

Spatial response to fibroblast growth factor signalling in *Xenopus* embryos

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SUMMARY

We have examined the spatial pattern of activation of the extracellular signal-regulated protein kinase (ERK) during *Xenopus* development, and show that it closely resembles the expression of various fibroblast growth factors (FGFs). Until the tailbud stage of development, all ERK activation domains are sensitive to the dominant negative FGF receptor, showing that activation is generated by endogenous FGF signalling. ERK is not activated by application of other growth factors like BMP4 or activin, nor is endogenous activation blocked by the respective dominant negative receptors. This shows that various domains of FGF expression, including the periblastoporal region and the midbrain-hindbrain boundary, are also sites of FGF signalling *in vivo*.

Wounding induces a transient (<60 minutes) activation of ERK which is not significantly reduced by the dominant negative FGF receptor.

An artificial FGF source, created by injection of eFGF mRNA into cleavage stage embryos, provokes ERK activation outside of its injection site over a range of several cell diameters. The range and extent of ERK activation outside the source region is unchanged by co-injection of a dominant negative form of Ras, which blocks ERK-activation within the source. This suggests that FGF protein can diffuse over several cell diameters.

Key words: MAP kinase, ERK, FGF, *Xenopus laevis*, cell signalling

INTRODUCTION

Many examples of regional specification in developing embryos are known to depend on the action of diffusible extracellular inducing factors and candidates exist for many of the factors responsible (Wolpert et al., 1998). But there are two problems in proving the function of an expression domain of a particular inducing factor within a developing embryo. Firstly the expression of the gene at the mRNA level does not guarantee the subsequent translation, secretion and correct processing of the protein. Secondly, the factor may be only one of many that can stimulate the same receptor, and the expression patterns of the others may be different or unknown. We have addressed both of these problems in *Xenopus* embryos by direct observation of the response to growth factor signalling using an antibody to the active form of the extracellular signal-regulated protein kinase (ERK) (Gabay et al., 1997a; Marais and Marshall, 1996). A similar approach in *Drosophila* has revealed a very dynamic ERK staining pattern in the early embryo which accounts for all known functions of receptor tyrosine kinases (RTKs) (Gabay et al., 1997a,b).

Recent work has suggested the importance of FGFs as posteriorising factors in early vertebrate development, necessary for the patterning of the trunk/tail part of the body (Isaacs et al., 1994; Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995; Pownall et al., 1996) and the fine patterning of the head (Crossley et al., 1996; Lee et al., 1997;

Lombardo and Slack, 1998a). But doubt has been raised about the significance of the posterior expression of some FGFs because of the simultaneous ubiquitous expression of others (Song and Slack, 1994, 1996) and also of certain non-FGF ligands for the FGF receptors (Kinoshita et al., 1996). FGFs are known to stimulate the MAP kinase signal transduction pathway during mesoderm induction in *Xenopus* (Hartley et al., 1994; LaBonne et al., 1995; Umbhauer et al., 1995; Whitman and Melton, 1992) leading to the transient diphosphorylation and hence activation of ERK. Furthermore it is known that ERK becomes activated in untreated embryos during gastrulation (LaBonne and Whitman, 1997).

We have extended the biochemical study of LaBonne and Whitman (1997) and examined the presence of the double phosphorylated form of ERK (dpERK) by immunostaining in wholemount embryos. We confirm that FGF but not activin or BMP-4 is able to activate ERK in the blastula stage embryo and show that the spatial distribution of activation in the untreated embryo is very reminiscent of the zygotic FGF expression patterns. We have used a dominant negative receptor to show that, remarkably, all of the observed domains of activation until an advanced stage of development are attributable to FGF. Moreover, neither the overexpression of a dominant negative BMP-4 receptor nor a dominant negative activin IIA receptor will block ERK activation in the embryo.

We have also used this system to investigate the range of

FGF signalling and show, contrary to previous belief, that FGF can diffuse over several cell diameters.

MATERIALS AND METHODS

Immunostaining

Xenopus embryos of the appropriate stage were fixed in 10% formalin in phosphate-buffered saline A (PBSA) for 2 hours at room temperature and then membranes were removed manually. Afterwards they were stored in methanol until use. Embryos were rehydrated in PBSA and treated with 0.1 M $K_2Cr_2O_7$ in 5% acetic acid for 30 minutes. This sensitises them to H_2O_2 and destroys endogenous alkaline phosphatase. They were then washed 3 times in PBSA and treated with 5% H_2O_2 in PBSA for 60-90 minutes, which both permeabilises and bleaches them. Embryos were washed again 3 times in PBSA before being blocked 2 times 1 hour in BBT (PBSA, 1% bovine albumin, 0.1% Triton X-100) and one time 1 hour in BBT plus 5% horse serum. Embryos were then incubated overnight in anti-dpERK antibody (diphosphorylated ERK 1&2, clone MAPK-YT; 1:10,000, Sigma). The next day, the embryos were washed 4 times 1 hour in BBT and 1 hour in BBT plus 5% horse serum. They were incubated in the secondary antibody overnight (anti-mouse IgG, AP conjugated, 1:1000) and were washed 1 hour in BBT and 4 hours in PBSA plus 0.1% Tween 20. For detection, embryos were incubated once for 3 minutes and then for 10 minutes in alkaline phosphatase buffer at room temperature (100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20). This buffer was then replaced with 1 ml BM Purple substrate (Boehringer Mannheim). The reaction was stopped by washing twice with PBSA and the stained embryos were stored in 10% formalin in PBSA.

