Mesoderm Induction by Heterodimeric AP-1 (c-Jun and c-Fos) and Its Involvement in Mesoderm Formation through the Embryonic Fibroblast Growth Factor/Xbra Autocatalytic Loop during the Early Development of Xenopus Embryos*

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We have previously demonstrated the involvement of AP-1/Jun in fibroblast growth factor (FGF) signaling by loss-of-function assay (Dong, Z., Xu, R.-H., Kim, J., Zhan, S.-N., Ma, W.-Y., Colburn, N. H., and Kung, H. (1996) J. Biol. Chem. 271, 9942–9946). Further investigations by gain-of-function are reported in this study. AP-1 transactivation activity was increased by the treatment of animal cap explants with FGF. Ectopic overexpression of two components of AP-1 (c-jun and c-fos together, but not alone) produced posteriorized embryos and induced mesoderm formation in animal cap explants, indicating that both AP-1 heterodimers are required for mesoderm induction. Since Ras/AP-1 functions downstream of FGF signal, we then tested the involvement of Ras/AP-1 in FGF signaling mediated by embryonic FGF/Xbra using dominant-negative mutants. Mesoderm maintenance mediated by embryonic FGF/Xbra was blocked by dominant-negative mutants of Ras/AP-1, and AP-1 enhanced the expression of Xbra. Further studies demonstrated the inhibition of Ras/AP-1-mediated mesoderm formation by dominant-negative mutants of the FGF receptor and Xbra. These results indicate that Ras/AP-1 and FGF/Xbra signals are involved in the mesoderm maintenance machinery and mesoderm formation through the synergistic action of the diversified signal pathways derived from the FGF/Xbra autocatalytic loop.

In early vertebrate development, one of the most important events is the mesoderm-inducing process, and several mesoderm-inducing factors have been identified using an amphibian embryo system (1–6). In Xenopus, normal mesoderm formation in the embryo largely depends on FGF1 signaling (7–13). Experiments using the dominant-negative FGF receptor demonstrate that FGF signaling is required for posterior mesoderm formation in whole embryos (7, 8) and is also required for mesoderm induction in response to members of the transform-
derm maintenance machinery mediated by eFGF/Xbra. Furthermore, we found that FGF induces mesoderm formation and maintenance through the synergistic action of the diversified signals derived from the FGF/Xbra autocatalytic loop.

**EXPERIMENTAL PROCEDURES**

**DNA and RNA Preparation**—The c-jun cDNA was inserted into pGEM (28), and c-fos and antisense c-fos were subcloned into the pSP65 vector (32). The dominant-negative FGF receptor (DN-FR) (7), Xbra,
FIG. 2. Mesoderm induction in blastula animal caps by AP-1 (c-jun/c-fos). a, morphological changes in animal cap explants. X. laevis embryos were obtained by in vitro fertilization (35). Embryos at the two-cell stage were injected in the animal pole with mRNAs of control β-galactosidase (1 ng), c-jun (1 ng), c-fos (1 ng), or c-jun (1 ng) plus antisense c-fos (1 ng) (panel A); c-jun/c-fos (0.25 ng each) (B); c-jun/c-fos (0.5 ng each) (C); or c-jun/c-fos (1 each ng) (D). c-jun, c-fos, and antisense c-fos were derived from rat clones (28, 32). Animal caps were dissected from...
and Xbra-Engrailed were inserted into the pSP64T vector (33, 34). Xbra-Engrailed (dominant-negative mutant of Xbra (DN-Xbra)) is a dominant-interfering Xbra generated by replacing the activation domain of Xbra with the repressor domain of the Drosophila Engrailed protein (34). DN-jun was subcloned into the pSP64TEN vector (26, 27). The constitutively active ras (Val12-Ha-Ras) and dominant interfering ras (DN-ras; Asn17[Ha-Ras]) cDNAs were inserted into the pSP64 vector (18). Each of the cDNAs were linearized and used for in vitro synthesis of capped mRNA using an Ambion transcription kit in accordance with the manufacturer’s instructions. The synthetic RNA was quantitated by ethidium bromide staining in comparison with a standard RNA.

