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Involvement of the MAP kinase cascade in *Xenopus* mesoderm induction

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Mitogen-activated protein kinase (MAPK) is activated by MAPK kinase (MAPKK) in a variety of signaling pathways. This kinase cascade has been shown to function in cell proliferation and differentiation, but its role in early vertebrate development remains to be investigated. During early vertebrate embryogenesis, the induction and patterning of mesoderm are thought to be determined by signals from intercellular factors such as members of the fibroblast growth factor (FGF) family and members of the transforming growth factor-β family. Here we show that the microinjection of either mRNA encoding a constitutively active mutant of MAPKK or mRNA encoding a constitutively active form of STE11, a MAPKK kinase, leads to the induction of mesoderm in ectodermal explants from *Xenopus* embryos. Moreover, the expression of MAPK phosphatase-1 (MKP-1, also called CL100) blocks the growth factor-stimulated mesoderm induction. Furthermore, injection of CL100 mRNA into two-cell stage embryos causes severe defects in gastrulation and posterior development. The effects induced by CL100 can be rescued by co-injection of wild-type MAPK mRNA. Thus, the MAPK cascade may play a crucial role in early vertebrate embryogenesis, especially during mesoderm induction.

Key words: MAP kinase/MAP kinase kinase/mesoderm induction/signal transduction/*Xenopus*

Introduction

MAP kinases (MAPKs) are serine/threonine kinases that are activated in a wide variety of signaling pathways from yeast to vertebrate (Blenis, 1993; Davis, 1993; Nishida and Gotoh, 1993; Errede and Levin, 1993; Marshall, 1994). Activation of MAPKs requires dual phosphorylation on threonine and tyrosine residues (Anderson *et al.*, 1990; Payne *et al.*, 1991) catalyzed by MAPK kinase (MAPKK) (Ahn *et al.*, 1991; Gomez and Cohen, 1991; Ashworth *et al.*, 1992; Crews and Erikson, 1992; Kosako *et al.*, 1992, 1993; Matsuda *et al.*, 1992; Seger *et al.*, 1992; Wu *et al.*, 1993b). In turn, MAPKK is activated by serine phosphorylation catalyzed by MAPKK kinase (Matsuda *et al.*, 1993). A number of protein kinases, including Raf-1 (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992), Mos (Nebreda and Hunt, 1993; Posada *et al.*, 1993; Shibuya and Ruderman, 1993) and MEKK (Lange-Carter *et al.*, 1993), have been demonstrated to function as MAPKK kinases. In addition, two activators for MAPKK, whose identities are not known, have been isolated from *Xenopus* oocytes (Itoh *et al.*, 1993; Matsuda *et al.*, 1993). The activation mechanism of MAPKK kinases is largely unknown, although binding of Ras to Raf-1 is thought to contribute to Raf-1 activation (Vojtek *et al.*, 1993; Warne *et al.*, 1993; Zhang *et al.*, 1993). The activation of MEKK was also shown to be dependent on Ras in some signaling pathways (Lange-Carter and Johnson, 1994). In the eye development of *Drosophila* and the vulval induction of *Caenorhabditis elegans*, Ras, Raf-1 and MAPK have been suggested to form a linear pathway (reviewed in Dickson and Hafen, 1994; Selfors and Stern, 1994). It is clear, however, that the Ras/Raf-1 pathway has many branches and is subject to regulation by other signaling pathways. In fact, Ras has effectors other than Raf-1 (such as PI3 kinase and rasGAP) (Martin *et al.*, 1992; Duchesne *et al.*, 1993; Rodriguez-Viciana *et al.*, 1994; and see review by Boguski and McCormick, 1993) and Raf-1 has substrates other than MAPKK (such as IκB) (Li and Sedivy, 1993). There also exist Ras-independent pathways for Raf-1 activation (Sozeri *et al.*, 1992; Fabian *et al.*, 1993; Kolch *et al.*, 1993; Macdonald *et al.*, 1993), and the Raf-1 activity can be suppressed by cAMP-dependent protein kinase (Cook and McCormick, 1993; Wu *et al.*, 1993a). MAPK can be activated without activation of Ras or Raf-1 (Lange-Carter *et al.*, 1993; Nebreda and Hunt, 1993; Posada *et al.*, 1993; Shibuya and Ruderman, 1993), and Raf-1 activation is not always accompanied by MAPK activation (Samuels *et al.*, 1993; Porras *et al.*, 1994). Thus, MAPK does not function solely downstream of Ras or Raf-1, and the functions of Ras or Raf-1 are not necessarily mediated by MAPK.

The fact that MAPK is activated in a particular process does not necessarily prove the functional role of MAPK in that process. Ras and Raf-1 were shown to play crucial roles in cell proliferation, and activation of MAPK by growth factors was described many years ago. Most recently, several lines of evidence for the essential role of the MAPK cascade in cell growth and transformation have been obtained from studies utilizing a number of methods that can induce the blockade of the MAPK cascade and/or its constitutive activation (Pages *et al.*, 1993; Cowley *et al.*, 1994; Mansour *et al.*, 1994). Functions of the MAPK cascade are still unknown in many biological processes. Particularly, the role of the MAPK cascade in vertebrate development remains to be clarified.