For double staining, the embryos were fixed as before but permeabilised with Proteinase K (10 $\mu g/ml$) for 6 minutes, washed in PBSA and postfixed in 10% formalin for 20 minutes before they were blocked with BBT as before. For the DIG-eFGF staining the anti-DIG-AP FAB fragment from Boehringer Mannheim was used with Magentaphos (175 $\mu g/ml$) as substrate and for the dpERK staining the protocol as above was followed but the Vector Red was used as the substrate.

RNA injection

DIG-labelled *eFGF* mRNA was transcribed *in vitro* with SP6 RNA polymerase (Krieg and Melton, 1984) from pCS2+ plasmid (Turner and Weintraub, 1994). Instead of normal nucleotides, 10 \times DIG-NTP-mix from Boehringer was used. 100 pg of synthetic mRNA was injected. 1 ng of the dominant negative FGF receptor mRNA which was first described by Amaya et al. (1991) was injected. 1 ng of the truncated BMP-4 receptor (XTFRII) which is described by Suzuki et al. (1994) and 2.5 ng of the truncated type IIA activin receptor (Δ STK+10) described by New et al. (1997) was injected. The dominant negative receptors were injected at the 2 cell stage into both cells. Both receptors were kindly provided by Dr L. Dale. Full-length RasN17, a gift from Dr K. Nobes, was linearised with *NotI* and transcribed with SP6 RNA polymerase, and 1 ng of mRNA was injected. BMP4 and activin B were both transcribed with SP6 polymerase and 100 pg of synthetic mRNA was injected.

Wounding

Wild-type or XFD-injected embryos were wounded with a fine needle at stage 11 and fixed 15 minutes later in 10% formalin for 2 hours. Normal ERK immunostaining followed. For the time series stage 9 and 11 were fixed at different times after wounding followed by ERK immunostaining.

Embryological methods

Fertilised eggs and embryos were obtained and cultured as previously

described (Slack, 1993) and staged according to Nieuwkoop and Faber (1967).

RESULTS

ERK activation

Synthetic mRNA for three growth factors, eFGF, BMP-4 and activin B, were tested for their ability to activate the MAP kinase pathway during blastula stages. Only *eFGF* injections resulted in a positive response; and neither BMP-4 nor activin were able to activate ERK (Fig. 1). We went on to examine ERK activity in whole embryos at different stages. ERK activity was first detected at stage 8 as a small patch in the dorsovegetal quadrant. When gastrulation starts this has become a crescent centred on the dorsal lip (Fig. 2A,B). During gastrulation ERK remains active in a ring around the blastopore, corresponding to the newly formed mesoderm. In the early gastrula the staining is stronger on the dorsal side (Fig. 2C) but this evens out by middle gastrula. ERK is still active in a ring around the blastopore at the yolk plug stage, extending anteriorly into the forming neural plate (Fig. 2D). By the end of gastrulation, strong ERK activation has occurred. A more intense and wider ring around the blastopore is seen, but also new domains emerge anteriorly (Fig. 2E,F). There, ERK activity is observed in a half crescent in the open neural plate, which will end up in the forebrain and the hatching gland region, in two patches in the prospective hindbrain (Eagleson and Harris, 1990) and a further patch in between, in the region of the forming notochord. Weaker activity is also detected outside the neural plate area in the prospective gill region. ERK activity decreases as neurulation proceeds. In the early neurula, active ERK domains are also seen on either side of the neural tube (Fig. 2H) which will end up in the midline as the neural tube closes (Fig. 2J). In the early tailbud embryo there is still prominent activity in the tailbud (Fig. 2G) and activity is also observed in the forebrain, the midbrain/hindbrain junction, the stomodeal anlage, the otic vesicle, the heart anlagen and the branchial region (Fig. 2L). ERK activity is maintained in all these regions until at least the prelarval stage 37 (Fig. 2K). By stage 31, ERK has also become activated in the dorsal part of the cement gland, the neural tube, notochord and somites (Fig. 2I).

The initial activation we observe is consistent with the biochemical study of LaBonne and Whitman (1997), who observed a low basal level of MAP kinase activity uniformly distributed in the embryo, followed by an increase after about stage 8.5 mainly in the vegetal and marginal zone. The subsequent spatial and temporal ERK staining pattern is very similar to various *FGF* expression patterns in the *Xenopus* embryo. Several *FGFs* are transcribed in the blastopore region from early gastrula stages onwards. In particular, *eFGF*, *FGF-3* and *FGF-8* expression is seen in the posterior similar to the detected ERK staining at late gastrula stages (Christen and Slack, 1997; Isaacs et al., 1995; Lombardo and Slack, 1998b). At early neurula stages, *FGF-3* is expressed in two stripes where future rhombomeres 3-5 will form (Lombardo and Slack, 1998b), and by late neurula, *FGF-8* appears in the ectoderm of the prospective hatching gland and in an epidermal crescent outside of the neural plate which is fated to become part of the gills (Christen and Slack, 1997). Also at tailbud stages several *FGFs* are expressed in the domains where ERK is activated. For example *FGF-3* mRNA is present in the otic

vesicle, stomodaeum and branchial clefts. *eFGF*, *FGF-3* and *FGF-8* are also expressed in the midbrain/hindbrain junction and tailbud (Christen and Slack, 1997; Isaacs et al., 1995; Tannahill et al., 1992). However, there are also some differences between *FGF* expression and ERK activity. Up to now no localised *FGF* expression has been found in the dorsal closure seam of the neural tube or in the heart in *Xenopus*.

