

Activin-mediated mesoderm induction requires FGF

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SUMMARY

The early patterning of mesoderm in the *Xenopus* embryo requires signals from several intercellular factors, including mesoderm-inducing agents that belong to the fibroblast growth factor (FGF) and TGF- β families. In animal hemisphere explants (animal caps), basic FGF and the TGF- β family member activin are capable of converting pre-ectodermal cells to a mesodermal fate, although activin is much more effective at inducing dorsal and anterior mesoderm than is basic FGF. Using a dominant-negative form of the *Xenopus* type 1 FGF receptor, we show that an FGF signal is required for the full induction of mesoderm by activin. Animal caps isolated from embryos that have been injected with the truncated FGF receptor

and cultured with activin do not extend and the induction of some genes, including cardiac actin and *Xbra*, is greatly diminished, while the induction of other genes, including the head organizer-specific genes *gsc* and *Xlim-1*, is less sensitive. These results are consistent with the phenotype of the truncated FGF receptor-injected embryo and imply that the activin induction of mesoderm depends on FGF, with some genes requiring a higher level of FGF signaling than others.

Key words: growth factors, *Xenopus*, mesoderm induction, FGF, activin, early gene expression

INTRODUCTION

While many studies have focused on the individual abilities of FGF and activin to induce mesoderm of various types from presumptive ectodermal explants and cells (for review, Kimelman et al., 1992; Sive, 1993), it is widely expected that these and other growth factors will act in combination to pattern the embryo. Indeed, current models of mesoderm patterning in *Xenopus* entail an initial induction of mesoderm in the equatorial region by signals emanating from the vegetal hemisphere with subsequent patterning of this mesoderm by signals originating in dorsal, and possibly ventral, organizing centers (Smith and Slack, 1983; Green et al., 1992; Kimelman et al., 1992; Sive, 1993). There is already ample evidence for the combinatorial effects of growth factors in vitro. bFGF combines synergistically with TGF- β 1 to induce the mesoderm-specific transcript muscle actin in animal caps (Kimelman and Kirschner, 1987), and the addition of low doses of bFGF lowers the concentration of activin necessary for the maximum induction of muscle actin in dissociated animal cap cells (Green et al., 1992). Two extracellular *Xenopus* proteins, noggin and Xwnt-8, enhance the formation of dorsal mesoderm in animal caps exposed to activin (Smith and Harland, 1991, 1992; Christian et al., 1992; Sokol and Melton, 1992) whereas the TGF- β family member BMP-4 inhibits the ability of activin to induce dorsal mesoderm (Dale et al., 1992; Jones et al., 1992). Understanding how the embryonic mesoderm becomes patterned will require the study of both the distribution of morphogens and the way cells react when they experience these signals individually and in concert.

Since little is known about the signal transduction process in *Xenopus* embryos, it is not yet clear how cells integrate different extracellular signals. Injection of RNA encoding a dominant-negative mutant of *raf*, a serine/threonine kinase activated by tyrosine kinase receptors, has been reported to eliminate only the FGF signaling pathway (MacNicol et al., 1993). Similarly, FGF can activate MAP kinase, a key regulator in the intracellular signal transduction process, whereas activin cannot (Graves, 1994; LaBonne and Whitman, 1994). While these studies suggest that FGF and activin operate through different intracellular pathways, it has been shown that a dominant-negative mutant of the ras protein can block the ability of both FGF and activin to induce at least some mesodermal markers (Whitman and Melton, 1992). Thus it is still unclear how the FGF and activin intracellular pathways are connected.

In order to explore possible interactions between activin and FGF, we have studied the response of ectodermal cells to activin under conditions where the FGF signaling pathway has been blocked. We find that several activin-responsive genes are induced by activin at reduced levels in animal caps explanted from embryos injected with RNA encoding the truncated FGF receptor. The activin induction of some dorsally expressed genes, including *Xbra*, cardiac actin and *Xnot*, is highly sensitive to inhibition by the truncated FGF receptor, while the induction of other genes, including the head organizer-specific genes, *gooseoid* and *Xlim-1*, is much less sensitive, a pattern consistent with the 'trunkless head' phenotype of embryos injected with the truncated FGF receptor (Amaya et al., 1991). These results suggest that activin signaling alone is insufficient

to induce some types of mesoderm, and that the fate of an individual embryonic *Xenopus* cell depends on the combination of growth factors to which it is exposed.

MATERIALS AND METHODS

Embryos

Fertilized embryos were obtained as previously described (Newport and Kirschner, 1982). Eggs were fertilized in 0.5× MMR (1× MMR is 0.1 M NaCl, 2 mM KCl, 1.0 mM MgSO₄, 2.0 mM CaCl₂, 5.0 mM Hepes and 0.1 mM EDTA). The jelly coat was removed with 2% cysteine in 0.1× MMR, pH 7.8, and eggs were rinsed in 0.1× MMR. Embryos were kept at 14°C to 23°C. Staging was done according to Nieuwkoop and Faber (1956).

Animal caps

Animal caps were dissected from stage 8-9 embryos with fine wire knives. Care was taken to remove any adherent vegetal cells. Caps were cultured in 1× MMR with 50 µg/ml gentamicin sulfate (Sigma) alone or in the presence of growth factors as follows. *Xenopus* basic FGF (bFGF) was prepared as previously described (Kimelman et al., 1988) and used at 100 ng/ml. Human activin A was a generous gift of Genentech (South San Francisco), and was used at 2.4 ng/ml, except as indicated, in the presence of 200 µg/ml BSA. Dispersed cell induction experiments were carried out exactly as in Green and Smith, 1990, except dispersed cells were incubated in media (0.5× NAM) with cations, with or without 2.4 ng/ml activin, for 1 hour before gentle centrifugation. Cells were always reaggregated before stage 10. Spreading assays were carried out exactly as in Smith et al. (1990), except that cells were incubated until stage 11.

RNA synthesis and microinjection

DNA templates encoding the various forms of the FGF receptor were used as previously described (Amaya et al., 1991). mRNA was synthesized and injected as published (Moon and Christian, 1989), except that a 2-fold greater guanosine cap concentration was used. A 100 µl reaction contained 40 mM Tris, pH 7.8, 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl, 0.5 mM each of ATP, CTP, and UTP, 50 µM GTP, 1.0 mM ⁷mG(5') ppp(5')G sodium salt (New England Biolabs, Beverly, MA), 1 µl of RNasin (40 units/µl), 3 µl of RNA polymerase (50-100 units/µl) and 3 µg of linearized DNA template. The reaction was incubated at 37°C for 1 hour, then 3 µl of RQ1 DNase (1 unit/µl) was added and the reaction was incubated a further 15 minutes at 37°C. Phenol-extracted RNA was separated from unincorporated nucleotides with an Amicon 100 microconcentrator (Amicon, Beverly, MA) and injected without further purification. RNA was diluted and injected in volumes of 20 nl or less into the animal pole of both cells of the 2-cell stage embryo. For titration experiments, RNA of a fixed concentration was injected in increasing volumes.