During early embryogenesis, a vegetal hemisphere (prospective endoderm) emanates signals that convert the equatorial region of the overlying hemisphere from an ectodermal to mesodermal fate. Several factors are likely

to be involved in the mesoderm-inducing signals, including members of the transforming growth factor (TGF)-β family and members of the fibroblast growth factor (FGF) family (reviewed in New *et al.*, 1991; Jessel and Melton, 1992; Kimelman *et al.*, 1992; Sive, 1993; Slack, 1994). Dominant-negative mutants of Ras or Raf-1 have been shown to block the induction of mesoderm markers by activin, a member of the TGF-β family, or by basic FGF (bFGF) (Whitman and Melton, 1992; MacNicol *et al.*, 1993), and an active mutant of Ras can induce mesoderm in animal cap explants (Whitman and Melton, 1992). Little is known, however, about the intracellular signal transduction pathways that lead to mesoderm induction. Although it can be hypothesized that the function of Ras and Raf-1 in mesoderm induction could be mediated through the MAPK cascade, this hypothesis must be tested because the spectrum of functions of the Ras/Raf-1 pathway is not the same as that of the MAPK pathway, as discussed above. In fact, activation of MAPK in early *Xenopus* development after fertilization has not been demonstrated so far, although MAPK has been shown to be activated in animal cap explants in response to bFGF (Graves *et al.*, 1994; Hartley *et al.*, 1994; Labonne and Whitman, 1994).

Here we have shown the existence of an active form of MAPK in early *Xenopus* embryogenesis. We then looked into the effects of constitutive activation of the MAPK cascade in animal cap explants and studied the effects of ectopic expression of MAPK phosphatase-1 (CL100) (Keyse and Emslie, 1992; Alessi *et al.*, 1993; Sun *et al.*, 1993) on mesoderm induction. The results obtained strongly suggest the involvement of the MAPK cascade in mesoderm induction.

Results

Detection of active MAPK during early embryogenesis

It is possible that MAPK is involved in *Xenopus* early embryogenesis especially in mesoderm induction, since MAPK has been shown to be activated in animal caps in response to bFGF (Graves *et al.*, 1994; Hartley *et al.*, 1994; Labonne and Whitman, 1994). However, activation of MAPK in early *Xenopus* development after fertilization has not yet been demonstrated clearly. To address this point, MAPK was immunoprecipitated with anti-MAPK antibody from extracts of animal hemispheres (containing marginal zone) from *Xenopus* embryos at early stages of development, and the kinase activity was assayed against myelin basic protein. A low, but detectable, MAPK activity was observed reproducibly in embryos from stage 4 to 11 (Figure 1A). Furthermore, when extracts from *Xenopus* embryos were analyzed by immunoblotting with anti-MAPK antibody, the electrophoretically retarded, activated form of MAPK was detected during blastula and gastrula (Figure 1B). On the other hand, little MAPK activity was detected in the anti-MAPK immunoprecipitate obtained from animal cap explants (lacking marginal zone) (Figure 1C). These results suggest the existence of the activated form of MAPK, probably in the marginal zone, during the time of mesoderm induction.

Activation of the MAPK cascade is sufficient for mesoderm induction

To examine whether activation of the MAPK cascade is capable of mesoderm induction, we utilized a constitutively

