Development 120, 463-472 (1994)
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Mesoderm induction by activin requires FGF-mediated intracellular signals

Carole LaBonne and Malcolm Whitman*

Program in Cell and Developmental Biology and Department of Cell Biology, Harvard Medical School, 25 Shattuck Street, Boston, MA 02115, USA

*Author for correspondence

SUMMARY

We have examined the role of FGF signaling during activin-mediated mesoderm induction in *Xenopus*. Using dominant inhibitory mutants of FGF signal transducers to disrupt the FGF-signaling pathway at the plasma membrane or in the cytosol prevents animal cap blastomeres from expressing several mesodermal markers in response to exogenous activin. Dominant inhibitory mutants of the FGF receptor, c-ras or c-raf inhibit the ability of activin to induce molecular markers of both dorsal and ventral mesoderm including *Xbra*, *Mix1* and *Xnot*. Some transcriptional responses to activin such as *goosecoid* and *Xwnt8* are inhibited less effectively than others, however, suggesting that there may differing requirements for an FGF signal in the responses of mesoderm-specific genes to activin induction. Despite the requirement for this signaling pathway during activin induction, downstream components of this pathway are not activated in response to activin, suggesting that activin does not signal directly through this pathway.

Key words: activin, FGF, mesoderm induction, signal transduction, *Xenopus*

INTRODUCTION

During early *Xenopus* development, cells in the equatorial region of the embryo adopt a mesodermal fate in response to inductive signals emanating from the underlying endoderm. As mesodermal subtypes are regionally specified during this process and since mesoderm plays a critical role in gastrula movements and neural induction, understanding the nature of the inductive signals and their response is crucial to understanding the molecular basis of pattern formation.

Several growth factors have been identified that can induce mesoderm in explanted animal pole cells (animal caps) (reviewed in Smith, 1989; Whitman and Melton, 1989). Two such factors, bFGF and activin, are present in the embryo during the time of mesoderm induction (Asashima et al., 1991; Kimelman et al., 1988; Slack et al., 1987). In animal cap assays, both FGF and low concentrations of activin are capable of inducing ventrolateral mesoderm such as mesenchyme, kidney and muscle, while only high concentrations of activin can efficiently induce dorsal mesodermal tissues such as notochord (Green et al., 1990; Slack et al., 1987; Sokol et al., 1990). A wide variety of mesodermal response genes have been identified during recent years, many of which can be induced by either FGF or activin. These include *Xbra* (Smith et al., 1991), *Xhox3* (Ruiz i Altaba and Melton, 1989), *Xwnt8* (Christian et al., 1991) and *Xnot* (von Dassow et al., 1993). However, a subset of mesodermal marker genes, such as goosecoid (Cho et al., 1991) and *Mix1* (Rosa, 1989), appear to respond only to activin. Marker genes that respond solely to FGF have yet to be identified in this system. Since graded doses of activin can induce the entire range of available mesodermal markers, this suggests an apparent redundancy between FGF and low level activin signaling.

The receptors for FGF and activin have been cloned and encode a protein tyrosine kinase and a protein serine/threonine kinase, respectively (Lee et al., 1989, Matthews and Vale, 1991). Expression of dominant inhibitory mutants of these receptors has been shown to block ligand-mediated signaling, and this effect is rescued by co-expression of wild-type receptor, indicating that these receptors are indeed responsible for inductive responses in embryonic cells (Amaya et al., 1991; Hemmati-Brivanlou and Melton, 1992). In embryos, expression of a dominant inhibitory FGF receptor perturbs posterior axial patterning (Amaya et al., 1991), while expression of a dominant inhibitory activin receptor can, in the most severe cases, completely block the formation of mesoderm (Hemmati-Brivanlou and Melton, 1992). The latter result is striking in light of the fact that FGF-signaling pathways are still functional in these embryos. Indeed, animal cap explants expressing a dominant inhibitory activin receptor show increased responsiveness to exogenous FGF (Hemmati-Brivanlou and Melton, 1992), making the lack of FGF-induced mesoderm in embryos expressing the dominant inhibitory activin receptor puzzling. These results suggest that although FGF is capable of inducing mesoderm in animal cap assays, it may not do so in whole embryos.