ERK staining is blocked in XFD embryos

With the above in mind and because we were specifically interested in FGFs and their contribution towards ERK activation, we studied the dpERK pattern in embryos that had their FGF signalling inhibited using the dominant negative FGF receptor XFD (Amaya et al., 1991). We have found that this reagent blocks all types of *Xenopus* FGF so far cloned (FGF-2,-3,-8, -9 and eFGF, *op. cit.*) and so were able to use it to determine which of the dpERK patches represented active FGF signalling centres. We found that the early activity at gastrula stages is entirely dependent on FGF signalling since all ERK activation is abolished in an XFD-injected embryo (compare Figs 2C,D and 3A,B). To our surprise, no activity could be detected in neurula stage embryos or early tailbud embryos either (compare Figs 2E-H,J and 3C-E). Only by the late tailbud stage was activity apparent in the head and trunk, representing a subset of the normal stage 40 pattern (Fig. 3F). Since embryos injected with XFD lose their tail and posterior trunk, we could not assess the posterior ERK domains and whether they are dependent on FGFs at later stages.

A requirement for an active FGF signalling pathway in activin induced mesoderm induction has been reported (Cornell and Kimelman, 1994; LaBonne et al., 1995) however it is not clear whether the opposite is true as well. Is activin or any other TGF- β type signalling needed for the full spectrum of FGF function? To test this we looked at the dpERK pattern in embryos that had been injected either with a truncated form of the BMP-4 receptor (XTFR II) or a dominant negative activin type IIA receptor (Δ STK+10) (New et al., 1997; SchulteMerker et al., 1994). In both cases early ERK staining looks normal, the ring around the blastopore at gastrula stages is similar to the one seen in uninjected control embryos (compare Fig. 3G,H with 3I). Also at early neurula stages the normal ERK domains are present in XTFR II and Δ STK+10 embryos. ERK is also activated along the closure line of the secondary neural tubes induced by the dominant negative receptors (data not shown).

ERK activation in wounding

In early experiments we noticed random spots or patches of activity that varied from embryo to embryo. These proved to be caused by a wound response to mechanical damage. To investigate the role of FGFs in this process, embryos injected at the 2-cell stage with 500 pg XFD RNA into each cell were wounded by pricking with a fine needle at around stage 11 and then scored for ERK activation 15 minutes later. The intensity of the ERK staining in XFD-injected embryos was the same as in control embryos, showing that, unlike the endogenous dpERK activation, this effect is not FGF dependent (compare Fig. 4A with 4B).

We also looked at the time course of ERK activation in embryos that were wounded either at stage 9 or 11. In both cases weak ERK staining was detected 2 minutes after

wounding and reached a plateau by 5 minutes. ERK activity remained strong for another 10 minutes and started to decrease rapidly thereafter. By 30 minutes after wounding it had become only barely detectable. If the wound was much larger, as achieved by cutting embryos into quarters, the rise of ERK activity followed a similar time course but the maximum intensity was greater and some activation was still visible at 60 minutes. The time course for both small and large wounds was examined in embryos injected with XFD and was unaffected.

eFGF diffusion

The extent of the dpERK posterior domain at the end of gastrulation exceeds that of the eFGF domain (Isaacs et al., 1995). This suggests that FGF protein may diffuse away from the source region. However this comparison is not conclusive, partly because of the dynamic nature of FGF expression and partly because of the possible existence of additional unknown FGFs in the larger domain. For this reason we decided to investigate the range of FGF signalling, using as the source, a region of the animal hemisphere of the blastula containing injected synthetic mRNA. This means that we know the source contains only the input FGF and that there is normally no detectable ERK activation in the vicinity. DIG-labelled *eFGF* RNA was injected into one cell at the 8 cell stage and embryos were fixed between the 64 cell stage and stage 9, then double-stained for DIG and dpERK. At all stages the injected DIG-*eFGF* message is clearly confined to a defined patch of cells (Fig. 5A-D). A dpERK signal did not appear until early stage 8 by which time it is already present in a patch, 2-3 cells wide, surrounding the source (Fig. 5A,B). By stage 9 the penumbra of dpERK staining extends up to 8-9 cells from the source (Fig. 5C). During this time cells are continuing to cleave so an increase in cell numbers need not correspond to an increase in distance. However our results clearly show that the activation does occur over a greater distance at the later stage (compare 5B and C), showing that the domain of activation has really expanded between stage 8 and 9. Co-injection of XFD into the same blastomere with the DIG-*eFGF* completely abolishes the ERK activation, confirming that this is, as expected, a response to FGF (Fig. 5D).