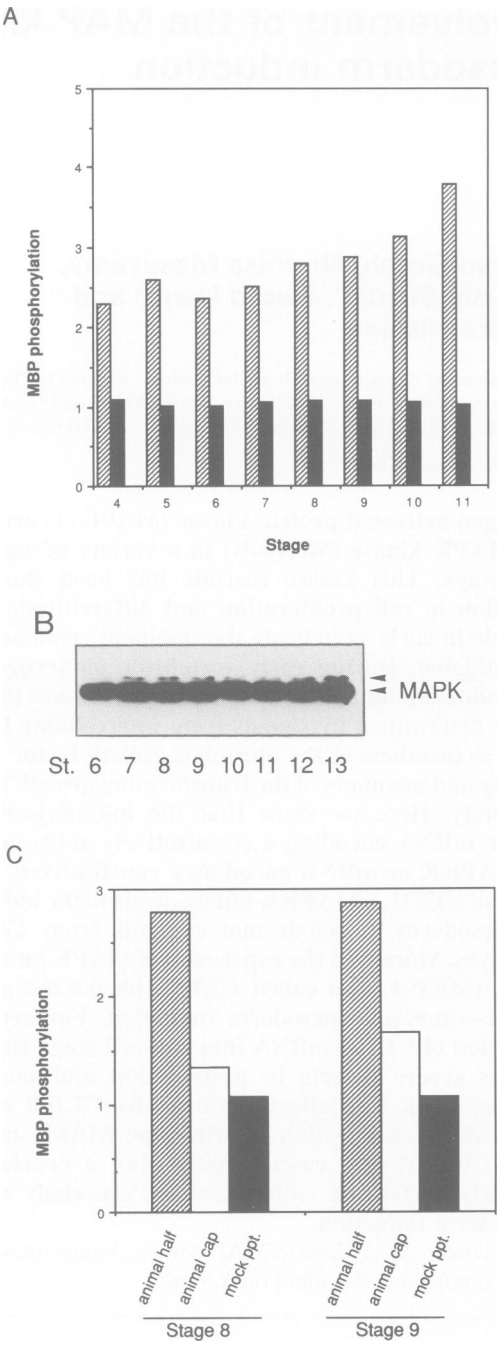

Fig. 1. Activity of MAPK in *Xenopus* embryos. (**A**) Activity of anti-MAPK immunoprecipitates. Animal hemispheres (containing marginal zone) from stage 4 to 11 were extracted and immunoprecipitated with anti-MAPK (hatched columns) or control IgG (mock precipitates, closed columns). These immunoprecipitates were assayed for MAPK activity toward myelin basic protein. (**B**) Immunoblotting analysis of MAPK in *Xenopus* embryos. Extracts were obtained from animal hemispheres including marginal zone at each stage of embryos, and were immunoblotted with anti-MAPK antibody. An electrophoretically retarded, activated form of MAPK (upper arrowhead) was detected. Activation of MAPK during early embryogenesis was detected reproducibly, but its extent was low and corresponded to ~3–8% of the full activation (in the case of unfertilized eggs). (**C**) Animal hemispheres containing marginal zone (animal half) or animal cap explants lacking marginal zone (animal cap) from stage 8 and 9 embryos were dissected and extracted. The animal half extracts (200 μg of protein) were subjected to immunoprecipitation by anti-MAPK antibody (animal half) and by control IgG (mock ppt), and the animal cap extracts (200 μg of protein) were subjected to immunoprecipitation by anti-MAPK antibody (animal cap). Then the immunoprecipitates were assayed for MAPK activity, which is shown as an arbitrary unit.

active mutant of *Xenopus* MAPKK [S218E/S222E (SESE)-MAPKK] and a constitutively active form (an N-terminal truncated form; Cairns *et al.*, 1992) of STE11, a yeast MAPKK kinase (Rhodes *et al.*, 1990). We have previously shown that phosphorylation of serine residues 218 and 222 activates *Xenopus* MAPKK (Gotoh *et al.*, 1994). Corresponding serine residues in mammalian MAPKK have also been shown to be activating phosphorylation sites (Alessi *et al.*, 1994; Zheng and Guan, 1994). Mutation of these serine residues to acidic amino acids produces a constitutively active form of MAPKK (Cowley *et al.*, 1994; Gotoh *et al.*, 1994; Mansour *et al.*, 1994). The *Xenopus* SESE-MAPKK had 16-fold higher activity than wild-type MAPKK when expressed in COS cells (K.Shirakabe *et al.*, unpublished). STE11 has been shown to phosphorylate and activate STE7, a yeast MAPKK (Neiman and Herskowitz, 1994). The recombinant N-terminal truncated STE11, when added to *Xenopus* oocyte extracts, induced prolonged activation of both MAPKK and MAPK (K.Takenaka *et al.*, unpublished), and the recombinant STE11 recovered from the extracts could phosphorylate and activate recombinant *Xenopus* MAPKK (Gotoh *et al.*, 1994).

Microinjection of SESE-MAPKK mRNA or STE11 mRNA, but not microinjection of wild-type MAPKK mRNA, into both blastomeres of two-cell embryos resulted in activation of MAPK in animal caps (Figure 2A). When animal caps were dissected at the blastula stage and cultured until siblings had reached the late neurula stage, the animal caps from embryos injected with either SESE-MAPKK mRNA or STE11 mRNA had elongated (Figure 2B) in a manner similar to that of animal caps treated with bFGF (see Figure 5B). Animal caps derived from embryos injected with water or wild-type MAPKK remained spherical (Figure 2B).

Upon histological examination, the animal caps expressing SESE-MAPKK showed the appearance of mesenchyme and coelomic cavities lined with mesothelium (Figure 3), like bFGF-treated animal caps. In contrast, control caps showed only atypical epidermis (Figure 3).

The induction of mesoderm was then assessed by examining the expression of early and late molecular markers for mesoderm; *Xenopus* brachyury (Xbra) (Smith *et al.*, 1991), which is expressed throughout the presumptive mesoderm of gastrula and is an immediate early marker for mesoderm, and muscle-specific actin (Mohun *et al.*, 1984), which is a late marker for mesodermal differentiation. Ectopic expression of either SESE-MAPKK or STE11 in animal caps induced both Xbra and muscle actin, as did bFGF treatment of animal caps (Figure 4). These results suggest that activation of the MAPK cascade is sufficient for the induction of mesoderm in animal cap explants.

Requirement for the MAPK cascade in FGF- and activin-induced mesoderm induction

To address whether the activation of the MAPK cascade is required for mesoderm induction, we utilized a dual-specificity phosphatase, MAPK phosphatase-1 (MKP-1, also called CL100), which is thought to act specifically on members of MAPK families (Keyse and Emslie, 1992; Alessi *et al.*, 1993; Sun *et al.*, 1993). Bacterially expressed