Embryo Injection and Explant Culture—Xenopus laevis embryos were obtained by in vitro fertilization (35). Developmental stages were designated according to Nieuwkoop and Faber (36). Embryos at the two-cell stage were injected in the animal pole with messenger RNA or cDNA as described in the figure legends. Animal caps were dissected from the injected embryos at stages 8.5–9 and cultured to various stages for further analysis as described in the figure legends.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Total RNA was extracted from cultured explants with TRIzol reagent (Life Technologies, Inc.) following the manufacturer’s instructions. RT-PCR was performed with a Superscript preamplification system (Life Technologies, Inc.). Primer sets and PCR conditions for Xbra, muscle actin, and EF-1a were as described previously (26, 36).

Analysis of AP-1 and NF-κB Activities in Animal Cap Culture—AP-1- or NF-κB-luciferase plasmid DNA (26, 37, 38) was injected alone or together with the designated RNA into two blastomers of the two-cell stage embryo as described in the figure legends. After injection, the animal caps were excised from the embryos (stages 8.5–9) and cultured in the presence of different dosages of bFGF (2–100 ng/ml) until stages 10.5 and 13. AP-1-dependent luciferase activity in the animal cap explants was measured after homogenization in lysis buffer as described previously (26).

RESULTS AND DISCUSSION

bFGF Stimulates AP-1 Activity in Animal Cap Explants during Mesoderm Induction (Stage 10.5) and Mesoderm Maintenance (Stage 13) Stages—Previously, we reported high levels of AP-1-dependent transactivation activity during Xenopus early embryonic development and inhibition of FGF-induced mesoderm formation in animal cap explants from embryos injected with DN-jun RNA (26). To determine whether AP-1 is a target transcription factor of the FGF signal, we performed the following experiments. Embryos were injected with AP-1- or NF-κB-luciferase reporter genes at the two-cell stage. The animal pole tissue was then dissected at stages 8.5–9 and incubated with different dosages of bFGF. The bFGF-treated animal caps were harvested at stages 10.5 and 13, and the luciferase activity in the animal caps was measured. The luciferase activity in the animal caps injected with the NF-κB-luciferase reporter gene was not affected by bFGF (Fig. 1B). On the other hand, the luciferase activity in the animal caps injected with the AP-1-luciferase reporter plasmid was increased 2–8-fold by bFGF treatment in a dose-dependent manner (Fig. 1A).

In separate experiments, AP-1-luciferase reporter plasmids were co-injected with the mRNAs encoding c-Jun and c-Fos, and the luciferase activity was compared with the animal cap explants treated with bFGF. AP-1 RNA (c-jun/c-fos)-injected animal cap explants showed similar luciferase activity as bFGF-treated animal cap explants at stages 10.5 and 13 (Fig. 1C). The AP-1 activity in FGF-treated animal cap explants was inhibited by DN-ras RNA injection (data not shown), suggesting that FGF signals through the Ras pathway for AP-1 activation. However, AP-1-dependent luciferase activity was not affected in animal cap explants injected with noggin or dominant-negative BMP-4 receptor mRNA (data not shown), which caused neutralization in the ectoderm.

Heterodimeric AP-1 Induces Mesoderm Formation—The mesoderm-inducing activity of AP-1 (c-jun and c-fos) was then examined by injection of RNA encoding c-Jun and/or c-Fos at the two-cell stage. The animal pole tissue was dissected at the blastula stage (stages 8.5–9) and incubated until the animal cap explants were harvested. Injection of β-galactosidase, c-jun, or c-fos RNA alone or c-jun plus antisense c-fos RNA did not induce any morphological changes in the animal cap explants cultured until the tadpole stage (Fig. 2a, panel A). On the other hand, animal caps from embryos injected with c-jun and c-fos RNAs together showed morphological changes (the animal caps were swollen and elongated) similar to those induced by FGF treatment in a dose-dependent manner (Fig. 2a, panels B–D).