The simplest explanation for the penumbra of ERK activation around the mRNA-filled source region is that it is a response to FGF protein diffusing from the source cells. However, it is also conceivable that FGF-expressing cells stimulate each other to emit a different ERK-activating factor which is diffusible. To exclude this possibility we made use of a dominant negative form of Ras (Ras^{N17}). Activation of Ras normally occurs between activation of the FGF receptor and phosphorylation of ERK. So, cells containing Ras^{N17} should be unable to phosphorylate ERK and unable to execute downstream responses, such as release of other factors.

When Ras^{N17} mRNA was coinjected with *eFGF* mRNA into the same cell we found, as expected, a reduction or abolition of ERK phosphorylation in the RNA-filled patch of cells. However, a band of ERK activation several cells wide developed around the source region. The diameter of the activated patch is similar whether or not the cells within the source region are able to respond (Fig. 5E,F). This shows that it must arise by diffusion of FGF protein made from the injected mRNA and not from some secondary consequence of FGF signalling within the source region.

DISCUSSION

In previous publications we have shown that FGFs are responsible for the formation of the trunk-tail part of the anteroposterior pattern in *Xenopus* by activation of a molecular pathway comprising *Cdx* and *Hox* genes (Isaacs et al., 1994, 1998; Pownall et al., 1996). We have shown that overexpression causes anterior defects, that inhibition causes posterior defects and that the normal expression of several FGFs during gastrulation is posterior. This is consistent with work of other labs which have also shown a posteriorising activity for FGFs (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995). But there is a problem because there are some FGFs and FGF receptor ligands known to have a ubiquitous expression (Kinoshita et al., 1996; Song and Slack, 1994, 1996), and there are certainly further FGFs in *Xenopus* that are not yet cloned. How do we know that the FGFs expressed in the posterior (periblastoporal) region are more important than those with ubiquitous expression? The analysis of ERK activation has enabled us to prove that they are. The periblastoporal domain of ERK phosphorylation is the first to appear during development, and it does not appear in the presence of the dominant negative FGF receptor. This shows that the spatially restricted FGFs in the periblastoporal region are effective in activating the MAP kinase pathway while other ubiquitously expressed FGFs do not seem to be signalling.

The biochemical study of LaBonne and Whitman (1997) clearly shows the post-MBT stage increase. It also shows a very low basal level of activation in the early stages. We do not see the early activity, presumably because the biochemical method is more sensitive to low, uniform signals than the immunocytochemical method. Their study also showed activity in both marginal and vegetal parts of late blastulae, by dissection. This is not necessarily inconsistent with our results since the initial activation we see is well within the vegetal hemisphere.

FGF does it all

In *Drosophila*, where activation of ERK by RTKs has been studied extensively, a very dynamic but spatially restricted activation pattern is found which is due to several different RTKs. (Gabay et al., 1997a,b) In different mutants different parts of the ERK pattern are absent and therefore can be attributed to the loss of the corresponding RTK. By contrast, in *Xenopus*, the FGF family seems to be responsible for the full pattern of activated ERK in early development since activation can be blocked completely by XFD up to tailbud stages. Also LaBonne and Whitman (1997) noticed that ERK

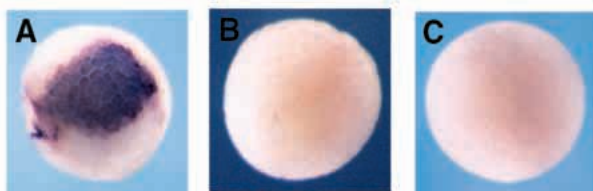


Fig. 1. ERK activation by growth factors. ERK activation after injection of various growth factors into an 8 cell embryo. ERK is phosphorylated in stage 8 embryos after injection with *eFGF* mRNA (A) but not with *BMP-4* (B) or *activin B* mRNA (C).

