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Fibroblast growth factor, but not activin, is a potent activator of mitogen-activated protein kinase in *Xenopus* explants

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ABSTRACT **Isolated explants from the animal hemisphere of *Xenopus* embryos were incubated with *Xenopus* basic fibroblast growth factor (XbFGF) or human activin A. XbFGF incubation resulted in the rapid activation of mitogen-activated protein kinase (MAPK) and ribosomal S6 protein kinase ($pp90^{rsk}$) in a dose-dependent manner with the highest levels of activation occurring at 50 ng/ml. Maximal activation occurred within 6–10 min after the addition of growth factor, and the activity of both kinases declined to unstimulated levels after 30 min. Activin was unable to activate either MAPK or $pp90^{rsk}$ in the *Xenopus* explants to a substantial level, although it induced dorsal mesoderm better than XbFGF under the same experimental conditions. The regulatory protein Xwnt-8 did not activate MAPK, nor did it enhance the activation of MAPK by XbFGF. XbFGF was able to activate MAPK through at least the midgastrula stage, suggesting that this family of growth factors may have a role in gastrula-stage events.**

Mitogen-activated protein kinase (MAPK) is an essential component of a protein kinase cascade linking signals at the cell surface to changes in intracellular phosphorylation and gene expression (reviewed in refs. 1–3). A wide array of mitogenic signals, including growth factors that signal through receptor tyrosine kinases, as well as activators of protein kinase C, G proteins, and Ras, lead to the phosphorylation of MAPK on both tyrosine and threonine residues and activate the enzyme. The MAPK cascade is found in cultured cells (4, 5) as well as *Xenopus* oocytes (6) and includes MAPK kinase (MAPKK; reviewed in ref. 7; also referred to as MEK) and its upstream activators Raf-1 (8–10), Mos (11), and MEK kinase (12). In addition, two activators of MAPKK have recently been isolated from *Xenopus* extracts (13, 14) that are potentially different than the other characterized MAPKK activators. Several substrates have been identified for MAPK, including ribosomal S6 protein kinase ($pp90^{rsk}$), MAPKAP-2, and phospholipase A_2 (reviewed in refs. 1 and 2). In addition, activated MAPK has been found to translocate to the nucleus (15) and has been implicated in transcriptional regulation through MAPK substrates such as c-Myc, c-Jun, c-Fos, NF-IL6, $p62^{TCF}$, and ATF-2 (1, 2).

A central focus of vertebrate developmental biologists over the past several decades has been to identify the intercellular signals that are responsible for inducing and patterning the mesoderm. Over 20 years ago, the experiments of Nieuwkoop and colleagues (16–19) suggested that the amphibian vegetal hemisphere signals the equatorial region of the overlying animal hemisphere to convert from an ectodermal to a mesodermal fate. During this inductive process, the mesoderm also becomes regionally patterned along the dorsal–ventral axis (16). Several factors that are likely to be involved in mesoderm induction have recently been identified, including members of the fibroblast growth factor (FGF), transforming growth factor β (TGF-β), and Wnt gene families and the product of the *Xenopus* gene noggin (reviewed in refs. 20 and 21). Two of these factors, basic FGF (bFGF) and activin (a member of the TGF-β family), are able to convert ectodermal cells to mesoderm, whereas noggin and several members of the Wnt family have the ability to modulate the type of mesoderm that forms but are not capable of inducing mesoderm themselves during the blastula stages (22, 23).

The inducing abilities of candidate signaling molecules have been studied by using either explants of the animal hemisphere or single cells isolated from the animal hemisphere. The explants or cells are cultured in the presence of potential inducing factors and then assayed for the presence of differentiated mesodermal cell types or the expression of mesoderm-specific genes (24–28). Activin and bFGF have been found to induce a similar array of mesodermal genes and cell types (26, 29, 30), although bFGF is a weaker inducer of dorsal mesoderm and is unable to induce anterior genes and cell types which can only be induced by activin (31–33). Injection of RNA encoding a dominant negative FGF receptor into fertilized *Xenopus* eggs leads to embryos that are missing all but anterior structures, presumably because the FGF and not the activin signaling pathway has been eliminated (34). Expression of a dominant negative form of the Raf-1 protein has been shown to elicit the same phenotype as found by expression of the dominant negative FGF receptor, indicating that Raf-1 is a component of the FGF signaling pathway in *Xenopus* embryos (35). Since Raf-1 is an integral part of the MAPK cascade in cultured cells (8–10), FGF is a potential activator of the MAPK pathway in *Xenopus* embryos.