To confirm the mesoderm induction in animal caps, we examined early and late molecular markers for mesoderm by RT-PCR. Xbra, an immediate-early marker for mesoderm, and muscle-specific actin, a late marker for mesodermal differentiation, were expressed in AP-1 (c-jun/c-fos)-injected caps, but not in β-galactosidase, c-jun, or c-jun RNA-injected caps or in c-jun plus antisense c-fos RNA-injected caps (Fig. 2b). Immunohistochemical analysis showed that the animal cap injected with c-jun and c-fos together generated unorganized muscle tissues similar to the tissues generated by treatment with bFGF. Actin was detected by specific antibody to muscle actin in the sections of c-jun- and c-fos-injected animal cap samples and bFGF (100 ng)-treated samples, but not in the other samples (Fig. 2c). For mesoderm formation, injection of 0.5 ng of c-jun RNA and 0.5 ng of c-fos RNA together was sufficient to induce Xbra at the early stage (stage 11) and muscle actin at the later stage (stage 24) in ectoderm cells. However, animal cap explants injected with up to 4 ng of c-jun alone did not show any detectable morphological changes or increased expression of the mesodermal markers Xbra and actin. A very small amount of Xbra was detected in c-jun-injected animal cap explants as determined by RT-PCR (Fig. 2d).

These results clearly demonstrate that heterodimeric AP-1

the injected embryos at stages 8.5–9 and cultured until stage 30 for the picture. The experiment was repeated three times in over 120 embryos with similar results. Developmental stages were designated according to Nieuwkoop and Faber (36). b, molecular markers of mesoderm induction induced by AP-1 (c-jun and c-fos together). Embryos were injected, and animal caps were dissected and cultured as described for a. RNA was isolated from animal caps with the following: lane 1, β-galactosidase (β-gal); lane 2, c-jun (1 ng); lane 3, c-fos (1 ng); lane 4, c-jun (1 ng) plus antisense c-fos (1 ng); lane 5, c-jun (1 ng) plus sense c-fos (1 ng); lane 6, control (No Injection). The RNA was analyzed by RT-PCR (26, 35) to determine the expression of the molecular markers Xbra and EF-1a with stage 11 animal caps and muscle actin and EF-1a with stage 30 animal caps. Embryos at equivalent stages were used as a positive control (lane 8). Immunohistochemical analysis of β-galactosidase RNA (1 ng) (panel A) or AP-1 RNAs (1 ng of c-jun and 1 ng of c-fos) (panel B) and by treatment with bFGF (100 ng/ml) (panel C). Animal caps were harvested and sectioned at stage 30. The slides were then fixed and stained with specific antibody to muscle actin. Panels B and C show the muscle actin-stained section (dark brown color) with unorganized muscle tissue. Panel A shows an unstained section with a typical epidermis structure. The animal caps from embryos injected with c-jun (1 ng), c-fos (1 ng), or c-jun (1 ng) plus antisense c-fos (1 ng) were examined with the same procedure as described for panels A, B, and C of Fig. 2c. Typical epidermis structures similar to β-galactosidase-treated animal caps were found. d, dose-dependent mesoderm formation as measured by expression of Xbra (stage 11) and actin (stage 24). Injection of c-jun alone (0.5–4 ng in lanes 1–4 in the upper panels), c-jun + c-fos (0.5–4 ng; 0.5 ng = 0.25 ng of each in lane 1, 1 ng = 0.5 ng of each in lane 2, 2 ng = 1 ng of each in lane 3, and 4 ng = 2 ng of each in lane 4 in the lower panels), and β-galactosidase (lane 5). Lane 6 is the positive control of the embryo, and lane 7 is the negative control with no reverse transcriptase. Embryos were injected, and animal caps were dissected and cultured as described for a. RT-PCR was performed as described for b. Expression of EF-1a was used as a control for equal loading of reverse transcriptase samples.
(c-\textit{jun} and \textit{c-fos}) is sufficient to induce mesoderm in animal cap explants, similar to FGF and its signal molecules, Ras/Raf/mitogen-activated protein kinase, but homodimeric AP-1 (c-Jun) is not able to induce mesoderm. The previous results (26) for the AP-1/Jun involvement in FGF-mediated mesoderm induction by loss-of-function assay with DN-\textit{jun} were possibly