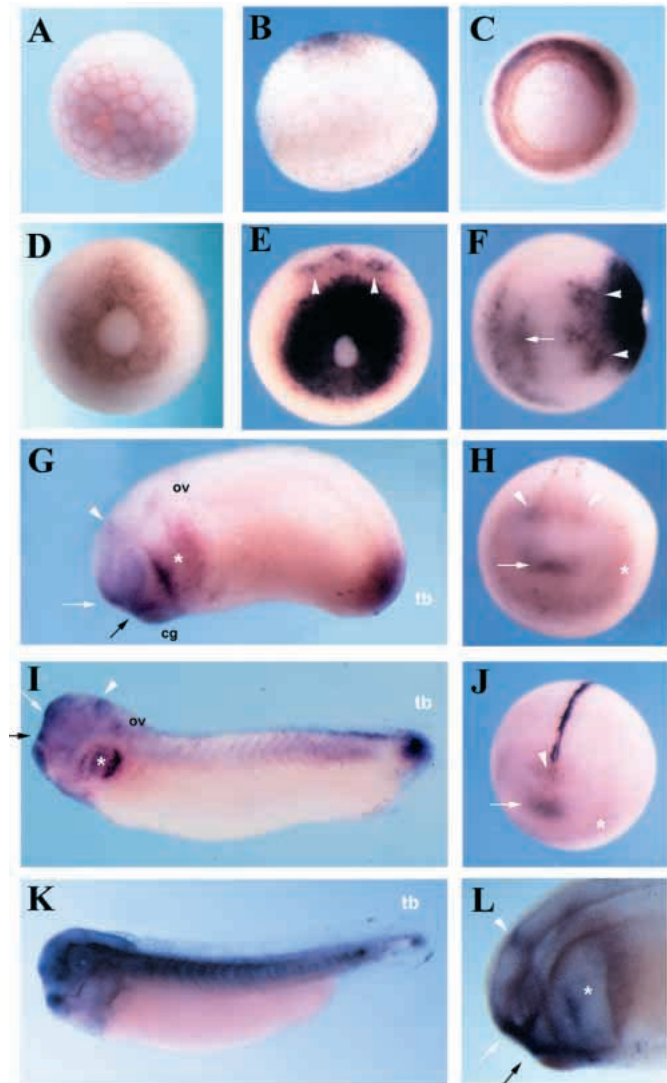


Fig. 2. Immunostaining for activated ERK in wild-type embryos. (A) Animal view of a stage 7 embryo; ERK has not been activated yet. (B) Stage 8, vegetal view; first activation of ERK is seen in a patch on the dorsal side where the blastopore will form. (C) Stage 10.5, vegetal view; ERK activation is seen around the blastopore, stronger dorsally. (D) Posterior view of a stage 12 embryo; a ring of dpERK is seen around the blastopore trailing into the neural tube. (E,F) Stage 12.5, posterior and dorsal view, respectively; strong burst of ERK activation. A very wide blastoporal ring is seen at the posterior (E) and several anterior domains in the region of midbrain/hindbrain junction (white arrowheads), in the dorsal midline, the forebrain (white arrow) and outside the anterior margin of the neural plate (F). (H,J), Anterior views of later neurulae (arrows indicate forebrain (white), stomodaeum (black); arrowheads, midbrain/hindbrain border and asterisks, gill region). At stage 14 ERK becomes active on either side of the neural tube (H) and as the neural tube closes, ends up in the dorsal midline (J). (G,I,L) At early and late tailbud stages, ERK is active in the tailbud (tb), the branchial arch region, the otic vesicle (ov), the stomodeal anlage, the forebrain and midbrain/hindbrain junction. During the tailbud stages it also becomes activated in the heart anlagen, the dorsal part of the cement gland (cg), the neural tube, notochord and somites. G shows a stage 24, I a stage 31 and L a stage 24 embryo after clearing. (K) At the prelarval stage 37 there is more generalised activity in the head and axial structures. All embryos are orientated anterior to the left and dorsal to the top unless otherwise stated.

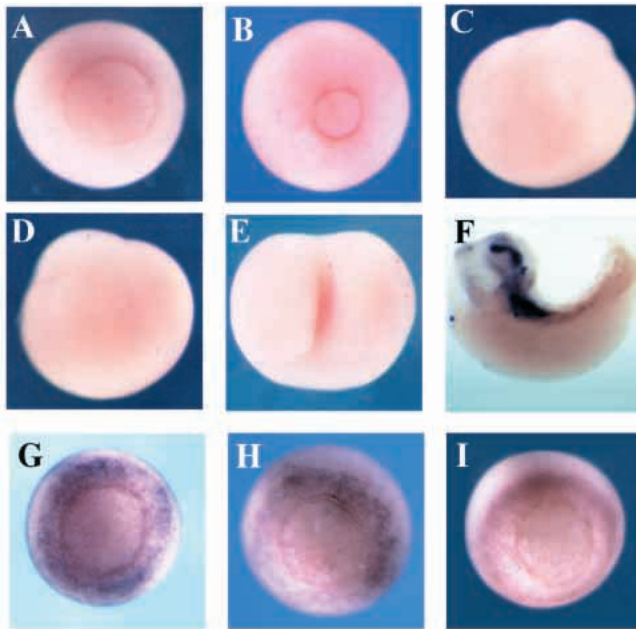


Fig. 3. Immunostaining for activated ERK in XFD-injected embryos. Embryos were injected at the 2 cell stage with 0.5 ng of *XFD* mRNA into each cell. (A) Stage 11 gastrula, vegetal view; activation of ERK around the blastopore is completely blocked. (B) Stage 12 embryo, vegetal view. (C,D) Stage 18 neurula, anterior and posterior view, respectively; no ERK activation is seen either anteriorly (C) or at the posterior (D). (E) Early tailbud embryo; still all ERK activation is blocked. (F) stage 40; some dpERK staining reappears in the head and trunk. ERK is activated around the blastopore in *XTFR II*- (G) and Δ *STK+10*-injected embryos (H). (I) Wild-type control for G and H.

activation during early gastrula stages is virtually abolished by XFD injection. This block by XFD must be specific since other dominant negative receptors do not have any inhibitory effects on ERK activation. This is in accordance with an interesting observation made by (Gabay et al., 1997b). They noticed that even though different RTKs signal through the same pathway there is no temporal overlap in tissues where more than one RTK is operating; meaning that there is never more than one RTK active in a specific tissue at a given time. Since several FGF receptors are co-expressed in various tissues in the *Xenopus* embryo we can not rule out that different FGF receptors are occupied by different FGFs and consequently signal at the same time in the same tissue via the MAP kinase pathway. We found, however, no evidence that any other RTKs are using the pathway at the same time as well.