One approach to elucidating the roles of FGF and activin during mesoderm induction is to examine and manipulate the early cytosolic responses to these growth factors. The activin receptor belongs to a novel family of transmembrane serine/threonine kinases and little progress has been made toward understanding how these receptors transmit signals

(reviewed in Massague, 1992). Tyrosine kinase signaling, however, is the subject of intense study in other systems (reviewed in Ullrich and Schlessinger, 1990). Experiments in cell culture suggest that the small GTP-binding protein p21ras and the cytoplasmic kinases Raf-1 (Morrison et al., 1988), MEK (Crews et al., 1992) and MAP kinase (Ray and Sturgill, 1988) act downstream of most or all protein tyrosine kinase receptors, including the FGF receptor (reviewed in Blenis, 1993; Roberts, 1992). In addition, homologues for these signal transducers play necessary roles in tyrosine kinase receptor-mediated cell fate decisions in *Drosophila* and *Caenorhabditis elegans* (Beitel et al., 1990; Biggs and Zipursky, 1992; Dickson et al., 1992). In *Xenopus*, it has previously been found that a dominant inhibitory allele of c-ras, p21(Asn17)^{ras,} blocks mesoderm induction by both activin and FGF (Whitman and Melton, 1992), suggesting that some components of the activin and FGF-signaling pathways may be shared. However, an activated ras allele, like FGF, induces mainly ventrolateral mesoderm and fails to induce the more dorsal mesoderm characteristic of activin (M. Whitman, unpublished data). While a requirement for ras activation during FGF signaling had previously been described in other systems (Cai et al., 1990; Szebernyi et al., 1990; Wood et al., 1992), such a necessary role for ras during activin signaling was unexpected. If the block to activin induction represents a direct block of its signaling pathways, this finding reveals a novel role for ras in signaling by serine/threonine kinase receptors. A second possibility, however, is that the block to activin signaling by dominant inhibitory ras is indirect. As ras is clearly involved in FGF signaling, this could indicate a cryptic requirement for FGF in activin induction. This possibility led us to examine other messengers implicated in tyrosine kinase signaling for evidence of cross-talk between the FGF- and activin-signaling pathways. We find that overexpressing dominant inhibitory mutants for several signal transducers in the FGF-signaling pathway, including the FGF receptor itself, can inhibit the induction of mesoderm by both FGF and activin in animal cap assays. FGF and not activin, however, strongly activates a downstream component of this pathway, MAP/ERK kinase. These results suggest that FGF activation of a ras-dependent signaling pathway may be required in vivo for activin-mediated mesoderm induction.

MATERIALS AND METHODS

Plasmids and in vitro transcriptions

Dominant negative FGF receptor (XFD), nonfunctional FGF receptor(d50) and full-length FGF receptor(FR3) clones were a kind gift of Enrique Amaya and Marc Kirschner and have been described previously (Amaya et al., 1991). Constitutively active ras (p21(Val12)$^{v\text{-}Ha\text{-}ras}$) and dominant inhibitory ras (p21(ASN17)$^{Ha\text{-}ras}$) have been described elsewhere(Whitman and Melton, 1992). Full-length human c-raf-1 clones in pSP64T was kindly provided by Deborah Morrison. The dominant inhibitory raf carries a serine-to-alanine mutation at serine 621, the activated raf carries a serine-to-alanine mutation at serine 259, and both have been described previously (Fabian et al., 1993, Morrison et al., 1993). We note that at very high doses (>10 ng) the Raf(S621A) seems to become less inhibitory and may itself have some mesoderm-inducing activities. Full-length activin receptor was a kind gift of Ali Hemmati-Brivanlou. All plasmids were linearized with the appropriate restriction endonuclease and transcribed with SP6 RNA polymerase as previously described. (Krieg and Melton, 1987)