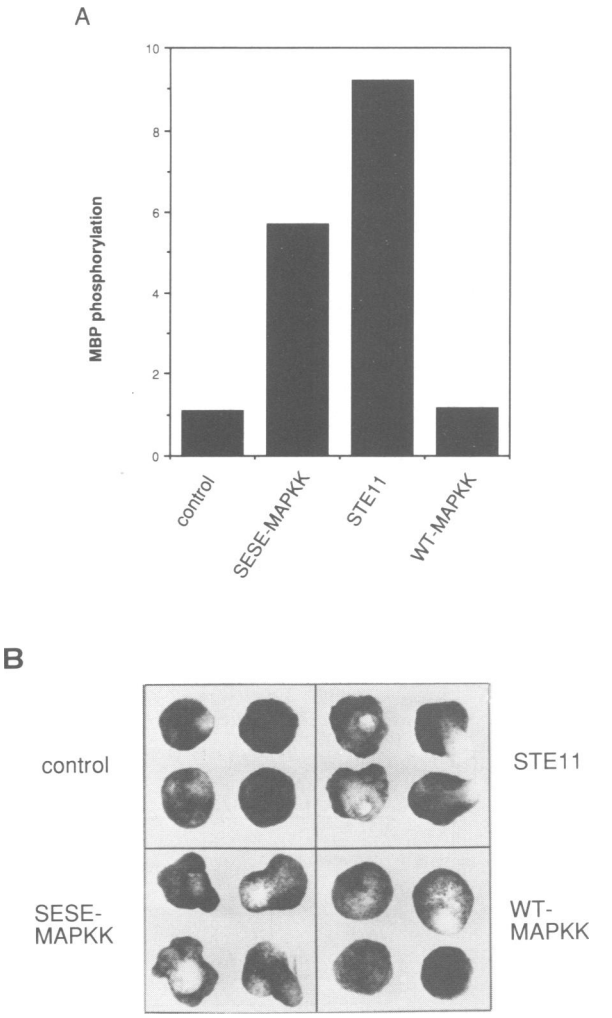

Fig. 2. Mesoderm induction by a constitutively active MAPKK or a MAPKK kinase (STE11). Animal caps were explanted at stage 8.5 from embryos that had been injected with water (control) or 2 ng of mRNA encoding a wild-type *Xenopus* MAPKK (WT-MAPKK), a constitutively active *Xenopus* MAPKK (SESE-MAPKK) or an N-terminal truncated *Saccharomyces cerevisiae* STE11 (STE11) at the two-cell stage. Some caps were extracted for immunoprecipitation of MAPK and assayed for MAPK activity (**A**), and some caps were cultured until siblings had reached stage 17. Typical caps for each condition are shown in (**B**).

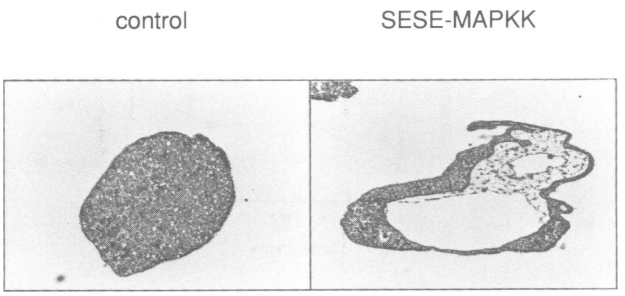

Fig. 3. Histological analysis of animal cap expressing a consitutively active MAPKK. Animal caps were explanted at stage 8.5 from embryos that had been injected with water (control) or a constitutively active MAPKK (SESE-MAPKK) at the two-cell stage, and cultured until siblings had reached stage 40. These animal caps were fixed, sectioned and stained with hematoxylin–eosin.

CL100 was able to dephosphorylate and inactivate not only purified *Xenopus* MAPK but also endogenous active MAPK in unfertilized egg extracts (K.Dell *et al.*, unpublished). Microinjection of CL100 mRNA into two-cell embryos inhibited the bFGF-induced activation of MAPK in animal cap explants that had been dissected at the blastula stage (Figure 5A). Less than 5% of animal caps derived from embryos injected with CL100 mRNA elongated in response to bFGF, whereas >95% of water-injected animal caps elongated in response to bFGF (Figure 5B). This inhibitory effect of CL100 on the mesoderm-inducing activity of bFGF could be almost completely rescued by co-injection of wild-type *Xenopus* MAPK mRNA (Figure 5B) but not by co-injection of a kinase-deficient mutant MAPK (K57D-MAPK) mRNA (data not shown). The elongation of animal cap explants by activin was also inhibited, although partially, by ectopic expression of CL100 (data not shown).

The bFGF-induced expression of muscle-specific actin in animal caps was inhibited by CL100 in a dose-dependent manner (Figure 6A). The bFGF-induced expression of Xbra was also inhibited by CL100, and these effects of CL100 could be rescued by co-injection of wild-type MAPK mRNA (Figure 6B). The induction of muscle-specific actin expression by activin was also inhibited by CL100, and the effect of CL100 could be rescued by co-injection of wild-type MAPK mRNA (Figure 7). Injection of wild-type MAPK mRNA alone did not induce expression of Xbra or muscle-specific actin (data not shown). These results, taken together, suggest that activation of the MAPK cascade may be required for the growth factor-stimulated induction of mesoderm.

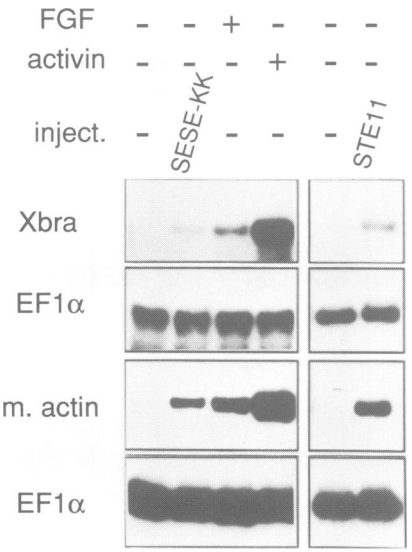

Fig. 4. Expression of markers for mesoderm by SESE-MAPKK or STE11. Two cell-stage embryos were injected with water (–) or 2 ng of mRNA encoding SESE-MAPKK or STE11. Then, animal cap explants were either left untreated or treated with 100 ng/ml bFGF or 10 ng/ml activin. To examine expression of molecular markers for the mesoderm induction, RNA was isolated from animal caps 3 h (Xbra) and 24 h (muscle actin) after explantation for analysis by RT-PCR. EF1α levels served as a control for template levels for each condition.