Wounding related MAP kinase activation

LaBonne and Whitman (1997) noticed first that dissection of a blastula embryo into animal, marginal and vegetal parts increased ERK activity drastically over the level of normal ERK activity in an intact blastula embryo. We also see a strong response to wounding. This is quite short lived, declining to a barely detectable level after 30 minutes for small wounds and 60 minutes for extensive ones. We did not find any evidence for FGF involvement in that XFD-injected embryos responded to wounding in the same way as uninjected ones. Contrary to

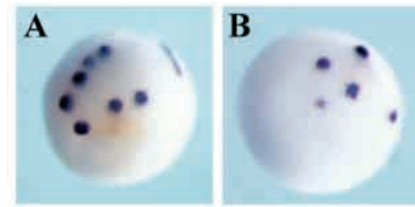


Fig. 4. Wound response. Embryos were wounded at stage 11 with a fine needle, fixed 15 minutes later and then immunostained for dpERK. (A) Control embryo shows strong ERK activation. This activation is not FGF dependent since it is not blocked in an *XFD*-injected embryo (B).

our result, LaBonne and Whitman (1997) reported that this wounding response was due to FGF signalling since they could block ERK activation by prior injection of the same dominant negative FGF receptor XFD. Neither wound size, stage of wounding nor animal-vegetal position seem to account for the different result. It is probably true that the biochemical method is both more sensitive and more quantitative than immunostaining, so we cannot exclude a small proportion of FGF-dependence, but our results do not support the idea that the bulk of the wound response is due to release of maternal FGF protein.

Another report by Dieckgraefe et al. (1997) provides a precedent for a similar effect not dependent on an extracellular signal. They have shown in a recent study on intestinal epithelial wound repair that ERK becomes activated following wounding but that the effect could not be transmitted to non-wounded cells by a soluble factor in the supernatant. It may be therefore that the wound effect does not depend on any extracellular factor but on a direct coupling between the cytoskeletal tension and the signal transduction pathway.

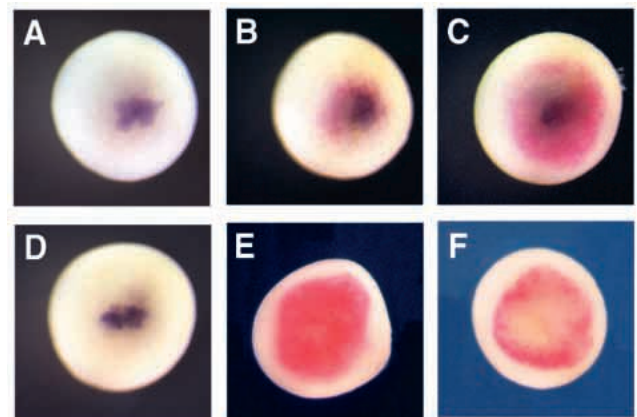


Fig. 5. eFGF activates ERK. Double labelling for DIG-*eFGF* (in purple) and ERK activation (in red) after injection with DIG-*eFGF* mRNA into an 8 cell embryo. (A) Stage 7 embryo; ERK activation has not yet occurred. (B) Stage 8 embryo (512-2048 cells); the eFGF signal has spread and activated ERK outside the injected cells. (C) Stage 9 embryo; the signal has spread even further and activated ERK in a bigger area. (D) *XFD*-injected stage 9 embryo; no ERK activation has occurred. (E,F) Effects of Ras^{N17}. E shows the effect of injecting just eFGF, while in F eFGF and Ras^{N17} were injected together, and the response is abolished in the source region.

Diffusion of FGF

Activin and dpp protein have recently been shown to be capable of diffusing over several cell diameters (Gurdon et al., 1995; Jones et al., 1996; Lecuit et al., 1996; McDowell et al., 1997; Smith, 1996). By contrast, the strong binding of FGFs to heparan sulphate has led many workers to consider this group of factors to be effectively insoluble and highly localised (Rifkin and Moscatelli, 1989).

Three features of our results suggest that eFGF can, in fact, diffuse over several cell diameters. Firstly, ERK activation occurs several cell diameters away from the source region that is making the eFGF. Secondly, the range of the ERK activation is unaffected when the response of the entire source region is abolished by co-expression of Ras^{N17}. Thirdly, following an early injection of eFGF mRNA, there is no ERK activation for several hours, until early stage 8, and when it occurs the band of activation is already several cells wide. Such a time course is unlikely to indicate a relay effect and suggests that FGF competence is acquired at about this time, as indicated also by ¹²⁵I-FGF binding studies (Gillespie et al., 1989).

The size of the activation domain increases substantially from stage 8 to stage 9. We have considered the possibility that this is due to factors other than diffusion, such as cell division or epibolic spreading of the animal cap, but we believe these alternative explanations are unlikely. Cell division is proceeding during these stages but the divisions are cleavages that reduce the size of the cells with each division. Over about 1 hour, the domain of activation approximately doubles in diameter, including many more cells than could be offspring of the original activated cells. Similarly, spread due to epiboly is unlikely because this would imply that the original activated cells are flattening out. Although epiboly is commencing by this stage (Keller, 1978) it cannot account for a doubling of diameter, i.e. fourfold increase in area, of the activated patch, since even by the end of gastrulation when the former animal hemisphere has covered the whole embryo, it has only approximately doubled in area.