Although the FGF receptor is a tyrosine kinase (36) whereas the activin receptor is a serine/threonine kinase (37), both receptors may activate a common signal-transduction pathway to induce mesoderm. This possibility was tested by examining MAPK regulation in response to *Xenopus* bFGF (XbFGF) and activin in embryonic *Xenopus* explants. We find that in these cells, XbFGF induces a rapid activation of MAPK, similar to that observed in cultured cells. Activin is unable to substantially activate MAPK in these explants, indicating that activin-mediated mesoderm induction operates primarily through alternative pathways. Finally, we show that XbFGF can activate MAPK through the midgastrula stages, demonstrating that the FGF intracellular signaling pathway is still functional even though XbFGF is unable to induce mesoderm in animal-hemisphere explants at these later stages.

MATERIALS AND METHODS

Embryos and Growth Factors. Fertilized eggs were prepared as described (38). After the jelly coat was removed with 2% cysteine (pH 7.8), the eggs were washed in 0.1× MMR

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Abbreviations: MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; bFGF, basic fibroblast growth factor; XbFGF, *Xenopus* bFGF; MBP, myelin basic protein.

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(1× MMR is 0.1 M NaCl/2 mM KCl/1 mM $MgSO_4$/2 mM $CaCl_2$/5 mM Hepes/0.1 mM EDTA, pH 7.8). Recombinant XbFGF was prepared as described (39) and used at 100 ng/ml except where indicated. Activin A, a gift of Genentech, was used at 2.4 ng/ml.

Animal-Cap Assay. The upper portion of the animal hemisphere, corresponding to roughly one-fourth of the embryo, was manually separated at stage 8–9, with care taken to remove adhering vegetal cells, and cultured in 1× MMR containing bovine serum albumin (100 μg/ml). Where applicable, XbFGF or activin A was added to the buffer. The explants were incubated in these solutions and harvested after 7 min unless otherwise indicated.

RNA Isolation and Analysis. RNA was prepared from animal-cap explants at stage 19 and examined by RNase protection (40) with a 500-bp antisense probe from the *Xenopus* cardiac actin gene (41) and an antisense probe transcribed from the *Xenopus* elongation factor EF-1α gene (42) synthesized with [^{32}P]UTP at reduced specific activity (43).

RNA Synthesis and Microinjection. RNA was synthesized and microinjected following published procedures (44), except that the RNA was purified and concentrated using a Microcon 100 spin column (Amicon).

Measurement of MAPK and S6 Peptide Kinase (pp90^{rsk}) Activity. Extracts of animal-cap explants were prepared by repeated pipetting in 100 μl of 20 mM Tris, pH 7.6/45 mM β-glycerophosphate/11 mM EGTA/10 mM $MgCl_2$/3 mM dithiothreitol/1 mM Na_3VO_4/1 mM phenylmethanesulfonyl fluoride. Homogenates were centrifuged at 100,000 × *g* for 20 min at 4°C. The supernatant was filtered through a small amount of glass wool. Aliquots (5 μl) of this extract were assayed for MAPK activity by measuring the phosphorylation of myelin basic protein (MBP) (45).

The pp90^{rsk} activity was assayed by measuring the phosphorylation of a peptide (Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala) patterned after one of the phosphorylation sites of the ribosomal S6 protein (46). pp90^{rsk} activity was calculated by subtracting the counts incorporated in the absence of S6 peptide from those obtained in the presence of peptide.

Antibodies to MAPK and pp90^{rsk} were developed as described (47).

Detection of MAPK Activity in SDS/Polyacrylamide Gels Containing MBP. MAPK activity was measured in SDS/polyacrylamide gels containing MBP (0.5 mg/ml), by a modification of the method of Gotoh *et al.* (48). One microgram of total protein was mixed with Laemmli sample buffer and loaded without boiling onto an SDS/polyacrylamide gel (10% acrylamide). After electrophoresis, the proteins were denatured and renatured according to the published procedure and the kinase reaction was performed with a modification of the amount of [γ-^{32}P]ATP included in the assay mixture (250 μCi/10 ml; 1 μCi = 37 kBq). To determine the amount of phosphorylation in the gel due to autophosphorylation, a gel lacking MBP was cast and the procedure was performed as described above.

