israel Chemicals Ltd., Rehovot, Israel, for participation in preparing the

activated MAPK (dp-ERK) antibody, and the following colleagues for providing antibodies and fly stocks: A. Brand, P. Fisher, M. Krasnow, S. Roth and T. Volk. We thank all members of the Shilo lab, R. Schweitzer and T. Volk for critical reading of the manuscript. R. S. is an incumbent of the Samuel and Isabela Friedman Career Development Chair. This work was supported by a grant from the Dr. Josef Cohn Minerva Center for Biomembrane Research to R.S., and by grants from the Tobacco Research Council, US-Israel binational fund, UK-Israel binational fund and Minerva Foundation to B. S.

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(Accepted 17 July 1997)