The Ras^{N17} experiment involves the blockade of FGF function within the source region. It therefore suggests that the spread of ERK activation is due to diffusion of FGF protein and not to the FGF-provoked emission of some other factor within the source region. At first we were a bit puzzled about the total block of ERK activation in embryos co-injected in one blastomere with XFD and eFGF mRNA. The cells at the edge of the patch will be exporting eFGF and although unable to respond to it themselves, might be expected to stimulate their neighbours, as is seen in the Ras^{N17} experiments. We now believe that the difference between two types of co-injection experiment lies in the ability of XFD to interact directly with eFGF protein. Because XFD is present in considerable excess over the endogenous receptor it will probably sequester all the free protein produced by the source region. By contrast, Ras^{N17} does not interact directly with eFGF and cannot prevent its diffusion out of the source region. A similar sequestration effect by a dominant negative receptor was shown by McDowell et al. (1997).

Our results show that FGF can diffuse over several cell diameters and is therefore potentially capable of acting as a morphogen like the TGF β -factors. To be a morphogen, an

inducing factor must also be able to induce different genes at different concentration thresholds, and whether FGF can do this in the *Xenopus* context awaits further investigation.

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REFERENCES

- Amaya, E., Musci, T. J. and Kirschner, M. W. (1991). Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in *Xenopus* embryos. *Cell* **66**, 257-270.
- Christen, B. and Slack, J. M. W. (1997). FGF-8 Is Associated with Anteroposterior Patterning and Limb Regeneration in *Xenopus*. *Dev. Biol.* **192**, 455-466.
- Cornell, R. A. and Kimelman, D. (1994). Activin-mediated mesoderm induction requires FGF. *Development* **120**, 453-462.
- Cox, W. G. and Hemmati-Brivanlou, A. (1995). Caudalization of neural fate by tissue recombination and bFGF. *Development* **121**, 4349-4358.
- Crossley, P. H., Martinez, S. and Martin, G. R. (1996). Midbrain development induced by FGF8 in the chick embryo. *Nature* **380**, 66-68.
- Dieckgraefe, B. K., Weems, D. M., Santoro, S. A. and Alpers, D. H. (1997). ERK and p38 MAP kinase pathways are mediators of intestinal epithelial wound-induced signal transduction. *Biochem. and Biophys. Research Comm.* **233**, 389-394.
- Eagleson, G. W. and Harris, W. A. (1990). Mapping of the presumptive brain regions in the neural plate of *Xenopus laevis*. *J. Neurobiol.* **21**, 427-440.
- Gabay, L., Seger, R. and Shilo, B.-Z. (1997a). In situ activation pattern of *Drosophila* EGF receptor pathway during development. *Science* **277**, 1103-1106.
- Gabay, L., Seger, R. and Shilo, B.-Z. (1997b). MAP kinase *in situ* activation atlas during *Drosophila* embryogenesis. *Development* **124**, 3535-3541.
- Gillespie, L. L., Paterno, G. and Slack, J. M. W. (1989). Analysis of competence: Receptors for fibroblast growth factor in early *Xenopus* embryos. *Development* **106**, 203-208.
- Gurdon, J. B., Michell, A. and Mahony, D. (1995). Direct and continuous assessment by cells of their position in a morphogen gradient. *Nature* **376**, 520-521.
- Hartley, R. S., Lewellyn, A. L. and Maller, J. L. (1994). MAP kinase is activated during mesoderm induction in *Xenopus laevis*. *Dev. Biol.* **163**, 521-524.
- Isaacs, H. V., Pownall, M. E. and Slack, J. M. W. (1994). eFGF regulates Xbra expression during *Xenopus* gastrulation. *EMBO J.* **13**, 4469-4481.
- Isaacs, H. V., Pownall, M. E. and Slack, J. M. W. (1995). eFGF is expressed in the dorsal midline of *Xenopus laevis*. *Int. J. Dev. Biol.* **39**, 575-579.
- Isaacs, H. V., Pownall, M. E. and Slack, J. M. W. (1998). Regulation of Hox gene expression and posterior development by the *Xenopus* caudal homologue Xcad3. *EMBO J.* **17**, 3413-3427.
- Jones, C. M., Armes, N. and Smith, J. C. (1996). Signalling by TGF- β family members: Short-range effects of Xnr-2 and BMP-4 contrast with the long-range effects of activin. *Curr. Biol.* **6**, 1468-1475.
- Keller, R. E. (1978). Time lapse cinematographic analysis of superficial cell behavior during and prior to gastrulation in *Xenopus laevis*. *J. Morphol.* **157**, 223-248.
- Kinoshita, N., Minshull, J. and Kirschner, M. W. (1996). The identification of 2 novel ligands of the FGF receptor by a yeast screening method and their activity in *Xenopus* development. *Cell* **83**, 621-630.
- Krieg, P. A. and Melton, D. A. (1984). Functional messenger RNAs are produced by SP6 *in vitro* transcription of cloned cDNAs. *Nucleic Acids Res.* **12**, 7057-7070.
- LaBonne, C., Burke, B. and Whitman, M. (1995). Role of MAP kinase in mesoderm induction and axial patterning during *Xenopus* development. *Development* **121**, 1475-1486.
- LaBonne, C. and Whitman, M. (1997). Localization of MAP kinase activity in early *Xenopus* Embryos: implications for endogenous FGF signaling. *Dev. Biol.* **183**, 9-20.
- Lamb, T. M. and Harland, R. M. (1995). Fibroblast growth factor is a direct neural inducer, which combined with noggin generates anterior-posterior neural pattern. *Development* **121**, 3627-3636.
- Lecuit, T., Brook, W. J., Ng, M., Calleja, M., Sun, H. and Cohen, S. M.