RESULTS

Activation of MAPK by XbFGF. Animal-hemisphere explants (the animal cap) dissected from *Xenopus* embryos at the late blastula stage develop into undifferentiated epidermis in the absence of growth factors (16). Treatment of these explants with bFGF induces a range of mesodermal cell types found in the ventral, lateral, and dorsal–lateral regions of the embryo (26, 49, 50). To examine whether addition of bFGF activates MAPK in embryos, groups of 10 *Xenopus* animal-cap explants were incubated with various concentrations of recombinant XbFGF for 7 min. MAPK activity was measured in protein extracts of these explants by the addition of [γ-^{32}P]ATP and MBP. At the lowest dose of XbFGF (0.4 ng/ml), the phosphorylation of MBP increased 18% above the control levels (Fig. 1). Addition of higher levels of XbFGF resulted in increased MAPK activity, reaching a maximum when XbFGF at 50 ng/ml was added to the explants. To ensure that MAPK was maximally activated in subsequent experiments, XbFGF was used at 100 ng/ml.

Rapid Activation of MAPK and pp90^{rsk} by XbFGF but Not by Activin. MAPK and pp90^{rsk} are rapidly activated and inactivated in cultured cells in response to growth factors (46). To examine the regulation of MAPK and pp90^{rsk} in *Xenopus* embryos, explants were incubated with XbFGF or activin for various lengths of time and assayed for MAPK and pp90^{rsk} activity. At the concentration of activin used here (2.4 ng/ml), a wide variety of mesoderm-specific genes were activated, including the dorsal–anterior gene goosecoid (31, 51). XbFGF addition resulted in an increase in MBP phosphorylation within 2 min, with the maximum level of MAPK activity occurring at 6–10 min (Fig. 2*A* and data not shown). The stimulation of MAPK activity was transient, with MBP phosphorylation declining after 10 min and returning to the basal level by 30 min (Fig. 2*A*). The activity of a substrate of MAPK, pp90^{rsk}, was also measured in the explant extracts by using a peptide substrate (S6 peptide) that has been shown to be phosphorylated by pp90^{rsk} (46). An S6 peptide kinase activity was found to rapidly increase and decrease in extracts of *Xenopus* explants following XbFGF addition (Fig. 2*B*). Immunoprecipitation experiments with anti-pp90^{rsk} antibodies demonstrated that this kinase activity was catalyzed by pp90^{rsk}. Additional experiments indicated that a MAPKK was also activated and inactivated in a similar manner (data not shown). Activin, in contrast to XbFGF, caused only a slight increase in the phosphorylation of MBP and little or no phosphorylation of the S6 peptide (Fig. 2 *A* and *B*). However, the concentration of activin used in these studies was able to induce the muscle-specific gene encoding cardiac actin, a marker of dorsal–lateral mesoderm, more effectively than XbFGF (Fig. 2*C*).

XbFGF-Stimulated MBP Phosphorylation Is Catalyzed by MAPK. To confirm that the MBP phosphorylation measured was due to MAPK, extracts from control, XbFGF-treated, or activin-treated explants were analyzed by an "in-gel" phosphorylation of MBP procedure (48). Incubation of the gel with [γ-^{32}P]ATP resulted in the phosphorylation of MBP by

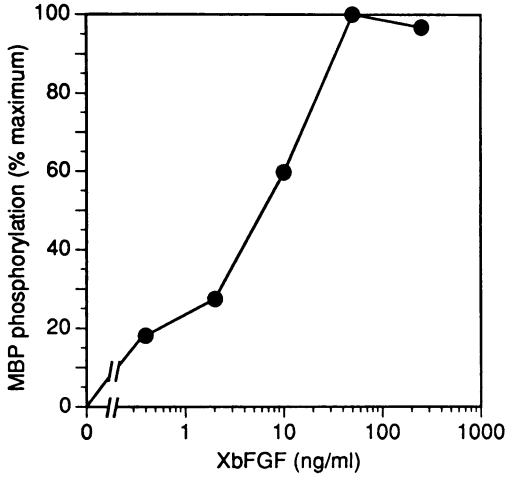

FIG. 1. Dose-dependent activation of MAPK by XbFGF. *Xenopus* animal-cap explants were cultured for 7 min in the presence of various amounts of XbFGF. Explants were homogenized and assayed for MAPK activity. The percent of maximum MBP phosphorylation is plotted on the *y* axis and was calculated from the mean of duplicate samples.