\textbf{FIG. 3.} \textit{Morphological changes in whole embryos induced by ectopic expression of AP-1 (c-\textit{jun} and c-\textit{fos}).} a, dose-dependent morphological changes in whole embryos by injection of AP-1 (0.125–1 ng of c-\textit{jun/c-fos}). Panel A, 0.125 ng each; panel B, 0.25 ng each; panel C, 0.5 ng each; panel D, 1 ng each. The RNAs of \textit{c-jun} and \textit{c-fos} were injected into the animal pole of the two-cell stage embryos. The injected embryos were allowed to develop until the tadpole stage. The embryos injected with AP-1 show posteriorized phenotypes with diminished anterior structures including cement gland and forebrain. The extent of morphological changes was dose-dependent. In the same experiments, the morphological changes were never observed in embryos injected with \(\beta\)-galactosidase (1 ng), c-\textit{jun} (1–4 ng), c-\textit{fos} (1–4 ng), or c-\textit{jun} (1 ng) plus antisense c-\textit{fos} (1 ng). b, gastrulation defect induced by injection of AP-1. The blastopore of an embryo injected with 1 ng of \(\beta\)-galactosidase was closed (panel A). The embryos injected with AP-1 (0.5 ng of c-\textit{jun} and 0.5 ng of c-\textit{fos}) developed normally through the blastula and early gastrula stages (not shown). However, the blastopore failed to close as shown by a round circle in the picture (panel B). Pictures were taken at stage 15. c, effects on embryonic development of injection of AP-1 into the VMZ or DMZ. AP-1 mRNAs (0.5 ng of c-\textit{jun} and 0.5 ng of c-\textit{fos}) were injected at the four-cell stage into the DMZ or VMZ. Injection of AP-1 into the VMZ caused a minor posterior defect (panel A, lower picture) compared with a normal tadpole (panel A, upper picture). In contrast, AP-1 injection into the DMZ of embryos caused a gastrulation delay and severe defects in body patterning including lack of head structure (~10% embryos) (panel B, lower picture).
caused by the blocking of heterodimeric AP-1 activity. Although we have demonstrated the requirement of both c-Jun and c-Fos for mesoderm formation, it remains to be determined whether the requirement of c-Jun/c-Fos can be replaced by other members of the Jun/Fos families. In the present study, whether the requirement of c-Jun/c-Fos can be replaced by other members of the Jun/Fos families. In the present study, we have focused only on the direct roles of c-Jun/c-Fos.

**AP-1 Causes Posteriorized Embryos**—Whole embryos injected with β-galactosidase, c-jun, or c-fos RNA alone at the two-cell stage did not show any morphological changes (data not shown). Another transcription factor (AP-2 RNA) was also injected, and no morphological change was observed (data not shown). However, c-jun and c-fos RNAs injected together caused morphological changes in a dose-dependent manner (Fig. 3a). Embryos injected with AP-1 mRNAs (0.5 ng of c-jun and 0.5 ng of c-fos) at the two-cell stage developed normally through the mid-gastrula stage, but the blastopore closure was delayed (Fig. 3b, panel B). A similar phenotype of abnormal gastrulation defects was observed with injection of other mesoderm inducers, Xbra (0.5 ng) or ras RNA (1 ng) (Refs. 12 and 33 and data not shown). Injection of low doses of AP-1 RNAs (0.125 ng of c-jun and 0.125 ng of c-fos) caused an enlarged proctodaeum at the posterior part of the embryo at the tadpole stage, which is a typical phenotype of CSKA-εFGF plasmid-injected embryos (17). The embryos injected with AP-1 exhibited a posteriorized phenotype with diminished anterior structures including cement gland and forebrain. The extent of morphological changes depends greatly on AP-1 dosages up to 2 ng. Histological analysis showed that unorganized muscle-like structure accumulated in the posterior part of AP-1-injected embryos (data not shown). Interestingly, in animal cap explants excised from embryos cojected with AP-1 and chordin, posterior neural markers were induced.2 The caudalization of neural tissues with FGF or Xbra has recently been reported (39–41), indicating that AP-1 is involved in a broad range of FGF activities.