RNA isolation

At least 10 animal caps were pooled for each treatment and homogenized in a buffer containing 50 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 0.5% SDS, and 125 µg/ml Proteinase K (Amresco, Solon, OH). This mixture was incubated for 1 hour at 37°C, then extracted with phenol/chloroform/iso amyl alcohol (25:24:1) 2-3 times. An ethanol precipitate of the aqueous layer was dissolved in water treated with diethylpyrocarbonate and precipitated again from 4 M LiCl for at least 4 hours at -20°C.

RNase protection assays

One animal cap equivalent of RNA for cardiac actin and 5-8 animal cap equivalents of RNA for all other markers were analyzed by the RNase protection assay. High specific activity antisense transcripts were prepared with ³²P-UTP following published protocols (Melton et al., 1984) and using the following constructs.

Cardiac actin. The subclone AC100 (*XmnI*-*EcoRI* fragment cloned into the *SmaI* and *EcoRI* sites of pSP64), kindly provided by Tom Sargent, was linearized with *PvuII* and transcribed with SP6 polymerase, yielding a probe which protects 510 bases of the 3' end of the cardiac actin transcript (Mohun et al., 1984).

Xbra. The 385 base *EcoRI*-*BamHI* fragment at the 5' end of the *Xbra* cDNA (Smith et al., 1991) was cloned into pBluescript SK+. This construct was linearized with *EcoRI* and transcribed with T3 polymerase.

MyoD. A *Xenopus MyoD* cDNA which ends at position 1418 of the published *MyoD*-1 sequence, kindly provided by Ralph Rupp, was linearized with *PvuII* and transcribed with T7 polymerase, resulting in a probe which protects 240 bases near the 3' end of the *MyoD* transcript (Hopwood et al., 1989).

Xnot. The *Xnot*-2 subclone was used as described (von Dassow et al., 1993).

Xcad-3. A *Xcad-3* (Northrop and Kimelman, 1993) clone was linearized with *HindIII* and transcribed with T3 polymerase, yielding a probe which protects 400 bases in the 3' untranslated region of the *Xcad-3* transcript.

Xwnt-8. A subclone of *Xwnt-8* (Christian et al., 1992), kindly provided by Jan Christian, contains the *EcoRI* fragment of *Xwnt-8* inserted into the *EcoRI* site of the pGEM1 vector. This construct was linearized with *AvaI* and transcribed with T7, yielding an antisense transcript which protects 256 bases spanning the 3' end of the *Xwnt-8* coding sequence.

Goosecoid. A subclone of *gsc* (Blumberg et al., 1991) containing the coding region oriented in the antisense direction in the pSP 64T vector (Krieg and Melton, 1985) was kindly provided by Jan Christian. This construct was linearized with *BglIII* and transcribed with SP6, yielding a probe which protects approximately the last 175 bases of the coding region of *gsc*.

Xlim-1. A clone of *Xlim-1* (Taira et al., 1992) containing the *PstI*-*SspI* fragment was linearized with *PstI*, yielding a probe which protects 254 bases within the coding region of the *Xlim-1* transcript.

Epidermal keratin. The subclone, pG3 (530 bases, *XmnI*-*SphI*, from 5' end of XK81A1), kindly provided by Tom Sargent, was linearized with *BamHI* and transcribed with SP6 (Jonas et al., 1985).

N-CAM. A subclone of N-CAM containing the approximately 220 bp *PvuII*-*PstI* fragment in the *EcoRV*-*PstI* sites of pBluescript KS+ was linearized with *HindIII* and transcribed with T7.

A probe for the ubiquitously expressed *EF-1α* gene (Krieg et al., 1989), synthesized to a lower specific activity (Sargent and Bennett, 1990), was included in every hybridization reaction to control for differences in sample processing. The samples were treated with 10 µg/ml and 0.5 µg/ml of RNase A (Sigma) and T1 (Sigma), respectively, for 1 hour at 30°C, and then RNases were inactivated by adding Proteinase K (Amresco, Solon, OH) to 0.2 mg/ml, SDS to 0.7% and incubating for 15 minutes at 37°C. Protected fragments were separated on 8% acrylamide gels with urea and the dried gels were exposed to Hybond film for 2-4 days (Amersham). The gels were scanned by a phosphorimager (Molecular Dynamics) and the signals from the protected fragments were quantitated.

RESULTS

The truncated FGF receptor interferes with activin-mediated extension in animal caps but not with activin-mediated spreading of animal cap cells

The three forms of the FGF receptor used in this study are presented schematically in Fig. 1. We used a truncated version of the FGF receptor (XFD) which is missing the intracellular tyrosine kinase domain and functions as a dominant-negative FGF receptor, presumably by forming heterodimers with the

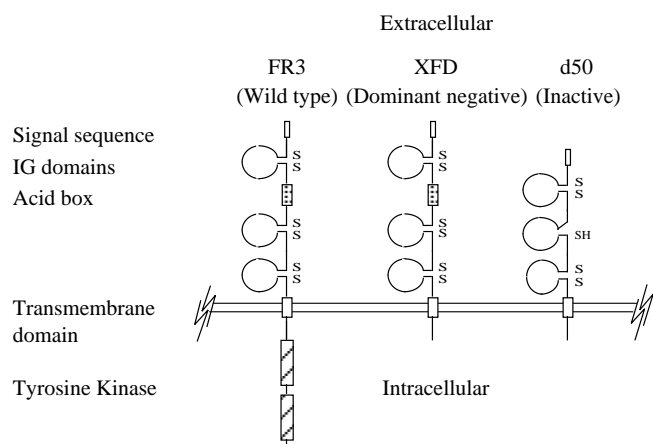


Fig. 1. Schematic representation of the wild-type and mutant FGF receptors used in this study. The structural elements of the FGF receptor include a signal sequence, three immunoglobulin-like domains that are thought to be stabilized by disulfide bonds (SS) between vicinal cysteine residues, a stretch of 8 conserved acidic amino acids (acid box), a transmembrane domain and an intracellular tyrosine kinase domain, which is separated by 14 amino acids. **FR3**, encodes the full-length chicken homolog of the flg (type I) FGF receptor (Lee et al., 1989). **XFD**, encodes a truncated version of the *Xenopus* flg homolog, which is missing the entire tyrosine kinase domain and has a dominant-negative effect. **d50**, is similar to XFD except that an additional 50 amino acids have been removed from the extracellular domain including the acid box and part of the immunoglobulin-like domain II. d50 was used as a negative-control.

endogenous FGF receptor and thus preventing it from being activated by FGF (Ullrich and Schlessinger, 1990; Amaya et al., 1991). To control for the potential non-specific effects of overexpressing an extracellular protein, we used a further truncated FGF receptor (d50), which is translated and glycosylated as efficiently as XFD, but does not function as a dominant-negative receptor (Amaya et al., 1991, 1993). Rescue experiments were performed with a wild-type chicken FGF receptor (FR3) (Amaya et al., 1991).