Involvement of the MAPK cascade in Xenopus early embryogenesis

We next examined the effect of injection of CL100 mRNA into two-cell stage embryos on early development. In the CL100-injected embryos, the lateral and ventral regions of the blastopore did not complete invagination, and the blastopore failed to close at the end of gastrulation (Figure 8). As a result, at the tailbud stage, the dorsal axis did not form normally and the embryos were bent dorsally. At the tadpole stage, severe defects in the posterior development were apparent in the CL100-injected embryos (Figure 9). These phenotypes induced by CL100 were dependent on the CL100 mRNA concentration and were rescued by co-injection of wild-type MAPK mRNA (Figure 8). Injection of wild-type MAPK mRNA alone did not have any marked effects on development (data not shown). Therefore, activation of MAPK may be involved in dorsal–posterior development of *Xenopus* early embryogenesis. Abnormalities in mesoderm induction in the CL100-injected embryos were observed by histological analysis (data not shown) and by examining the expression of molecular markers for mesoderm. In the CL100-injected embryos, the Xbra expression at stage 11 and the muscle-specific actin expression at stage 22 were markedly reduced

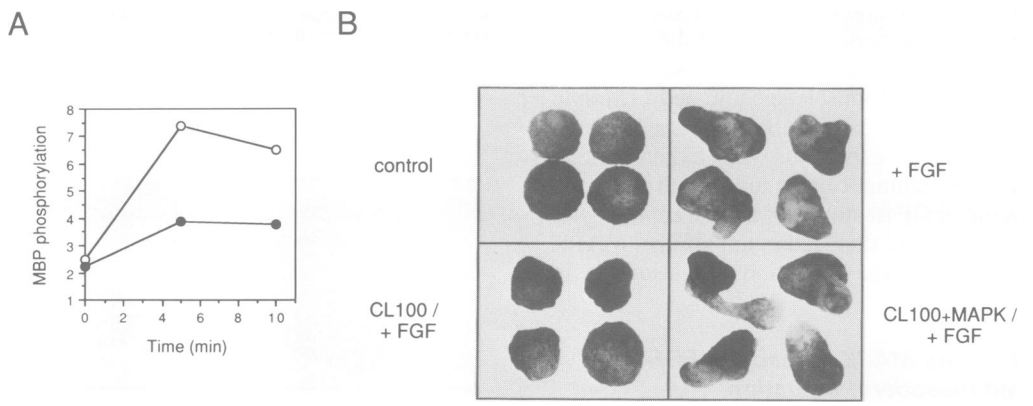

Fig. 5. Effects of MAPK phosphatase-1 (CL100) on mesoderm induction in animal cap explants. (**A**) The effect of CL100 on the bFGF-induced activation of MAPK. Animal caps derived from embryos injected with water (○) or CL100 mRNA (0.5 ng per embryo, ●) were treated with 100 ng/ml bFGF for the indicated times. The MAPK activity of the extract was assayed as described in Materials and methods. (**B**) Animal caps were explanted at stage 8.5 from embryos that had been injected with water (control) or mRNA encoding CL100 (0.5 ng per embryo) and/or mRNA encoding wild-type *Xenopus* MAPK (4.0 ng per embryo) at the two-cell stage, and were either left untreated or treated with 100 ng/ml bFGF. Animal caps after 10 h incubation are shown.

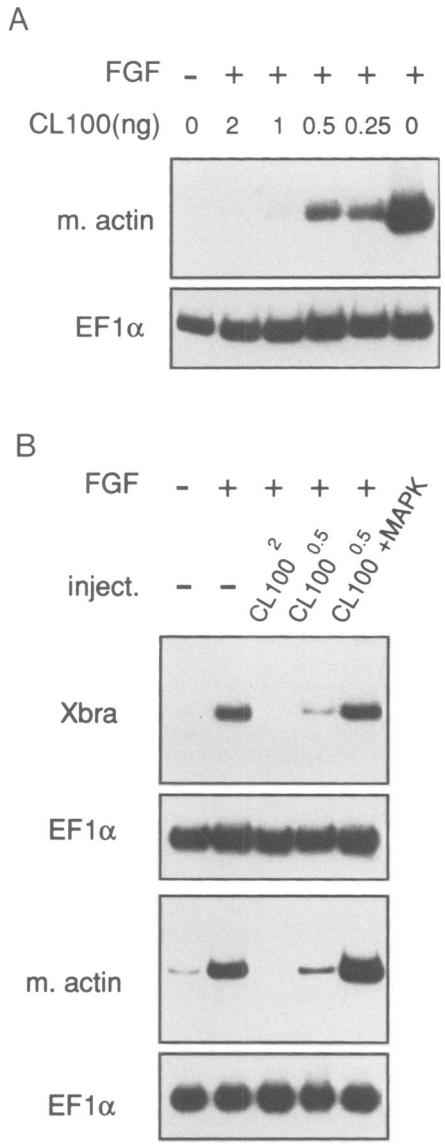

Fig. 6. Effects of MAPK phosphatase-1 (CL100) on bFGF-induced gene expression. (**A**) Dose-dependence of CL100. Animal caps were explanted from embryos that had been injected with water (–) or various amount of mRNA encoding CL100 (0.25–2.0 ng per embryo) and incubated with or without 100 ng/ml bFGF for 3 h to detect Xbra and for 24 h to detect muscle actin. (**B**) Effect of co-injection of MAPK. The experiment was performed as in (A), except that mRNA encoding wild-type MAPK (4 ng per embryo) was co-injected with CL100 where necessary.

(Figure 10). These data suggest that the phenotype observed in CL100-injected embryos may be attributed, at least in part, to a deficiency in mesoderm induction.