Microinjection and animal cap assays

Embryos were collected from *Xenopus laevis* females and fertilized as previously described (Newport and Kirschner, 1982). Fertilized eggs were placed in 3% Ficoll/0.1× MMR for injection. Injection was carried out at the 2-cell stage in the animal pole of both blastomeres with 10-15 nl of synthetic mRNA. For blocking experiments, injected dominant inhibitory mutant RNAs were 200 pg/nl or as noted. Animal caps were dissected at the 4,000-cell stage (stage 8, Nieuwkoop and Faber, 1967) in 1× MMR and incubated in the presence of 100 pM recombinant activin, 50 ng/ml human recombinant FGF (Gibco/BRL) or 0.25× PIF (Sokol et al., 1990). Animal caps were harvested following a 1.5-2 hour incubation for PCR analysis of early markers or 36 hours for muscle actin blots. Staging of *Xenopus* embryos was done according to Nieuwkoop and Faber (1967).

Northern blots

Total RNA was isolated from whole embryos and animal caps as previously described (Krieg and Melton, 1987). RNA samples were electrophoresed on 1% agarose/formaldehyde gels, blotted onto nylon membranes and hybridized in 50% formamide, 5× SSPE, 5% SDS and 100 μg/ml denatured salmon sperm DNA with an antisense RNA probe. Hybridization was carried out overnight at 55–60°C. Blots were washed the next day for 2–1 hour in 0.1× SSPE, 0.1% SDS at 65–75°C.

cDNA synthesis and PCR

Total RNA was isolated from whole embryos and animal caps as previously described(Krieg and Melton, 1987). Cellular DNA was removed by treatment with DNAse1(Promega) for 30 minutes at 30°C. Reverse transcription was carried out on RNA from 10 animal cap equivalents using MMLV reverse transcriptase (100U, Pharmacia) at 42°C for 30 minutes in a 20 μl reaction containing 50 mM Tris pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM of each dNTP, 10 U RNAsin (Promega) 0.1 mg/ml BSA and 0.5 OD units oligo(dT) (Pharmacia). 2 μl of RT sample was used per PCR reaction. PCR reactions were carried out in a 25 μl reaction volume in the presence of trace ^{32}P[αdATP] as described previously (Rupp and Weintraub, 1991), using an annealing temperature of 55°C and 22-25 cycles. Linearity was tested on serial dilutions of cDNA prepared from whole embryo RNA. Primer pairs utilized for RT-PCR were as follows:

Xwnt-8: 5′AGATGACGGCATTCCAGA3′, 5′TCTCCCGATATCTCAGCA3′
Xbra: 5′GGATCGTTATCACCTCTG3′, 5′GTGTAGTCTGTAGCAGCA3′
EF1α: 5′CAGATTGGTGCTGGATATGC3′, 5′ACTGCCTTGATGACTCCTAG3′
Mix1: 5′AATGTCTCAAGGCAGAGG3′, 5′TGTCACTGACACCAGAA3′
gscd: 5′AGAGTTCATCTCAGAGAG3′, 5′TCTTATTCCAGAGGAACC3′
XNot 5′ATACATGGTTGGCACTGA3′, 5′CTACACCTTGACATCCTC3′

Immunoprecipitations and western blot analysis

Animal caps were explanted from stage 8 embryos and dissociated in CMFM as previously described (Green and Smith, 1990). Following a 10-minute incubation Ca^{2+} and Mg^{2+} were added back to the medium and cells were treated with FGF or activin for various time points. Cells were then lysed and electrophoresed on 10% polyacrylamide gels and blotted to nitrocellulose, or were immunoprecipitated overnight with an antibody that recognizes MAP/ERK kinase (provided by J. Blenis), electrophoresed and blotted to nitrocellulose. Blots were probed sequentially with either the anti-phosphotyrosine

antibody 4G10 or with the MAP/ERK kinase-specific antibody overnight at 4°C. Following a 1 hour incubation with HRP-conjugated secondary antibody, signal was visualized using ECL (Amersham).