Injection of AP-1 RNAs (c-jun and c-fos) into the dorsal marginal zone (DMZ) or the ventral marginal zone (VMZ) in embryos at the four-cell stage resulted in different phenotypes (Fig. 3c). Injection of AP-1 into the VMZ caused a minor posterior defect with normal head structure (Fig. 3c, panel A). However, injection of AP-1 RNAs into the DMZ caused a gastrulation delay and consequently more severe defects in the anterior, including lack of a head structure, but rather normal posterior structure (Fig. 3c, panel B).

While this work was in progress, an implantation experiment with eFGF beads in gastrulas was published (42). When an eFGF bead was implanted into the dorsal lip of a stage 11.5 embryo, it resulted in the loss of eyes and forebrain as well as other anterior structures, with a normal proctodaeum in the tail bud-stage embryo. However, when an eFGF bead was implanted into the ventral lip at stage 11.5, the embryos developed normally, including the head structure. The results were similar to those of the present study of AP-1 (see above). The
similarity in morphological changes further suggests that AP-1 (c-Jun/c-Fos) may be a mediator in the FGF signaling pathway.

**Ras/AP-1 Functions Downstream of FGF Signaling in Mesoderm Formation**—AP-1 transactivation activity in animal cap explants was increased by treatment with bFGF in culture media. Furthermore, AP-1 induced mesoderm formation and the expression of Xbra and muscle actin similar to bFGF. These results suggest that AP-1 may be a target molecule of FGF signaling in the nucleus. Although *Xenopus* bFGF produced from the injected mRNA has a potent mesodermalizing effect on animal hemisphere cells, bFGF has no signal sequence that may be necessary for cell-cell signaling events such as mesoderm induction. Furthermore, virtually no phenotypic change by bFGF treatment is observed in intact embryos (43). In contrast to bFGF, eFGF plays an important role in maintaining the properties of mesoderm in the gastrulas of *Xenopus* embryos in addition to its mesoderm-inducing activity during the blastula stages. The expression of eFGF increases significantly during gastrulation. In addition, eFGF caused similar morphological changes in whole embryos (posteriorization) as observed with AP-1 mRNA injection. Although Xbra and eFGF have been reported to require each other for mesoderm maintenance (13, 17), the roles of Ras/AP-1 have not been clearly demonstrated in mesoderm maintenance mediated by eFGF/Xbra. Therefore, it is interesting to test whether signaling components of FGF (Ras/AP-1) are involved in mesoderm maintenance mediated by the FGF/Xbra autocatalytic loop.

The injection of Xbra RNA or the CSKA-eFGF plasmid into
animal caps caused morphological changes (swelling and elongation) as shown in Fig. 4a. The CSKA-eFGF plasmid becomes transcriptionally active after mid-blastula transition (44), and eFGF mRNA does not accumulate until stage 10 (17). The animal cap explants start to lose competence for mesoderm induction in response to bFGF after stage 10. However, the animal cap injected with the CSKA-eFGF plasmid showed a consistently potent mesoderm-forming ability, indicating that the period of competence of ectoderm cells for mesoderm induction might be longer than what is usually expected.

Coinjection of RNAs encoding DN-Ras significantly reduced elongation movements of animal cap explants in response to eFGF or Xbra during the gastrulation stages (data not shown), suggesting that maintenance of mesoderm depends on the downstream FGF signal, Ras. As expected, coinjection of DN-jun with Xbra or eFGF inhibited elongation movements similar to DN-ras (Fig. 4a). Since the maintenance of mesoderm in the gastrulating embryo is crucial for the formation of differentiated mesoderm-like muscle (45), we examined the expression of muscle actin. Animal caps derived from coinjection of DN-ras or DN-jun with eFGF or Xbra were cultured until the tadpole stage and assayed for muscle actin expression by RT-PCR. Consistent with the morphological data, DN-ras and DN-jun significantly inhibited the expression of muscle actin in animal caps derived from coinjected samples (Fig. 4b). Since Ras/AP-1 activates Xbra expression, and Xbra is able to induce the differentiated mesoderm, it was unclear whether Ras/AP-1 was involved only in mesoderm induction or also in later events after mesoderm induction. The results suggest that Ras and AP-1 are involved in mesoderm maintenance in addition to mesoderm induction.