Treatment of explants of the blastula animal hemisphere (the animal cap) with bFGF or activin diverts them from their epidermal fate and causes them to elongate and express a variety of mesodermal genes (see references in Kimelman et al., 1992; Sive, 1993). To determine whether FGF has a role in activin-mediated mesoderm induction, 0.5 ng of synthetic RNA encoding the truncated FGF receptor was injected into the animal pole of each cell of a 2-cell stage embryo. Injection of this amount of XFD RNA into the marginal zone of the 2-cell embryo resulted in tadpoles with the previously reported phenotype of severely truncated bodies and relatively normal heads (Amaya et al., 1991), in about half of the injected embryos (data not shown). Animal caps excised at the late blastula stage from embryos injected in the animal pole were cultured with or without activin and examined for changes in gross morphology when sibling embryos reached the late neurula stage. Uninjected animal caps incubated in media without activin remained spherical (Fig. 2A), while uninjected caps treated with activin extended, indicating the formation of mesoderm (Fig. 2B). Surprisingly, activin-treated animal caps from embryos injected with the truncated FGF receptor RNA did not extend (Fig. 2C), and more closely resembled

uninduced animal caps, while those injected with the d50 control RNA extended normally (Fig. 2D). These results suggest that an FGF signal is required for some aspect of activin-mediated mesoderm induction.

To assess further the effects of the truncated FGF receptor on the morphological response of presumptive ectoderm cells to activin, we asked whether XFD-injected, dissociated animal cap cells would spread on fibronectin-coated plates. Dissociated animal cap cells adhere to but do not spread on fibronectin-coated tissue-culture plastic, while marginal zone cells and animal cap cells treated with the activin-containing supernatant from the XTC cell line spread and migrate on this surface (Smith et al., 1990). Animal caps were explanted from uninjected embryos or from embryos injected with a total of 1.0 ng of XFD RNA and dissociated in calcium- and magnesium-free media. The epithelial layer was discarded, and the remaining cells were seeded in normal calcium-containing media with or without 2.4 ng/ml activin onto tissue-culture plates which had been incubated with 50 μ g/ml fibronectin and blocked with 0.5% BSA. The cells were examined when sibling embryos reached stage 11. As previously reported, most cells from uninjected animal caps incubated without activin adhered to the plastic but did not spread (Fig. 3A). A small fraction of cells did not adhere to the dish, but this fraction did not noticeably change with addition of growth factor or the injection of XFD RNA. When cells from uninjected animal caps were incubated with activin, about 70% ($n=300$) spread out as large sheets, extending cellular processes (Fig. 3B). Cells from XFD-injected embryos behaved the same way as cells from the uninjected embryos, adhering but not spreading in the absence of activin (Fig. 3C), while 74% ($n=300$) spread and extended cellular processes in the presence of activin (Fig. 3D). This result demonstrates that, while the truncated FGF receptor blocks activin-induced animal cap elongation, it does not seem to affect another activin-inducible behavior of animal cap cells, that of spreading on fibronectin-coated plates.

The truncated FGF receptor blocks the activin- and FGF-mediated induction of a muscle specific gene

We compared the sensitivity of activin- and bFGF-mediated mesoderm induction to inhibition by the truncated FGF receptor. Increasing doses of XFD RNA were injected into each cell of 2-cell stage embryos, and animal caps explanted at the blastula stage were cultured with bFGF or activin until sibling embryos reached the late neurula stage. RNase protection assays were carried out using a probe specific for cardiac actin, which is expressed throughout muscle tissue in the late neurula stage embryo (Mohun et al., 1984). Interestingly, a similar dose of injected XFD RNA prevented the induction of cardiac actin transcripts by either bFGF or activin. Although activin was more effective than bFGF at inducing expression of the cardiac actin gene (Fig. 4A, lanes 1 and 6, and Table 1A), injection of a total of 0.15 ng of XFD RNA resulted in an approximately 50% decrease in the levels of cardiac actin induced by either growth factor (Fig. 4A, lanes 2 and 7, and Table 1A). Injection of 1.0 ng of XFD RNA prevented about 90% of the normal induction of cardiac actin by either bFGF or activin (Fig. 4A, lanes 4 and 9, and Table 1A). Since 1.0 ng of XFD RNA was only slightly more effective at blocking activin-mediated induction of cardiac actin than 0.3 ng, we believe that some of the animal cap cells

were not exposed to the dominant-negative receptor due to the limited diffusion of injected RNA (Smith and Harland, 1991; Amaya et al., 1993). Injection of 1.5 ng of the control d50 RNA had little or no effect on the response of animal caps to bFGF or activin (Fig. 4A, lanes 5 and 10, and Table 1A). These results demonstrate that the truncated FGF receptor has similar effects on both the FGF and activin signaling pathways and suggest that a signal from the FGF receptor is necessary for the activin-mediated induction of cardiac actin.

One of the roles proposed for FGF in the *Xenopus* embryo is to enhance the ability of activin to induce dorsal mesoderm (Green et al., 1992). We therefore asked whether an increased concentration of activin could overcome the ability of the truncated FGF receptor to inhibit the activin-mediated induction of cardiac actin. As shown in Fig. 4B, increasing the activin concentration 3-fold did not increase the levels of actin transcripts induced in animal caps explanted from embryos injected with 1.0 ng of XFD RNA (Fig. 4B, lanes 2 and 3, and Table 1B). This result is consistent with the hypothesis that activin requires an FGF signal to induce the cardiac actin gene.

The truncated FGF receptor inhibits the activin-mediated induction of several mesodermal genes

To determine whether all mesodermal genes depend on a functional FGF receptor in order to respond to activin, the expression of several activin-responsive genes was examined in activin-treated animal caps isolated from embryos injected with a total of 1.0 ng XFD RNA. Similar results were seen for each marker in at least three experiments. The autoradiograms for one representative experiment are presented in Fig. 5.

In addition to cardiac actin, the activin-mediated inductions of three other mesodermal genes, *Xbra*, *MyoD* and *Xnot*, were highly sensitive to inhibition by the truncated FGF receptor. *Xbra* and *MyoD* are expressed throughout dorsal and ventral mesoderm in the midgastrula stage embryo (stage 11), with later expression restricted to the notochord and posterior mesoderm (*Xbra*, Smith et al., 1991), and muscle (*MyoD*, Hopwood et al., 1989; Frank and Harland, 1991). Since we had other markers of the muscle and notochord, we measured the expression of *Xbra* and *MyoD* at stage 11 when their expression is primarily pan-mesodermal. *Xnot* is expressed within the pre-notochord region from the middle of gastrulation throughout the neurula stages, with later expression restricted to the posterior notochord (von Dassow et al., 1993). Activin was an effective inducer of *Xbra*, *MyoD* and *Xnot* in animal caps from

uninjected embryos (Fig. 5), while animal caps from XFD-injected embryos had significantly reduced expression of these three genes in response to activin (Fig. 5). Phosphoimager analysis of the gels used to produce the autoradiograms in Fig. 5, revealed that the expression of *Xbra*, *Xnot* and *MyoD* was reduced at least 8-fold by the injection of the dominant-negative FGF receptor (data not shown). Injection of 1.0 ng of the control RNA, d50, did not reduce the ability of activin to induce any of these genes (Fig. 5). Thus genes expressed in both dorsal and ventral regions of the embryo depended on FGF in order to respond to activin.