- (1996). Two distinct mechanisms for long-range patterning by the secreted TGF- β signaling molecule Decapentaplegic in *Drosophila* wing. *Nature* **381**, 387-393.
- Lee, S. M. K., Danielian, P. S., Fritsch, B. and McMahon, A. P.** (1997). Evidence that FGF8 signalling from the midbrain-hindbrain junction regulates growth and polarity in the developing midbrain. *Development* **124**, 959-969.
- Lombardo, A. and Slack, J. M. W.** (1998a). Postgastrulation effects of fibroblast growth factor on *Xenopus* development. *Dev. Dyn.* **212**, 75-85.
- Lombardo, A. and Slack, J. M. W.** (1998b). Expression and functions of FGF-3 in *Xenopus* development. *Int. J. Dev. Biol.* **42**, 1101-1108.
- Marais, R. and Marshall, C. J.** (1996). Control of the ERK MAP kinase cascade by Ras and Raf. *Cancer Surveys* **27**, 101-125.
- McDowell, N., Zorn, A. M., Crease, D. J. and Gurdon, J. B.** (1997). Activin has a direct long-range signalling activity and can form a concentration gradient by diffusion. *Curr. Biol.* **7**, 671-681.
- New, H. V., Kavka, A. L., Smith, J. C. and Green, J. B. A.** (1997). Differential effects on *Xenopus* development of interference with type IIA and type IIB activin receptors. *Mech. Dev.* **61**, 175-186.
- Nieuwkoop, P. D. and Faber, J.** (1967). *Normal Table of Xenopus laevis* (Daudin). North-Holland, Amsterdam.
- Pownall, M. E., Tucker, A. S., Slack, J. M. W. and Isaacs, H. V.** (1996). *eFGF*, *Xcad3* and *Hox* genes form a molecular pathway that establishes the anteroposterior axis in *Xenopus*. *Development* **122**, 3881-3892.
- Rifkin, D. B. and Moscatelli, D.** (1989). Recent developments in the cell biology of fibroblast growth factor. *J. Cell Biol.* **109**, 1-6.
- SchulteMerker, S., Smith, J. C. and Dale, L.** (1994). Effects of truncated activin and FGF receptors and of follistatin on the inducing activities of BVg1 and activin: Does activin play a role in mesoderm induction? *EMBO J.* **13**, 3533-3541.
- Slack, J. M. W.** (1993). Embryological assays for growth factors. In: *Growth Factors- A Practical Approach*. IRL Press, Oxford.
- Smith, J. C.** (1996). How to tell a cell where it is. *Nature* **381**, 367-368.
- Song, J. and Slack, J. M. W.** (1994). Spatial and temporal expression of basic fibroblast growth factor (FGF-2) mRNA and protein in early *Xenopus* development. *Mech. Dev.* **48**, 141-151.
- Song, J. and Slack, J. M. W.** (1996). XFGF-9: A new fibroblast growth factor from *Xenopus* embryos. *Dev. Dynam.* **206**, 427-436.
- Suzuki, A., Thies, R. S., Yamaji, N., Song, J. J., Wozney, J. M., Murakami, K. and Ueno, N.** (1994). A truncated bone morphogenetic protein receptor affects dorsal-ventral patterning in the early *Xenopus* embryo. *Proc. Natl. Acad. Sci. USA* **91**, 10255-10259.
- Tannahill, D., Isaacs, H. V., Close, M. J., Peters, G. and Slack, J. M. W.** (1992). Developmental expression of the *Xenopus* int-2 (FGF-3) gene: activation by mesodermal and neural induction. *Development* **115**, 695-702.
- Turner, D. L. and Weintraub, H.** (1994). Achaet-Scute homolog-3 in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes Dev.* **8**, 1434-1447.
- Umbhauer, M., Marshall, C. J., Mason, C. S., Old, R. W. and Smith, J. C.** (1995). Mesoderm induction in *Xenopus* caused by activation of MAP kinase. *Nature* **376**, 58-62.
- Whitman, M. and Melton, D. A.** (1992). Involvement of p21(ras) in *Xenopus* mesoderm induction. *Nature* **357**, 252-254.
- Wolpert, L., Beddington, R., Brockes, J., Jessell, T., Lawrence, P. and Meyerowitz, E.** (1998). *Principles of Development*. Oxford University Press, Oxford.