RESULTS

FGF signaling is essential for normal activin induction

If a functional FGF signal transduction pathway is required for activin induction, then this would be most effectively demonstrated by blocking this pathway at the level of the FGF receptor. Amaya et al. (1991) have reported that a truncated FGF receptor lacking most of the intracellular domain competitively inhibits the function of endogenous FGF receptors. Overexpression of this dominant inhibitory receptor prevents FGF-mediated mesoderm induction in animal cap assays, while overexpression of non-functional control receptor, which lacks an additional 50 amino acids in the extracellular domain, has no effect. These mutant FGF receptors have been shown to be expressed and glycosylated to comparable levels in *Xenopus* oocytes (Amaya et al., 1991).

To examine the effects of a dominant inhibitory FGF receptor on activin induction, synthetic mRNAs encoding either the truncated FGF receptor or a non-functional FGF receptor were injected into *Xenopus* embryos at the 2-cell stage. Animal caps explanted from these embryos at stage 8 were treated with FGF or activin, and the degree of induction was assessed by both an examination of gross morphology and a measure of the expression of several tissue-specific genes. The mesodermal marker genes that have been cloned to date can be divided into two classes, based upon their induction profile in animal cap assays. Some genes, such as *Xbra*, *Xhox3*, *Xnot* and *Xwnt8*, can be induced by either activin or FGF, while others, such as goosecoid and *Mix1*, can be induced by activin but not by FGF. We wished to examine whether the dominant inhibitory FGF receptor affected the earliest transcriptional responses to activin and, if so, whether these two classes of genes were differentially affected. RT-PCR was chosen as a sensitive assay of gene expression for time points less than 2 hours postinduction (Rupp and Weintraub, 1991). Fig. 1 shows the results using primers specific for several early response genes and *EF1α* (Krieg et al., 1989), a ubiquitously expressed gene used as a control for template level. The levels to which FGF and/or activin could induce *Xbra*, *Xnot* and *Mix1* in animal caps expressing the dominant inhibitory FGF receptor were dramatically reduced, while all were inducible by the appropriate factor(s) in caps expressing a non-functional control receptor. Similar results were obtained for *Xhox3* expression (not shown). Overexpression of the dominant inhibitory FGF receptor was also found to inhibit activin induction of goosecoid, a marker for the earliest involuting dorsal mesoderm (Cho et al., 1991). While a nearly complete block to goosecoid induction was observed in the experiment shown in Fig. 1, this experiment represents the strongest inhibition of activin induction that we have observed. Unlike most other early markers examined, the completeness of the goosecoid block was not consistently seen, even when high doses of dominant inhibitory receptor mRNA (8 ng) were injected. Overall, however, the dominant inhibitory FGF receptor partially or completely blocked activin induction of all marker genes examined, regardless of whether they themselves were FGF inducible. The ability of the dominant inhibitory FGF receptor to inhibit induction of *Mix1* and goosecoid indicated that a functional FGF-signaling pathway is required for induction of both dorsal and ventrolateral mesoderm by activin. The dominant inhibitory FGF receptor was unable completely to block activin induction of *Xwnt8*, however, even in experiments in which goosecoid induction was completely blocked (Fig 1). This suggests that some components of the activin-signaling pathway may be FGF independent.