The activin-mediated induction of several other mesodermal genes was moderately or only weakly sensitive to inhibition by this dose of the truncated FGF receptor. Genes of this group include *Xcad-3*, which is expressed throughout the mesoderm at the midgastrula stage (Northrop and Kimelman, 1993), *Xlim-1* and *gsc*, which are expressed in the organizer region of the midgastrula stage embryo (Cho et al., 1991; Taira et al., 1992), and *Xwnt-8*, which is expressed in ventral and lateral mesoderm of the midgastrula stage embryo (Christian et al., 1991; Smith and Harland, 1991). As shown in Fig. 5, the truncated FGF receptor inhibited the expression of *Xcad-3* 3-fold, whereas expression of *Xlim-1* and *Xwnt-8* was unchanged. In some other experiments, *Xlim-1* and *gsc* were reduced 2-fold by the injection of the truncated FGF receptor (not shown). However, the expression of these genes was never reduced as much as the expression of *Xbra* or cardiac actin, which were analyzed in the same experiments. For instance, the analysis of

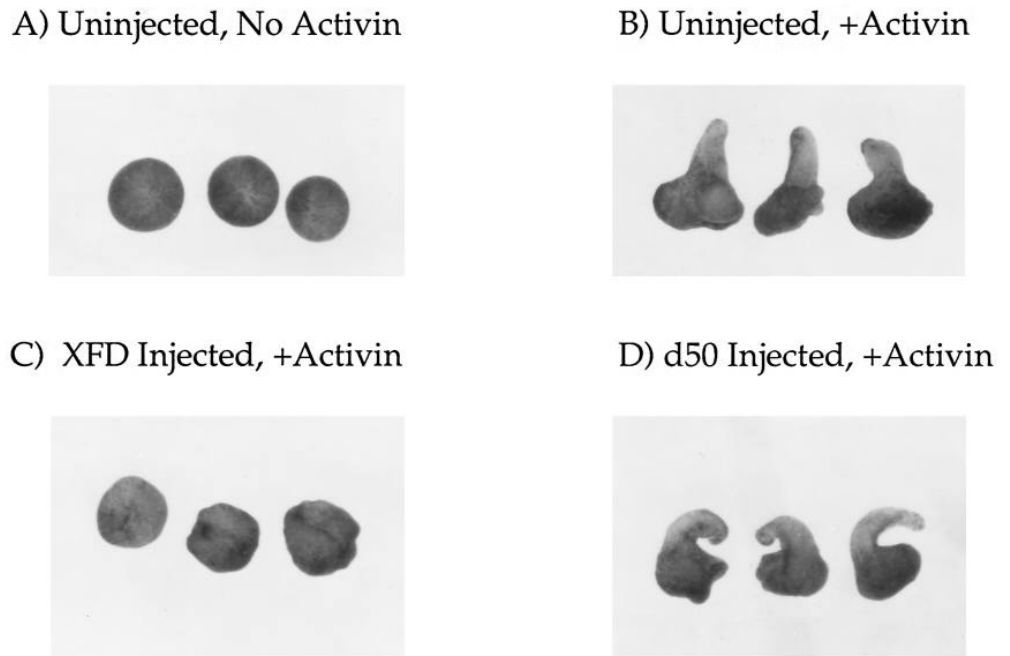


Fig. 2. Morphology of activin-treated animal caps derived from embryos injected with the truncated FGF receptor RNA. Animal caps were explanted at the blastula stage from uninjected embryos, or from embryos injected with synthetic RNA into both cells of the 2-cell stage embryo, then cultured overnight with or without activin and examined for extension when sibling embryos reached stage 17 (neurula). (A) Animal caps derived from uninjected embryos, cultured in media not containing activin. (B) Animal caps derived from uninjected embryos, cultured in media containing activin. (C) Animal caps derived from embryos injected with 1.0 ng of RNA encoding the truncated FGF receptor, cultured in media containing activin. (D) Animal caps derived from embryos injected with 1.0 ng of RNA encoding the control construct, d50, cultured in media containing activin.