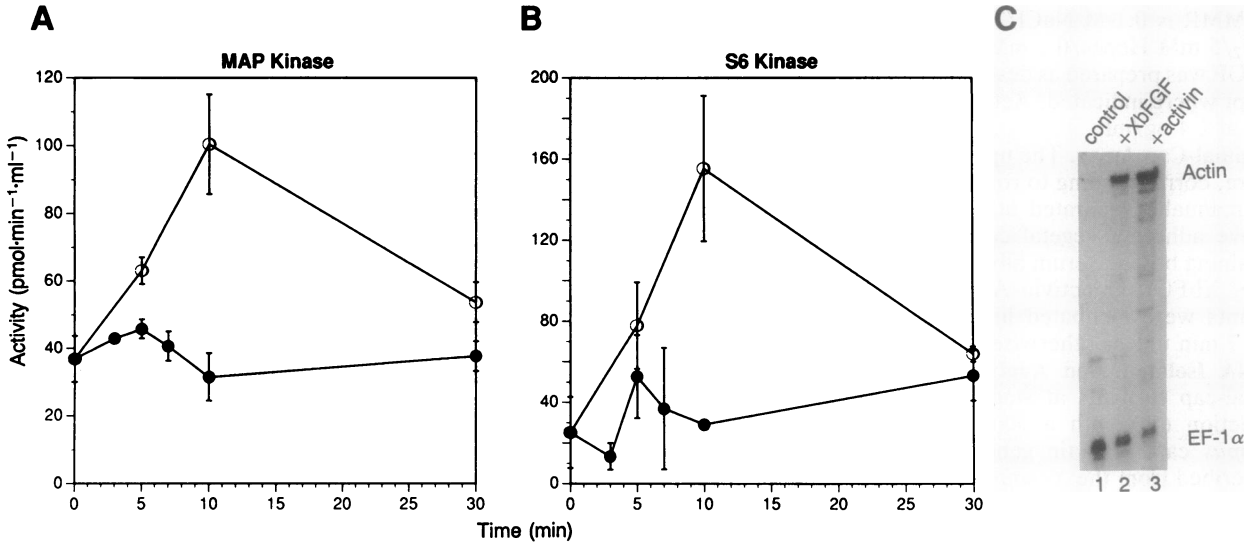

FIG. 2. (*A* and *B*) MAPK and S6 peptide kinase (pp90^{rsk}) activity from explants incubated with XbFGF or activin. Animal-cap explants were treated with XbFGF at 100 ng/ml (○) or activin at 2.4 ng/ml (●) for various times, homogenized, and assayed for MAPK (*A*) or pp90^{rsk} (*B*) activity. MAPK and pp90^{rsk} activity is represented as the mean of duplicate samples ± SEM. (*C*) Animal-cap explants were incubated in buffer alone or with XbFGF (100 ng/ml) or activin (2.4 ng/ml) until control embryos reached stage 19. Expression of the muscle-specific gene encoding cardiac actin and the ubiquitously expressed gene EF-1α, which served as a control for the amount of RNA in each sample, was measured by RNase protection analysis.

several protein kinases. Addition of XbFGF to explants resulted in the stimulated phosphorylation of MBP by a 42-kDa protein (Fig. 3, lanes 1 and 2), which migrated similarly to active recombinant MAPK (Erk-2; Fig. 3, lane 4). Immunoblotting experiments with anti-MAPK antibodies confirmed that the 42-kDa protein kinase was MAPK, or a kinase highly similar to MAPK, and demonstrated that this enzyme comigrated with recombinant Erk-2 (data not shown). An additional minor MBP phosphorylating activity was observed at 75 kDa (Fig. 3, lane 2). These phosphorylated proteins were not present when MBP was not included in the polyacrylamide gel (data not shown). Addition of activin to the *Xenopus* explants did not stimulate the phosphorylation of MBP by the 42-kDa protein kinase (Fig. 3, lanes 1 and 3).