AP-1-induced Mesoderm Is Inhibited by DN-Xbra—FGF/Ras/AP-1 had an activity to induce an early mesodermal marker, Xbra. While ectopic overexpression of Xbra itself is enough to generate the differentiated mesoderm, Xbra still requires endogenous FGF/Ras/AP-1 signals for mesoderm maintenance and differentiated mesoderm formation. On the other hand, FGF-mediated mesoderm formation and maintenance are dependent on Xbra. Since AP-1 was found to be involved in the mesoderm maintenance machinery mediated by eFGF/Xbra, we examined whether Xbra is required for AP-1-mediated mesoderm formation. AP-1-mediated mesoderm formation was abolished by coinjection of DN-Xbra. DN-Xbra inhibited the expression of muscle actin as well as Xbra itself in animal caps derived from coinjected samples (Fig. 5). The RT-PCR product of Xbra shown in Fig. 5 has been designed not to detect injected DN-Xbra. The results indicate that intact Xbra is required for its own expression and mesoderm induction. Furthermore, the effect of DN-Xbra was not reversed by AP-1. Although it remains to be investigated how Xbra regulates its own transcription, we postulate that Xbra is required to keep the mesoderm character of the tissue and that DN-Xbra blocks the competence of the animal cap for mesoderm induction. We extended the mesoderm maintenance machinery of eFGF/Xbra to include Ras/AP-1, and the blocking of one of the components is enough to abolish the machinery of mesoderm formation, suggesting that positive feedback is necessary to maintain mesoderm (see Fig. 7).

Mesoderm Formation by FGF Signaling Is a Synergistic Action of Diversified Signal Pathways—FGF and Xbra require each other for mesoderm formation and maintenance, and the downstream signal molecules of the FGF receptor are involved in these events. However, it is not clear whether FGF signals for mesoderm induction and maintenance through a linear array of signals or through the synergistic action of the diversified signals. These two possibilities were examined by coinjecting an activated form of Ras with DN-FR. Constitutively active ras was coinjected with DN-FR, and the animal cap explants were analyzed. The elongation movement of animal cap explants caused by the overexpression of the constitutively active form of ras was inhibited by coinjection of DN-FR (Fig. 6a). Consistent with morphological data, the expression level of the early mesodermal marker (Xbra) and the differentiated mesodermal marker (muscle actin) was reduced or totally abolished by coinjection of DN-FR (Fig. 6b). This result suggests that Ras-mediated mesoderm formation requires endogenous FGF signaling and that FGF triggers at least two different signals including Ras for mesoderm formation. Consistently, AP-1-induced mesoderm formation was also inhibited by coinjection of DN-FR (Fig. 6c). Furthermore, AP-1-induced mesoderm formation was inhibited by coinjection of DN-ras (Fig. 6c).

Our results suggest that mesoderm formation induced by FGF signaling is the synergistic action of the diversified signals. Based on these results, a model is proposed. As shown in Fig. 7, the signals derived from the FGF/Xbra autoregulatory loop contribute synergistically to the formation of mesoderm. The blocking of any signals by dominant-negative mutants resulted in inhibited mesoderm formation. In this paper, we show, for the first time, that heterodimeric AP-1 (c-Jun/c-Fos) induces mesoderm formation in animal cap explants of Xenopus embryos. Additionally, we found that eFGF- or Xbra-mediated mesoderm maintenance was dependent on the downstream signals of FGF, Ras, and AP-1. Also, AP-1-mediated mesoderm formation required FGF/ras/Xbra, and Xbra expression was activated by AP-1. Furthermore, we found that FGF induced mesoderm formation and maintenance through the synergistic action of the diversified signals derived from the FGF/Xbra autoregulatory loop.

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