Discussion

Although Ras and Raf-1 have been shown to be involved in *Xenopus* mesoderm induction (Whitman and Melton, 1992; MacNicol *et al.*, 1993), another key question is whether or not the MAPK cascade is necessary and/or sufficient for mesoderm induction. Here we have shown that constitutive activation of MAPKK (SESE-MAPKK) is sufficient for mesoderm induction in animal caps. Activation of the MAPK cascade by a yeast MAPKK

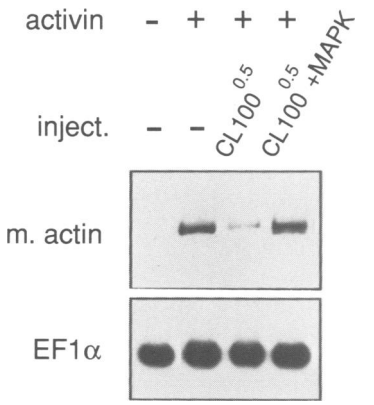

Fig. 7. Effects of CL100 on activin-induced gene expression. Animal caps explanted from embryos that had been injected with water (–) or 0.5 ng of CL100 mRNA with or without 4 ng of MAPK mRNA were treated with 10 ng/ml activin. RNA was isolated 24 h after explantation for detection of muscle actin.

kinase, STE11 (Rhodes *et al.*, 1990), also led to mesoderm induction. In previous detailed analyses concerning the concentration-dependent effects of bFGF on animal cap explants, low levels of bFGF were found to induce ventral mesoderm, while higher levels of bFGF were able to induce more lateral and dorsal mesoderm (such as muscle) (Green *et al.*, 1990). Although SESE-MAPKK induced only ventral mesoderm tissues in animal cap explants, as detected by histological analysis, SESE-MAPKK and STE11 induced expression of a detectable level of muscle-specific actin mRNA, suggesting that the MAPK cascade, if activated strongly, could induce dorsal–lateral mesoderm as well as ventral mesoderm.

To examine the requirement for the MAPK cascade in mesoderm induction, we utilized CL100 (MKP-1) as a tool for inactivation of MAPK. Ectopic expression of CL100 inhibited the bFGF-stimulated mesoderm induction; it suppressed animal cap elongation, mesodermal tissue formation as detected by histological analysis (data not shown) and expression of molecular markers for mesoderm, and caused severe defects in dorsal–posterior development. CL100 is a dual-specificity phosphatase which acts preferentially on MAPK compared to other phosphorylated proteins, such as receptor tyrosine kinases and cdc2 kinase (Alessi *et al.*, 1993; Sun *et al.*, 1993). Recently, CL100 was shown to be able to dephosphorylate and inactivate a MAPK-related molecule, RK (MPK2 in *Xenopus*) (Rouse *et al.*, 1994). Our results showing that co-expression of wild-type MAPK cancelled the inhibitory effects of CL100 strongly suggest that the effects of CL100 were mediated through the inhibition of MAPK activation. However, we cannot exclude the possibility that other targets of CL100 are involved in mesoderm induction and embryogenesis. Expression of CL100 partially inhibited activation of MAPK by bFGF in animal caps, whereas it almost completely blocked the bFGF-induced elongation of animal caps. If CL100 localizes predominantly to nuclei, it is possible that inactivation of nuclear MAPK would be sufficient for blockade of mesoderm induction. Of course, there are other possibilities and future work is necessary to answer this question.

Ectopic expression of CL100 inhibited activin-induced muscle actin expression and partially inhibited activin-