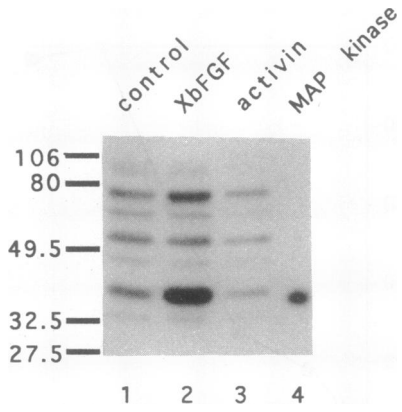

FIG. 3. MBP in-gel phosphorylation analysis of XbFGF- and activin-treated explants. Animal-cap explants were incubated with or without XbFGF or activin for 7 min and homogenized. Approximately 1 μg of extract protein was electrophoresed in an SDS/polyacrylamide gel containing MBP (0.5 mg/ml). The proteins in the gel were renatured and incubated with [γ-^{32}P]ATP to assay for MBP kinase activity. Extracts were from untreated animal caps (lane 1), animal caps incubated with XbFGF at 100 ng/ml (lane 2), or with activin at 2.4 ng/ml (lane 3). Lane 4, purified active recombinant MAPK (Erk-2). Molecular size (kDa) markers are at left.

XbFGF Can Activate MAPK During Gastrulation. In animal-cap explants, XbFGF can induce the formation of mesoderm from the 32-cell stage until the end of the blastula stage (stage 9) (26). By the start of gastrulation (stage 10), XbFGF is no longer capable of inducing mesoderm in these explants. To determine whether the animal cap cells were still able to receive the XbFGF signal after stage 9, the XbFGF-mediated activation of MAPK was measured by using animal-cap explants derived from embryos at stages 9, 10, and 11 (midgastrula). XbFGF was able to activate MAPK during the gastrula stages (Fig. 4), despite its inability to induce mesoderm after stage 9 (26). The XbFGF-stimulated activity was demonstrated to be MAPK by the in-gel phosphorylation of MBP performed on samples from stages 9–11 (data not shown). Although the total level of XbFGF-stimulated MBP phosphorylation was higher in stages 10 and 11, the unstimulated level of MBP phosphorylation also increased during the later stages (Fig. 4). This result demonstrates that the

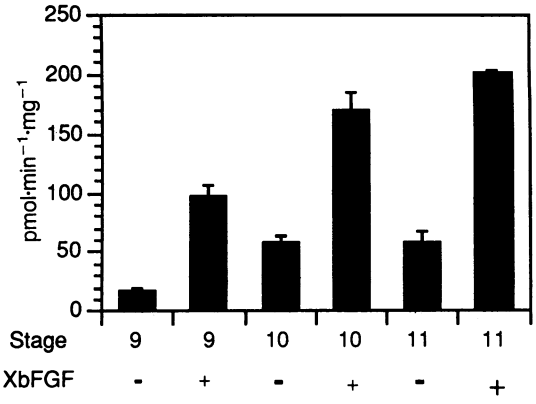

FIG. 4. XbFGF-stimulated MAPK activity during gastrulation. Animal caps were dissected at various stages and treated with or without XbFGF (100 ng/ml) for 7 min and the extracts were assayed for MAPK activity. MBP phosphorylation was corrected for differences in total protein in each sample to account for differences in the size of the animal caps at different stages. MAPK activity is expressed as the mean + SEM of duplicate samples.

ability of XbFGF to activate the MAPK signal-transduction pathway is maintained during gastrulation.

Xwnt-8 Does Not Alter the Activation of MAPK. Xwnt-8 is a secreted protein of the Wnt family that can induce the formation of a dorsal axis in ventralized *Xenopus* embryos when injected into one- or two-cell embryos (52, 53). In addition, Xwnt-8 enhances the dorsal mesoderm-inducing properties of XbFGF (23). This effect might be due to a potentiation of the FGF intracellular signaling pathway, since increasing concentrations of XbFGF induce more dorsal-type mesoderm in explant cultures (26). Therefore, we examined the effects of Xwnt-8 on XbFGF-induced MAPK activity. Since Xwnt-8 is not available as a soluble factor, animal caps were isolated from embryos that were injected twice at the two-cell stage with 100 pg of *in vitro* synthesized RNA encoding Xwnt-8. When the same amount of Xwnt-8 RNA was injected into the equatorial region, >90% of the embryos displayed the bifurcated axis that is typical of Xwnt-8-injected embryos (54) (data not shown). Animal caps obtained from Xwnt-8-injected embryos had a slightly higher level of MBP phosphorylation than animal caps from control embryos (Fig. 5). However, Xwnt-8 did not increase the FGF-mediated activation of MBP phosphorylation. These results indicate that Xwnt-8 does not enhance the formation of dorsal mesoderm in animal caps treated with FGF by increasing the activity of MAPK.