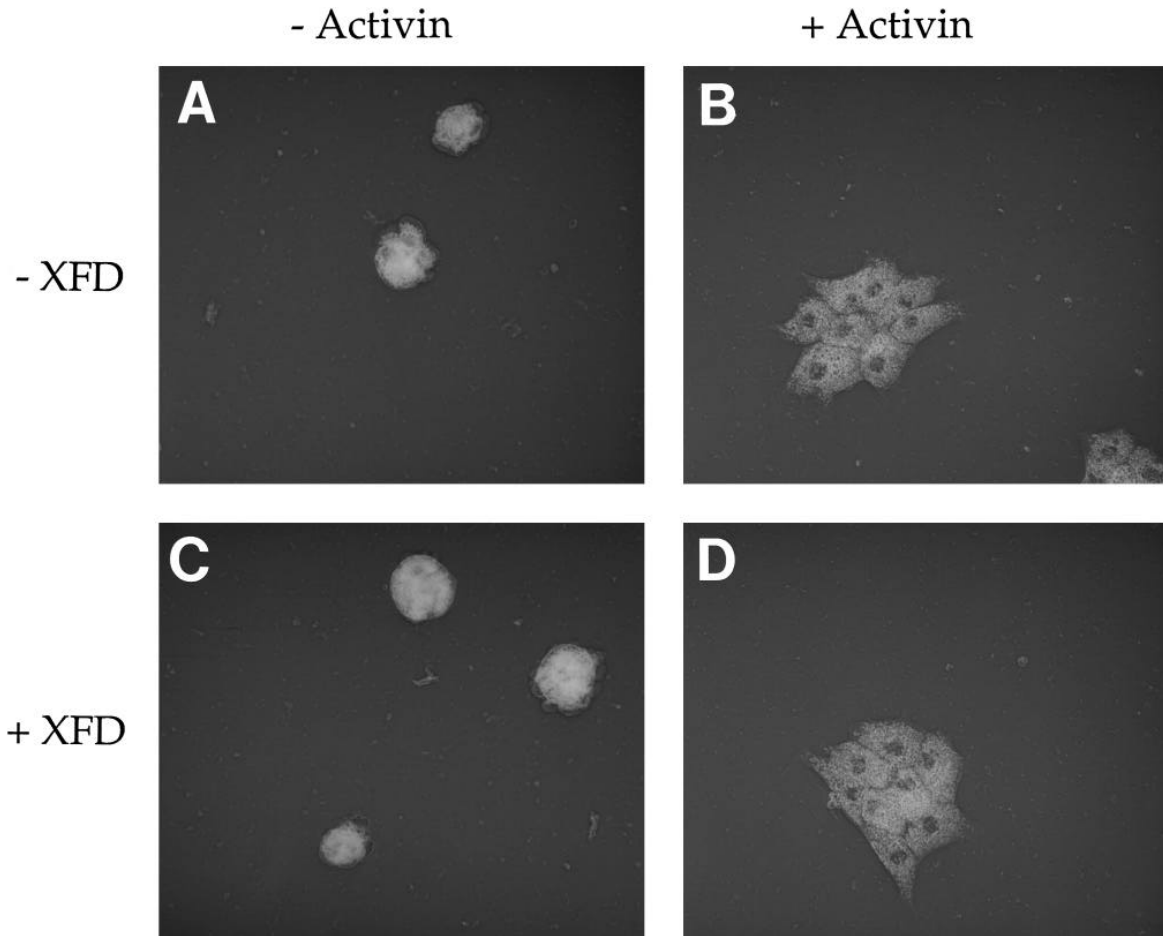


Fig. 3. Spreading of activin-treated cells on fibronectin-coated plates. Animal caps were explanted at the blastula stage and dissociated in calcium- and magnesium-free media, then seeded in cation-containing media on plates that had been treated with 50 $\mu\text{g/ml}$ fibronectin and blocked with 0.5% BSA, and photographed when sibling embryos reached stage 11 (midgastrula). (A) Animal cap cells from uninjected embryos seeded in media without activin. Cells have adhered but not spread on the fibronectin-treated plastic. (B) Animal cap cells from uninjected embryos seeded in media with activin. (C) Animal cap cells from embryos injected with 1.0 ng of XFD RNA, seeded in media without activin. (D) Animal cap cells from embryos injected with 1.0 ng of XFD RNA, seeded in media with activin.

Xbra and *Xlim-1* expression shown in Fig. 5 used the same RNA sample, demonstrating that the truncated FGF receptor has a markedly different effect on these two genes.

Since the expression of several mesodermal markers was greatly reduced in the absence of FGF signaling, we asked whether activin-treated animal caps explanted from XFD-injected embryos would be able to express an epidermal marker. For this experiment, animal caps were dissociated in calcium- and magnesium-free media in order to remove their epithelial layer, which does not respond well to inducing factors (Green and Smith, 1990). Animal cap cells isolated from uninjected or XFD-injected embryos were treated in cation-containing media with or without activin, reaggregated by gentle centrifugation before stage 10 and incubated until stage 22, when epidermal keratin and cardiac actin are expressed at high levels (Mohun et al., 1984; Jonas et al., 1985). As shown in Fig. 6, keratin, but not cardiac actin, was expressed in uninjected animal caps incubated without activin (Fig. 6, lane 1), while the reverse was true in uninjected animal caps incubated with activin (Fig. 6, lane 2). Keratin was also expressed at at least normal levels in animal caps explanted

from embryos injected with XFD RNA and incubated without activin (Fig. 6, lane 3, see below). Interestingly, when XFD-injected caps were treated with activin, the keratin expression was nearly eliminated, even though there was no detectable activin-mediated induction of cardiac actin (Fig. 6, lane 4). This result demonstrates that despite the absence of FGF signaling, activin can still divert animal cap cells from their epidermal fate.

We did notice a small but reproducible increase in keratin transcript levels in animal cap cells incubated without activin from embryos injected with XFD relative to the levels in uninjected animal cap cells (Fig. 6, compare lanes 1 and 3). The enhancement of keratin expression varied between experiments, but was typically less than 2-fold (data not shown). Thus FGF may have a role in regulating the expression of keratin.

Wild-type FGF receptor RNA reverses the effects of the truncated FGF receptor

We wanted to confirm that the intact extracellular domain did not non-specifically interfere with the ability of activin to

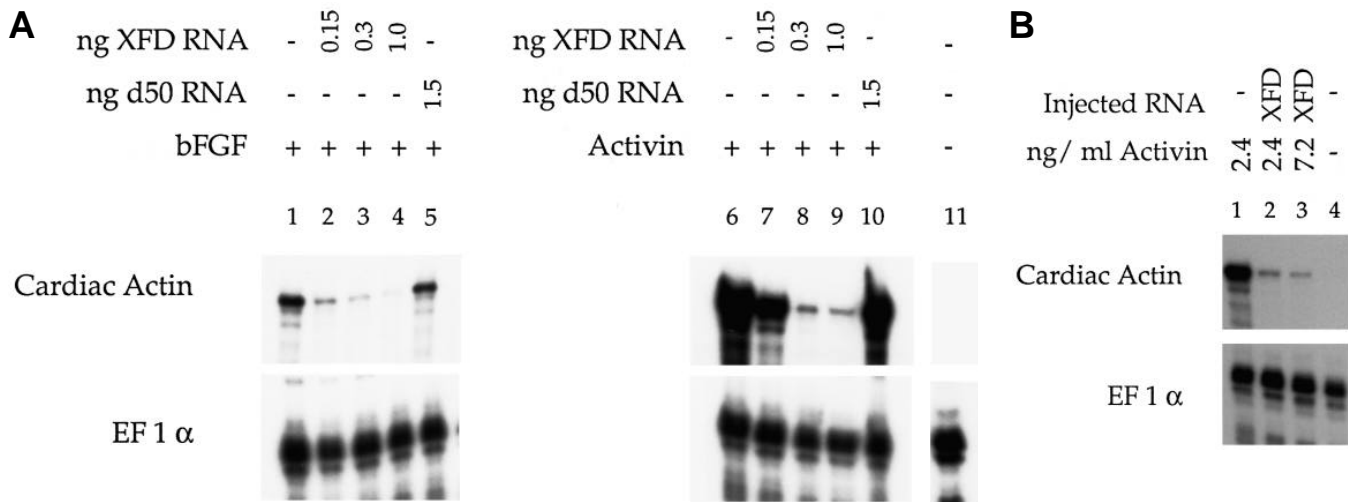


Fig. 4. (A) Cardiac actin transcript levels in FGF- and activin-treated animal caps explanted from embryos injected with the truncated FGF receptor RNA. At least 10 animal caps per treatment were explanted at the blastula stage and cultured with or without bFGF or activin. When sibling embryos reached stage 17 (neurula), caps were pooled and RNA was isolated. RNase protection analysis was performed on one animal cap equivalent of RNA using a mixture of probes specific for cardiac actin and *EF-1 α* . Lanes 1-5, animal caps, incubated in media containing bFGF, explanted from embryos injected at the 2-cell stage with: lane 1, 0.0 ng; lane 2, 0.15 ng; lane 3, 0.3 ng; or lane 4, 1.0 ng of XFD RNA; or lane 5, 1.5 ng of d50 RNA. Lanes 6-10, animal caps, incubated in media containing activin, explanted from embryos injected at the 2-cell stage with: lane 6, 0.0 ng; lane 7, 0.15 ng; lane 8, 0.3 ng; or lane 9, 1.0 ng of XFD RNA; or lane 10, 1.5 ng of d50 RNA. Lane 11, animal caps, incubated in media without growth factor, explanted from uninjected embryos. Phosphorimager analysis of the gels used to produce these autoradiograms is presented in Table 1A. (B) Induction of cardiac-actin by two doses of activin in animal caps explanted from embryos injected with the truncated FGF receptor RNA. Lane 1, animal caps from uninjected embryos, incubated with 2.4/ng ml activin; lane 2, animal caps from embryos injected with 1.0 ng of XFD RNA, incubated with 2.4/ng ml activin; lane 3, animal caps from embryos injected with 1.0 ng of XFD RNA, incubated with 7.2/ng ml activin; lane 4, animal caps from uninjected embryos, incubated without growth factor. Phosphorimager analysis of the gels used to produce these autoradiograms is presented in Table 1B.