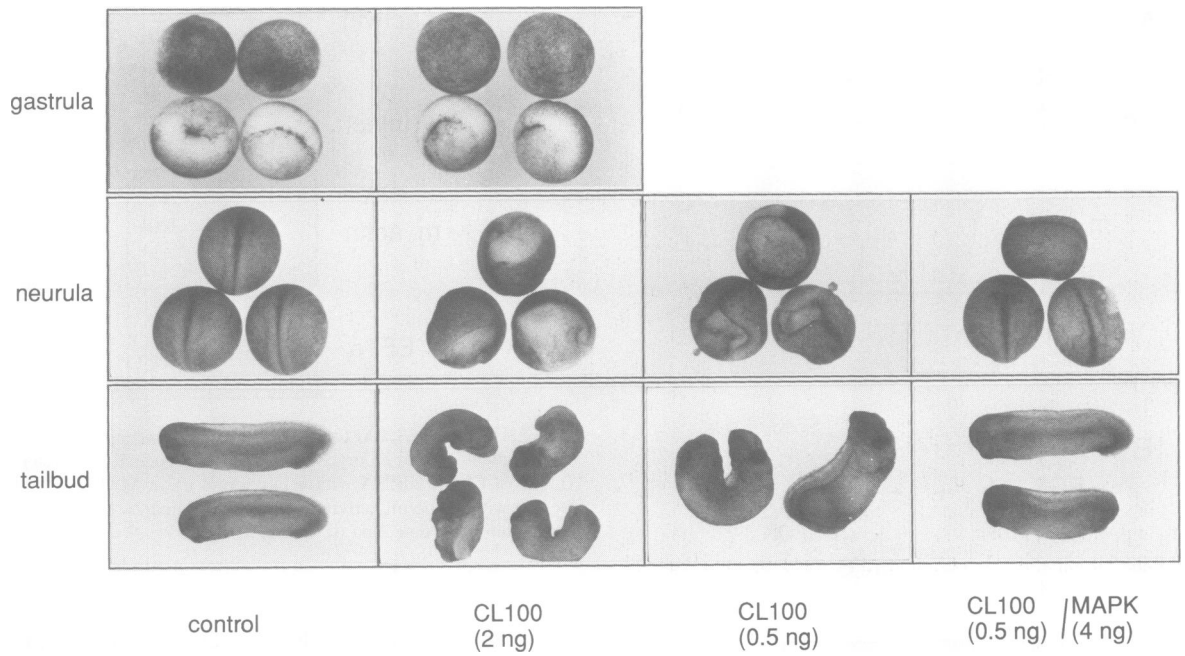

Fig. 8. Effects of MAPK phosphatase-1 (CL100) on *Xenopus* embryogenesis. Two-cell stage embryos were injected at the marginal zone of both blastomeres with water (control), 2 ng of CL100 mRNA, 0.5 ng of CL100 mRNA or 0.5 ng of CL100 mRNA plus 4 ng of wild-type MAPK mRNA, and were cultured. Photographs were taken at stage 10–11 (gastrula), stage 18–19 (neurula) or stage 26–28 (tailbud). Magnifications are: ×13.8 for gastrula and neurula, ×10.7 for tailbud, except for CL100 (2 ng)-injected embryos (×8.6) and CL100 (0.5 ng)-injected tailbud embryos (×13.8).

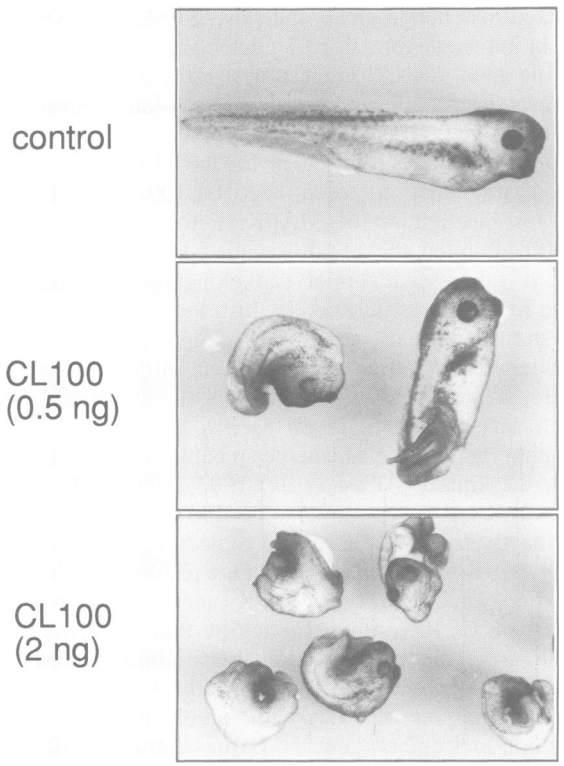

Fig. 9. MAPK phosphatase-1 (CL100)-induced phenotypes of *Xenopus* tadpole. Tadpoles (stage 35–38) derived from embryos injected with water (control), 0.5 or 2.0 ng of CL100 mRNA are shown.

induced elongation of animal caps. In recent reports, activin, unlike bFGF, did not induce activation of MAPK in animal caps (Graves *et al.*, 1994; Labonne and Whitman, 1994). This may suggest that MAPK does not lie down-

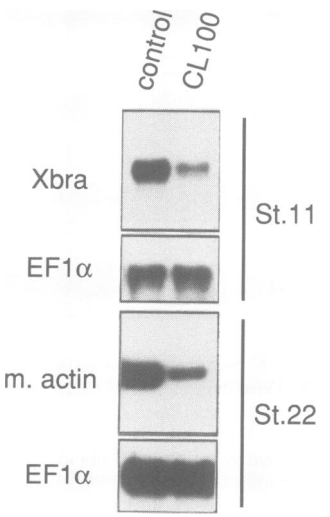

Fig. 10. Effect of MAPK phosphatase-1 (CL100) on expression of mesoderm markers *in vivo*. RNA was prepared at stages 11 and 22 from embryos that had been injected with water (control) or 2 ng (per embryo) of CL100 mRNA. RT-PCR of Xbra and muscle actin was carried out as described in Materials and methods.

stream of the activin signaling. It has been shown that dominant-negative FGF receptor (Cornell and Kimelman, 1994; Labonne and Whitman, 1994), dominant-negative Ras (Whitman and Melton, 1992) or dominant-negative Raf-1 (Labonne and Whitman, 1994) inhibited the activin-stimulated mesoderm induction as well as the bFGF-stimulated induction. Thus, signaling components downstream of FGF receptor may be required for activin to induce mesoderm. The bFGF-stimulated signaling pathway and the activin-stimulated pathway may be basically independent, and both may be required for normal meso-

derm induction. Recently, Maller and his collaborators reported that activin can synergize with bFGF to induce activation of MAPK (Hartley *et al.*, 1994). Thus, the possibility that MAPK works downstream of both activin and bFGF should not be ruled out. The phenotypes observed in CL100-injected embryos are similar to those in embryos injected with a dominant-negative FGF receptor or a dominant-negative Raf-1 (Amaya *et al.*, 1991; MacNicol *et al.*, 1993). Therefore, in early *Xenopus* development the MAPK cascade may mediate the bFGF receptor- and Raf-1-stimulated signaling pathways.

This study illustrates the central role of MAPKK and MAPK in mesoderm induction. In invertebrates such as *Drosophila, C.elegans* and yeast, the MAPK cascade has been shown to have essential roles in a number of biological processes including development. This study, by presenting evidence for the involvement of the MAPK cascade in *Xenopus* mesoderm induction, has revealed a hitherto unidentified function of the MAPK cascade in vertebrates.