Endogenous Levels of MAPK. MAPK in *Xenopus* is phosphorylated on tyrosine in unfertilized eggs but is rapidly dephosphorylated after fertilization (55). To extend the study of MAPK regulation into the blastula stages, MAPK activity was measured at various stages after fertilization by the standard procedure used above and by the in-gel MBP phosphorylation assay. A 42-kDa protein kinase which cross-reacted with anti-MAPK antibodies was observed to be the predominant MBP-phosphorylating species prior to fertilization. Within minutes after fertilization, MAPK activity rapidly decreased, reached a basal level after 1 hr, and remained at a low level throughout the blastula stages (Fig. 6). Similar results were obtained when MAPK activity was measured by the phosphorylation of MBP in solution (data not shown). MAPKK activity paralleled that of MAPK, with high levels in unfertilized eggs and a rapid decline to low levels after fertilization. Neither MAPK nor MAPKK abundance decreased during this time as determined by immunoblotting with anti-MAPK and anti-MAPKK antibodies (data not shown).

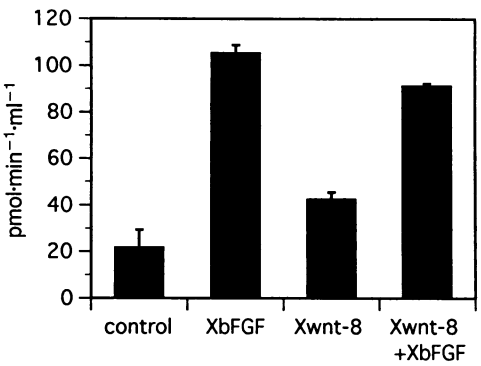

FIG. 5. MAPK activity after Xwnt-8 injection. Embryos at the two-cell stage were injected with 100 pg of RNA encoding Xwnt-8 and then were allowed to develop until the blastula stage. Animal-cap explants were isolated from Xwnt-8-injected or uninjected embryos and incubated with or without XbFGF (100 ng/ml), and the extracts were assayed for MAPK activity. MAPK activity is expressed as the mean + SEM of duplicate samples.

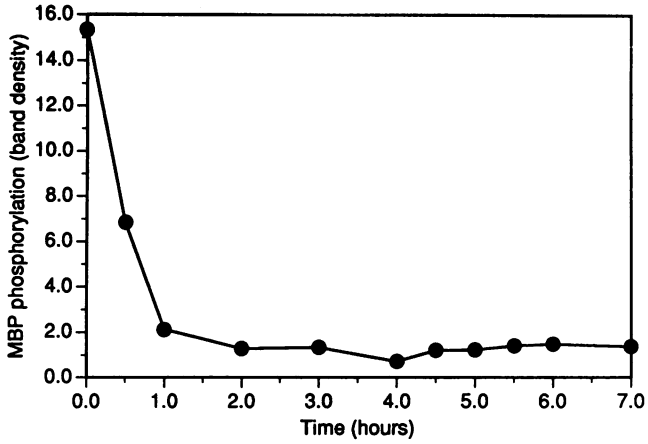

FIG. 6. MBP in-gel phosphorylation analysis of MAPK activity after fertilization. Ten *Xenopus* embryos were collected prior to and at various times after fertilization. The embryos were dejellied and homogenized, and 1 μg of each extract was assayed for MBP phosphorylation by the in-gel procedure. The radioactive gel was dried and exposed to film, and the intensity of the bands was quantitated by scanning the exposed film with a Bio-Rad GS-670 densitometer. Band volume was determined as the band intensity/area according to the manufacturer's instructions. Only the species migrating at 42 kDa is plotted.