induce mesoderm by demonstrating that the wild-type FGF receptor can rescue the phenotype caused by the truncated FGF receptor. As previously described, XFD RNA alone or in combination with a 2-fold molar excess of the wild-type chicken FGF receptor RNA was injected into 2-cell embryos (Amaya et al., 1991). Animal caps were harvested from blastula stage embryos and cultured in activin until the desired stage. Fig. 7 shows that the activin-mediated inductions of two mesodermal markers, *Xbra* and cardiac actin, which were severely inhibited by injected XFD RNA, were restored when the wild-type FGF receptor RNA was injected simultaneously. Animal caps explanted from embryos that had been injected with the wild-type FGF receptor RNA alone and then cultured without activin did not express muscle actin, nor did they express greater than normal levels of actin in response to submaximal doses of activin (data not shown). These results argue that the truncated FGF receptor interferes with the activin response by interfering with the endogenous FGF receptor.

The truncated FGF receptor blocks the formation of neural tissue

Animal caps treated with high levels of activin give rise to neural tissue as well as mesoderm, presumably as a result of the neural-inducing ability of dorsal mesoderm (Green and Smith, 1990). To determine whether FGF is involved in this process, late blastula stage animal caps explanted from embryos injected with 1.0 ng of XFD or d50 RNA were treated as described above and analyzed at stage 20 (late neurula) for expression of the neural specific gene, N-CAM (Kintner and Melton, 1987). Whereas uninjected or control RNA-injected

caps expressed high levels of the neural marker N-CAM in response to activin (Fig. 8, lane 1), animal caps containing the truncated FGF receptor expressed only slightly more N-CAM than the maternal levels present in uninduced animal caps (Fig. 8, compare lanes 2 and 4). These results demonstrate that FGF is required for the activin-mediated induction of neural ectoderm.

DISCUSSION

The truncated FGF receptor specifically interferes with the normal activin response

The experiments presented here demonstrate that the response of embryonic *Xenopus* cells to activin is mediated in part through the FGF receptor. Several controls argue strongly that the inhibition of the activin-response by the truncated FGF receptor cannot be explained by interference with the activin receptor. First, the d50 control RNA, which encodes a partially truncated form of the dominant-negative FGF receptor that is translated and glycosylated with efficiency equal to that of the dominant-negative mutant (Amaya et al., 1993), had no effect on the activin response. Second, co-injection of the truncated FGF receptor with a 2-fold molar excess of the wild-type FGF receptor restored the response to activin. Finally, the activin-mediated induction of several genes was normal in the presence of the truncated FGF receptor.

Amaya and Kirschner found that animal caps derived from embryos injected with XFD RNA responded normally to activin but not to FGF (1991), but two technical details

Table 1. Inhibition of cardiac actin expression by the truncated FGF receptor

(A) Analysis of Fig. 4A

ng RNA/ embryo	Type of RNA	Growth Factor	Percent Induction
Uninjected	-	bFGF	12.4
0.15	XFD	bFGF	6.0
0.3	XFD	bFGF	2.4
1.0	XFD	bFGF	1.6
1.5	d50	bFGF	12.4
Uninjected	-	Activin	100.0
0.15	XFD	Activin	42.6
0.3	XFD	Activin	8.4
1.0	XFD	Activin	8.0
1.5	d50	Activin	89.0
Uninjected	-	None	0.0

(B) Analysis of Fig. 4B

ng RNA/ embryo	Type of RNA	Activin ng/ml	Percent Induction
Uninjected	-	2.4	100.0
1.0	XFD	2.4	11.3
1.0	XFD	7.2	6.0
Uninjected	-	None	0.0

Phosphoimager analysis of the autoradiograms used in Fig. 4A and Fig. 4B was performed to quantitate the levels of cardiac actin expression in each sample. Differences in loading were accounted for by scaling cardiac actin levels according to the EF-1 α levels in each sample. 100% induction was defined as the level of cardiac-actin in uninjected caps cultured with activin.

probably explain the difference between their results and those presented in this study. Previously, a single dose of RNA was injected into the animal hemisphere, whereas we injected embryos twice into either side of the animal hemisphere. As diffusion of RNA in injected embryos is limited and variable (Smith and Harland, 1991), a single injection is likely to yield variable results. Indeed, Amaya and Kirschner found that penetrance of the effect in individual caps was variable (1991), whereas we routinely see a 10-fold reduction in the expression of cardiac actin. Secondly, our injected mRNA was synthesized with a 2-fold greater concentration of the guanosine cap analog, and the guanosine cap significantly alters the half-life and translatability of synthetic RNA injected into *Xenopus* embryos (Krieg and Melton, 1984). We have found that RNA synthesized with a lower concentration of guanosine cap analog is not as effective at inhibiting FGF- or activin-mediated mesoderm induction (data not shown).

Different classes of activin-responsive genes

Our data are consistent with the previously reported analysis of embryos that have been injected in the marginal zone with RNA encoding the truncated FGF receptor. These embryos have normal heads, but are deficient in most of the body axis (Amaya et al., 1991, 1993). By in situ hybridization analysis, these embryos are seen to express relatively normal levels of *gsc* RNA, but greatly diminished levels of *Xbra*, cardiac actin (Amaya et al., 1993) and *Xnot* (von Dassow et al., 1993). In our experiments, the truncated FGF receptor strongly diminished the activin-mediated induction of *Xbra*, cardiac actin and *Xnot*, whereas *gsc* and another head-specific gene, *Xlim-1*, were relatively unaffected. Similarly, in whole embryos, the truncated FGF receptor was much more effective at eliminat-