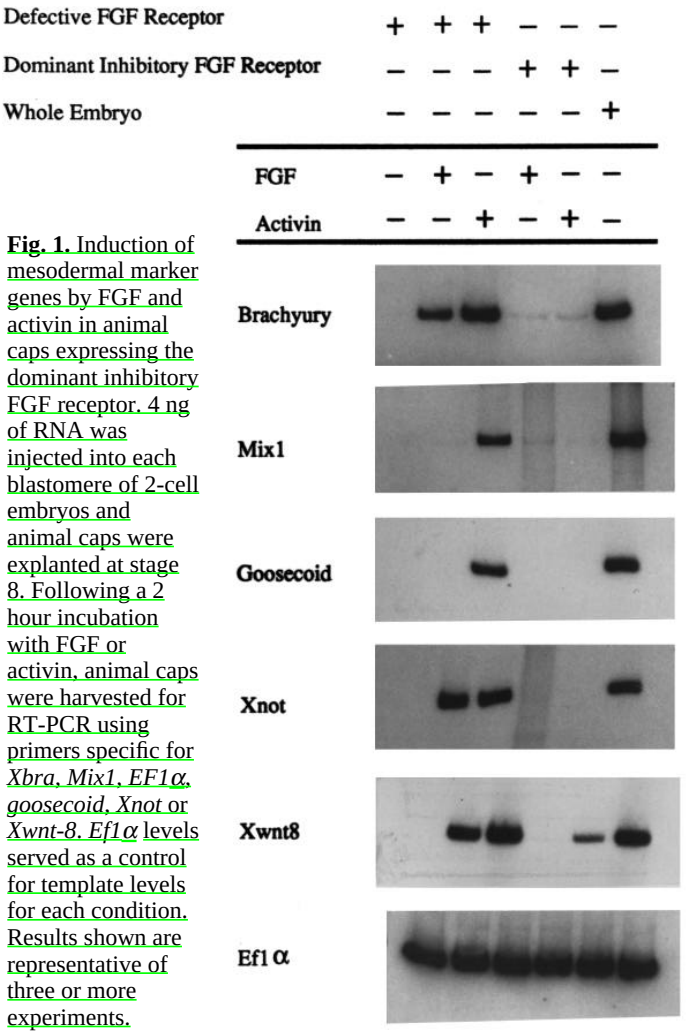
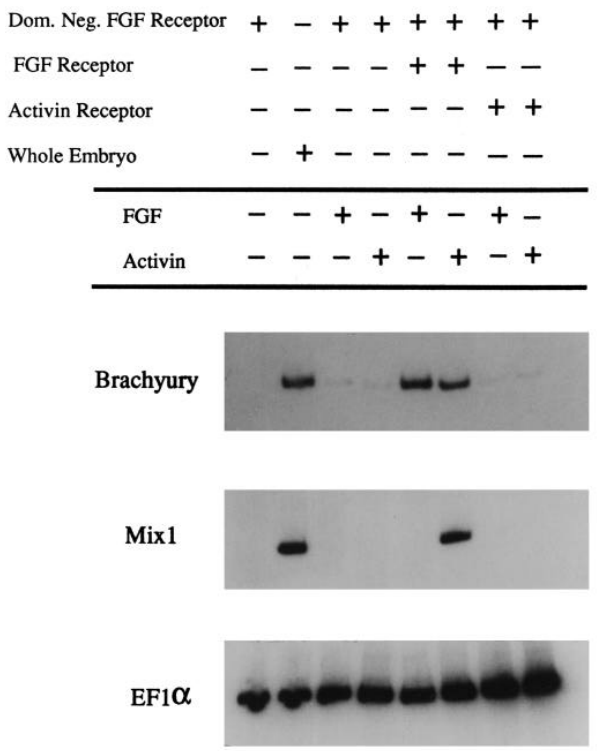
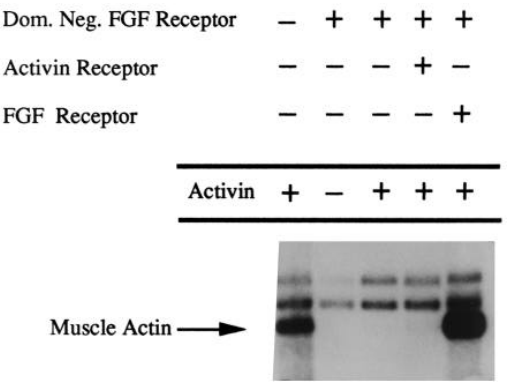