DISCUSSION

We have shown that XbFGF induces a rapid activation of MAPK and pp90^{rsk} activity in animal-cap explants from late-blastula *Xenopus* embryos. A stimulation of MAPK activity was detected with XbFGF concentrations as low as 0.4 ng/ml, and a dose-dependent increase in activity was observed up to 50 ng/ml, a concentration of XbFGF that resulted in the maximal stimulation of MAPK and pp90^{rsk} activity. In a detailed study of the effects of XbFGF on animal-cap explants, XbFGF at 0.1 ng/ml was found to induce the formation of ventral mesoderm (26). At higher levels of XbFGF, increasingly more lateral and dorsal–lateral mesoderm (muscle) was induced. At the highest concentration of XbFGF tested (48 ng/ml), the ability of XbFGF to induce muscle was still increasing, demonstrating that XbFGF can induce mesoderm in animal-cap explants between at least 0.1 ng/ml and 48 ng/ml. Since we observe a progressive increase in MAPK levels over a similar range of XbFGF concentrations, we suggest that the type of response of animal-cap explants to XbFGF may be mediated in part by the extent of MAPK activation, with higher levels of MAPK activity leading to the formation of more dorsal-type mesoderm. Interestingly, the ability of Xwnt-8 to augment the dorsal character of mesoderm induced by XbFGF (23) is not due to amplification of the MAPK activity but may occur by a separate event.

Activin and FGF induce a similar spectrum of mesodermal genes and cell types in *Xenopus* animal-cap explants, although activin is a more effective inducer of dorsal mesoderm than FGF, and only activin can induce anterior mesoderm (26). Increased concentrations of both factors shift the response to more dorsal-type mesoderm, although XbFGF is typically a weak inducer of muscle, a marker of dorsal mesoderm, in these assays (26, 56). Using a concentration of activin that strongly induces dorsal and anterior mesoderm, we did not detect a substantial activation of MAPK or pp90^{rsk}, indicating that activin-mediated mesoderm induction uses a different intracellular pathway. Similarly, human bFGF, but not activin, stimulates the tyrosine phosphorylation of MAPK in *Xenopus* explants (57). Consistent with these results is the finding that a dominant negative Raf-1 inhibited FGF-stimulated, but not activin-stimulated, mesoderm formation (35). Although we cannot rule out the pos-

sibility that activin weakly activates MAPK as part of its intracellular signaling pathway, it is clear that activin is not a stronger inducer of dorsal mesoderm than XbFGF simply because it is more effective at activating MAPK. We do not know whether the two pathways intersect to activate a common set of transcription factors or whether the set of genes that are induced by both factors can be independently activated by XbFGF-regulated and activin-regulated transcription factors.

The rapid inactivation of MAPK and MAPKK after fertilization may be necessary for mesoderm induction to occur. By reducing the activity of MAPKK and MAPK to a low level, the embryo is able to respond to exogenous signals from the FGF receptor, causing a transient rise in MAPKK and MAPK activity. Overexpression of Raf-1 (35) or expression of a constitutively active form of Ras (58), both of which are expected to activate the MAPK pathway, leads to the induction of ectopic mesoderm in animal caps. These results suggest that the disappearance of the c-Mos protein, a MAPKK kinase which may be responsible for the activation of MAPK and MAPKK in unfertilized eggs (59), must occur prior to the early embryonic stages in order to allow the embryo to respond to mesoderm-inducing signals.

The competence for XbFGF-mediated mesoderm induction ends at the start of gastrulation (26). Since the XbFGF receptor protein is detected throughout the gastrula stages, it has been suggested that the end of competence may be due to the elimination of an accessory protein (60). However, we find that XbFGF is able to activate MAPK during the gastrula stages, suggesting that the elimination of competence for XbFGF-mediated mesoderm induction occurs through alterations in a subsequent step. In addition, the ability of XbFGF to activate MAPK during gastrulation indicates a potential role for a FGF in regulating some aspects of gastrula-stage embryonic development. One possibility is that there is a requirement for FGF signaling in neural induction, which most likely occurs during these stages. Interestingly, a dominant negative FGF receptor blocks the expression of the neural marker N-CAM in *Xenopus* explants treated with activin (51). These results suggest a possible role for the MAPK signaling pathway in the induction of neural ectoderm.

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