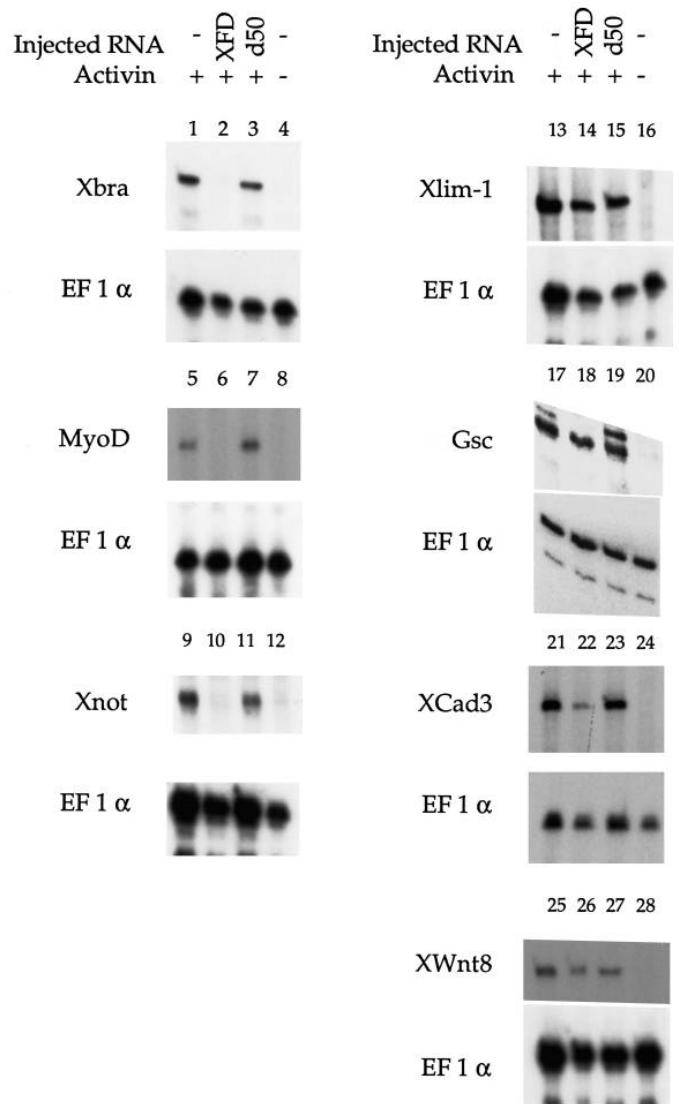


Fig. 5. Induction of seven mesoderm-specific genes by activin in animal caps explanted from embryos injected with the truncated FGF receptor. 0.5 ng of RNA was injected into each cell of the 2-cell stage embryos as indicated below. At least 10 animal caps per treatment were explanted at the late blastula stage and cultured with or without activin until sibling embryos reached the appropriate stage (stage 11 for *Xbra*, *MyoD*, *Xcad-3*, *Xlim-1*, *gsc*, and *Xwnt-8*, and stage 16 for *Xnot*). RNase protection analysis on 5-10 animal cap equivalents of RNA was performed using a mixture of antisense probes specific for the inducible gene and *EF-1 α* simultaneously. Caps were from uninjected embryos treated with activin (lanes 1, 5, 9, 13, 17, 21, 25); from XFD-injected embryos cultured with activin (lanes 2, 6, 10, 14, 18, 22, 26); from d50-injected embryos cultured with activin (lanes 3, 7, 11, 15, 19, 23, 27); and from uninjected embryos cultured without activin (lanes 4, 8, 12, 16, 20, 24, 28). The autoradiograms were analyzed with a phosphoimager, and differences in RNA loading between different lanes was accounted for by measuring *EF-1 α* levels.

ing the expression of *Xbra* than the expression of *Xcad-3* (Northrop and Kimelman, 1993), paralleling our results with activin-treated animal caps. Together these results argue that FGF is crucial for the expression of dorsal and ventral mesoderm genes throughout the trunk mesoderm, and that

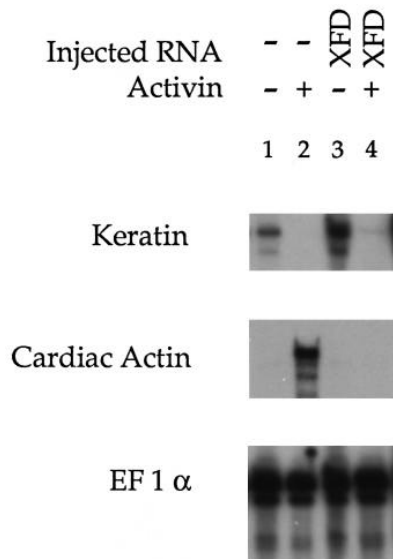


Fig. 6. Expression of cardiac actin and epidermal keratin in the presence of activin and the truncated FGF receptor. 0.5 ng of XFD RNA was injected into each cell of 2-cell embryos as indicated. Animal caps were dissected at stage 9 and dissociated in cation-free media. Cells were incubated in cation-containing media with or without activin for 1 hour then reaggregated by gentle centrifugation and incubated until stage 22. RNA was harvested and half of it was subjected to RNase protection analysis with probes specific for epidermal keratin and EF-1 α , and the other half with probes specific for cardiac actin and EF-1 α . The EF-1 α signals were equivalent in both assays but only the samples from the keratin assay are shown. Lane 1, animal cap cells from uninjected embryos incubated without activin; lane 2, animal cap cells from uninjected embryos incubated with activin; lane 3, animal cap cells from XFD-injected embryos incubated without activin, lane 4, animal cap cells from XFD-injected embryos incubated with activin.

activin but not FGF is involved in the induction of head markers. Interestingly, the activin-mediated induction of the ventral-lateral mesoderm marker *Xwnt-8* was not inhibited by the truncated FGF receptor, which suggests that activin is involved in the induction of genes outside of the presumptive head as well.

We have shown that there are different classes of activin-responsive genes in the *Xenopus* embryo which vary in the extent to which they depend on FGF signaling. It is interesting that the differential regulation of *gsc* and *Xbra* has also been observed in experiments with dissociated animal cap cells, where the activin-induced expression of *gsc* remains constant in dispersed cells, while *Xbra* expression declines if the cells are not reaggregated (Jeremy Green, personal communication). We note that the results described previously and those presented here may depend on the levels of truncated FGF receptor in the embryo. At very high doses of the truncated FGF receptor, head abnormalities are observed (J. Northrop and D. K., unpublished results), and the activin-mediated induction of *gsc* is severely impaired (LaBonne and Whitman, 1994).

The inhibition of one class of genes by the dominant-negative FGF receptor does not cause mesodermal cells to revert to an ectodermal state and express the keratin gene. Furthermore, activin-treated animal cap cells derived from

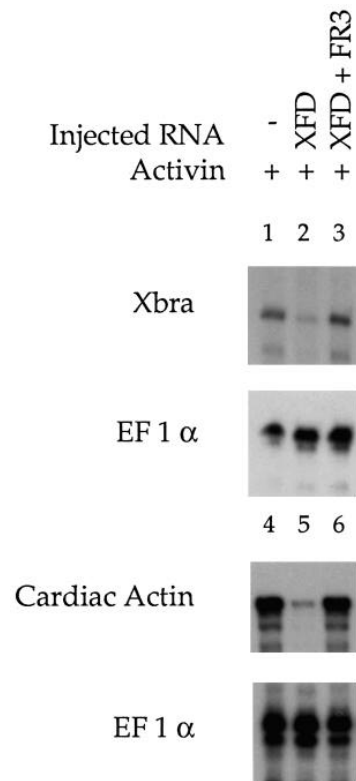


Fig. 7. Induction of two mesoderm-specific genes by activin in animal caps explanted from embryos injected with the truncated FGF receptor alone or together with wild-type FGF receptor RNA. Embryos were injected with 1.0 ng of the truncated FGF receptor RNA (XFD) alone or with 4.0 ng of the full-length chicken FGF-receptor RNA (FR3) at the 2-cell stage. The molar ratio of FR3 to XFD is approximately 2 to 1. Animal caps were explanted from blastula stage embryos and cultured with 2.4 ng/ml activin until sibling embryos reached the appropriate stage (stage 11 for *Xbra*, and stage 17 for actin). RNA was isolated for RNase protection analysis using probes for the specific mesodermal gene and EF-1 α . Animal caps were from uninjected embryos were cultured with activin (lanes 1, 4); from XFD-injected embryos cultured with activin (lanes 2, 5); and from XFD/FR3 coinjected embryos cultured with activin (lanes 3, 6).

embryos injected with the truncated FGF receptor display a spreading behavior that is characteristic of mesodermal, not ectodermal, cells. Therefore activin can inhibit the ectodermal pathway of differentiation even when aspects of mesodermal development are blocked.