Materials and methods

Materials

STE11 cDNA and CL100 cDNA are kind gifts from Dr B.Errede (University of North Carolina) and Dr S.Keyse (Biomedical Research Centre, University of Dundee), respectively. Moloney murine leukemia virus (MMLV) reverse transcriptase was purchased from Gibco-BRL. SP6 polymerase and *Taq* polymerase were from Takara. Recombinant human activin A was kindly provided by Drs H.Shibai and Y.Eto (Ajinomoto Co. Inc.). Recombinant bovine bFGF was purchased from Boehringer Mannheim, Biochemica.

Immunoblotting analysis of Xenopus MAPK

Extraction of *Xenopus* embryos was carried out as described (Gotoh *et al.*, 1991) using a buffer (HB) consisting of 20 mM Tris–HCl, pH 7.5, 20 mM β-glycerophosphate, 15 mM NaF, 10 mM EGTA, 2 mM $MgCl_2$, 50 mM NaCl, 6 mM dithiothreitol, 1 mM orthovanadate, 1 mM phenylmethyl sulfonylfluoride (PMSF), 0.5% aprotinin, 20 μg/ml leupeptin, 20 μg/ml pepstatin and 20 μg/ml chymostatin. After the embryo extracts were subjected to SDS–PAGE, proteins were transferred to polyvinylidene difluoride membrane (Immobilon P, Millipore). Membranes were incubated with anti-MAPK antibody (Gotoh *et al.*, 1991) in 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20 and subsequently with horseradish peroxidase-conjugated anti-rabbit IgG antibody. Immunoreactive bands were detected by the ECL Western blotting detection system (Amersham).

Immunoprecipitation and MAPK assay

Extracts (200 μg of total protein) from *Xenopus* embryos or animal cap explants were incubated with 1/50 vol of anti-MAPK antiserum or control IgG (mock precipitate) at 4°C for 2 h, and the immune complex was precipitated with protein A–Sepharose (Pharmacia) and washed with HB. MAPK activity in the immunoprecipitates was assayed by using myelin basic proten (Sigma, final concentration 2 mg/ml) as a substrate in the presence of 100 μM [γ-^{32}P]ATP (1 μCi/sample), 10 mM $MgCl_2$ and 20 mM Tris–HCl, pH 7.5.

In vitro mutagenesis

An *Eco*RI fragment of *Xenopus* MAPKK cDNA (Kosako *et al.*, 1993) was subcloned into M13mp18. The mutagenesis of Ser218 to Glu and Ser222 to Glu in *Xenopus* MAPKK was performed by the method of Kunkel *et al.* (1987) using a mutagenic primer 5′-TCTTGTCCCAACAAACTCCCATTTGCCATCTCGTCTATGAGTTGC-3′ to yield SESE-MAPKK. Phosphorylation of Ser218 and Ser222 activates *Xenopus* MAPKK and their replacement by glutamic acid made MAPKK constitutively active (Cowley *et al.*, 1994; Gotoh *et al.*, 1994; Mansour *et al.*, 1994). The whole sequence of MAPKK was analyzed to ensure that only the desired mutation had been introduced.

Plasmid construction

All constructs for *in vitro* transcription and injection were inserted into the pSP64T vector (Krieg and Melton, 1984). Synthesis of capped mRNA was performed with SP6 RNA polymerase essentially as described by Moon and Christian (1989).

Embryo injections

Embryos were dejellied 20 min after fertilization and kept at 17°C. At the two-cell stage, embryos were transferred to a solution containing 3% Ficoll–0.1× modified Barth's saline [8.8 mM NaCl, 0.1 mM KCl, 0.24 mM $NaHCO_3$, 1 mM HEPES, pH 7.5, 0.082 mM $MgSO_4$, 0.033 mM $Ca(NO_3)_2$, 0.041 mM $CaCl_2$]–10 ng/ml kanamycin. Each blastomere of two-cell embryos was injected with 5 nl of mRNA (0.025–0.4 mg/ml) at the animal pole or at the marginal zone. A few hours after injection, embryos were transferred to water. Embryos were staged according to Nieuwkoop and Faber (1967).

Animal cap assays and RT-PCR

Animal caps were dissected from stage 8.5 embryos and incubated in a solution containing 1× Steinberg [58 mM NaCl, 0.67 mM KCl, 0.34 mM $Ca(NO_3)_2$, 0.83 mM $MgSO_4$, 4.6 mM Tris–HCl, pH 7.6], 10 ng/ml kanamycin and 0.1% crystalized BSA in the presence or absence of bFGF and activin at 22°C. Total RNA was isolated from at least five animal caps using guanidium thiocyanate as described (Suzuki *et al.*, 1994). Reverse transcription was carried using MMLV reverse transcriptase. PCR reactions were performed using an annealing temperature of 58°C for muscle actin and elongation factor (EF)1α, and 60°C for Xbra for 18–22 cycles. Linearity was tested on serial dilutions of cDNA and cycle numbers. Primer pairs used here were; 5′-GGATCATCTTCTCAGCGCTGTGGA-3′ and 5′-TGGACTTTGTGGCAGCCGACAAC-3′ (Xbra), 5′-TCCCTGTACGCTTCTGGTCGTA-3′ and 5′-TCTCAAAGTCCAAAGCCACATA-3′ (muscle actin), 5′-CCCTGAATCACCCAGGCCAGATTGGTG-3′ and 5′-GAGGTAGTCTGAGAAGCTCTCCACG-3′ (EF1α).

Histology

Animal caps were fixed, dehydrated and embedded in paraffin as described previously (Suzuki *et al.*, 1994). Sections (6 μm) were cut on a rotary microtome and stained with hematoxylin–eosin.

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