The source of the FGF signal

The FGF-signal required for activin to induce mesoderm in animal cap cells could come from the same cell (autocrine) or from nearby cells (paracrine). As *Xbra* is inducible by activin in dissociated animal cap cells (Smith et al., 1991), FGF must be able to function through an autocrine pathway, whether or not it does so in vivo. Activin induces the expression of at least one FGF-family member, int-2 (Tannahill et al., 1992), which could act in either autocrine or paracrine fashion. However, since *Xbra* can be induced by activin in the presence of an inhibitor of translation (Smith et al., 1991), we believe that there is a constitutive FGF signal that can complement the

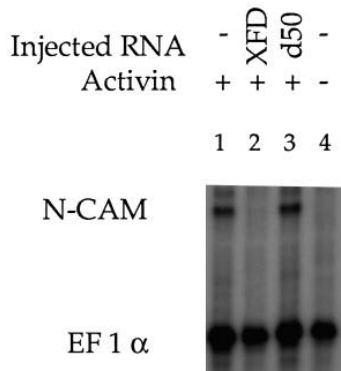


Fig. 8. N-CAM transcript levels in activin-treated animal caps explanted from embryos injected with the truncated FGF receptor RNA. 1.0 ng of synthetic RNA was injected into both cells of the 2-cell stage embryo. At least 10 animal caps per treatment were explanted from blastula stage embryos and cultured with 2.4 ng/ml activin. When sibling embryos reached stage 22 (tailbud), RNase protection analysis was performed on 5-10 animal cap equivalents of RNA using a mixture of probes specific for N-CAM and EF-1 α . Lane 1, animal caps isolated from uninjected embryos cultured with activin; lane 2, animal caps isolated from XFD-injected embryos cultured with activin; lane 3, animal caps isolated from d50-injected embryos cultured with activin; lane 4, animal caps isolated from uninjected embryos cultured without activin.

activin signal. Candidates for this FGF family member include XeFGF and basic FGF, both of which are encoded by maternal transcripts and are present throughout early development (Kimelman and Kirschner, 1987; Kimelman et al., 1988; Slack and Isaacs, 1989; Isaacs et al., 1992). We have measured a significant amount of bFGF activity in the animal cap, and have found that transcripts encoding bFGF are recruited to polysomes in early blastula stage embryos (T. Schuh, H. Farr, S. Hauschka, and D. K., unpublished data).

The role of *ras* and *raf* in mesoderm induction

Overexpression of a dominant-negative *ras* mutant was shown to inhibit the activin and FGF-mediated induction of cardiac actin, suggesting that *ras* is necessary for both intracellular pathways (Whitman and Melton, 1992). However, since FGF is necessary for the activin-mediated induction of cardiac actin, we suggest that *ras* may only be directly involved in the FGF intracellular pathway. Indeed, embryos injected with the dominant-negative *ras* mutant have the same reported phenotype as embryos injected with the truncated FGF receptor (Whitman and Melton, 1992).

A dominant-negative *raf* mutant was reported to inhibit the induction of *Xbra* by FGF but not by activin (MacNicol et al., 1993). This result is difficult to reconcile with our results since *raf*, like *ras*, is thought to act in the FGF signaling pathway. However, a second dominant-negative *raf* construct has been generated that blocks induction of *Xbra* by both FGF and activin, consistent with a role for FGF signaling in the activin-mediated induction of mesoderm (LaBonne and Whitman, 1994).

FGF and neural induction

Although the identity of the neural-inducing signal is not yet known, FGF has not been considered a likely candidate since it is unable to induce the expression of the neural marker N-

CAM in animal cap explants (Green et al., 1990). Our results demonstrate, however, that FGF is required for the expression of the N-CAM gene. FGF may be required for the production of the neural-inducing signal, or it may be necessary for the presumptive neural cells to respond to the neural-inducing signal. It will be interesting to test these possibilities when the neural-inducing signal is identified.

Mesoderm patterning by FGF and activin

While it has been suggested that FGF has a synergistic effect on activin, it seems now that there is a more complex relationship between these two factors. Green and colleagues have shown that the addition of activin to dissociated cells induces different mesoderm genes within narrow concentration windows, with the highest doses of activin necessary for the induction of head-mesoderm markers such as *gsc* (1992). Addition of bFGF at a threshold concentration for induction of the cardiac actin gene was found to lower the absolute dose of activin necessary for maximal induction of this gene, suggesting that the FGF and activin signals act additively or perhaps synergistically (Green et al., 1992). Our data can not be explained by this model. First, we find that an increased dose of activin does not increase the level of muscle actin in an activin-treated cap explanted from an embryo injected with the truncated FGF receptor. Second, if FGF were important for increasing the ability of activin to induce more dorsal-anterior markers, then elimination of the FGF signaling pathway would be expected to have the most dramatic effect on the *gsc* gene. However, we find that the activin-mediated induction of *gsc* is much less sensitive to inhibition by XFD than that of *Xbra*, a gene that is induced by a lower dose of activin. These results suggest that the activin and FGF signaling pathways may interact at several levels. For instance, low levels of FGF may be required for some genes to respond to activin while higher levels may amplify the activin response.

Experiments with the dominant-negative FGF receptor suggest an expanded role for both FGF and activin in normal development. Because bFGF induces primarily ventral-type mesoderm when added to animal caps, it has been predicted that FGF would be especially important for ventral mesoderm development. However, the results presented here, and those of LaBonne and Whitman (1994), demonstrate that FGF is necessary for the induction of dorsal and ventral mesoderm. It becomes an open question of whether FGF is a primary inducer or is used only as a partner in the activin pathway. But why is an FGF signal required for the induction of mesodermal genes by activin? We have observed that the animal cap contains a significant amount of bFGF activity, yet it does not produce mesoderm when cultured in isolation. The marginal zone, however, contains higher levels of bFGF activity than the animal cap (T. Schuh, H. Farr, S. Hauschka, and D. K., unpublished data). By requiring two factors to be present at high levels within the marginal zone in order to induce mesoderm, it may be possible to create sharper boundaries between mesodermal and non-mesodermal regions. A similar idea has been proposed for the induction of the organizer, using a combination of activin and a *wnt* or *noggin* signal (Kimelman et al., 1992; Sive, 1993). It will now be interesting to study the intracellular regulation of these factors to determine how they interact at the molecular level.

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