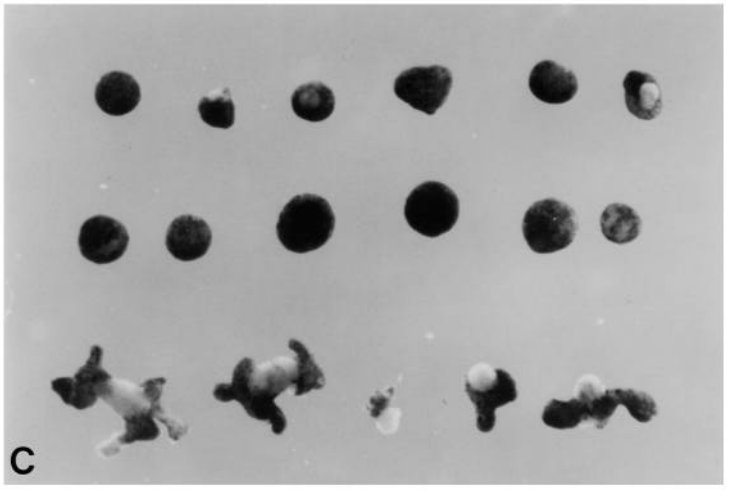
Fig. 1. Induction of mesodermal marker genes by FGF and activin in animal caps expressing the dominant inhibitory FGF receptor. 4 ng of RNA was injected into each blastomere of 2-cell embryos and animal caps were explanted at stage 8. Following a 2 hour incubation with FGF or activin, animal caps were harvested for RT-PCR using primers specific for *Xbra*, *Mix1*, *EF1α*, *goosecoid*, *Xnot* or *Xwnt-8*. *Ef1α* levels served as a control for template levels for each condition. Results shown are representative of three or more experiments.

Animal caps isolated at stage 8 and cultured in isolation heal into a ball of ciliated epidermis (Nieuwkoop, 1969). When these explants are cultured in medium containing FGF or activin, however, they elongate and differentiate a wide variety of mesodermal tissues (Nieuwkoop, 1969; Slack et al., 1987; Smith, 1987; Sokol et al., 1990). Animal caps expressing the control FGF receptor underwent extensive elongation in response to activin, and formed embryoids containing a wide range of tissues including muscle, cement gland and occasionally eyes. However, animal caps expressing the dominant inhibitory FGF receptor failed to elongate in response to activin and, in most cases, formed atypical epidermis (Fig. 2 and not shown). Although in the initial report of Amaya et al.

Fig. 2. The dominant inhibitory FGF receptors block to activin induction can be rescued by co-injection with wild-type FGF receptor but not activin receptor. Embryos were injected at the 2-cell stage with 1 ng of dominant inhibitory FGF receptor RNA alone, or were coinjected with 4 ng of FGF receptor or activin receptor RNA. Animal caps were explanted at stage 8, treated with activin and cultured for 36 hours. (A) Wild-type FGF receptor, but not activin receptor rescued activin induction of immediate early response genes *Xbra* and *Mix1*. *EF1α* levels were used as a control for template levels across RT-PCR reactions. (B) Animal caps expressing the dominant inhibitory FGF receptor alone fail to express muscle-specific actin in response to activin (lane 3). Coinjection of wild-type FGF receptor (lane 5) but not activin receptor (lane 4) rescues activin-mediated induction of muscle-specific actin to control levels (lane 1). (C) Animal caps expressing the dominant inhibitory FGF receptor alone or co-expressing the activin receptor fail to elongate when treated with PIF, a source of activin A, while animal caps co-expressing wild-type FGF receptor undergo significant morphogenetic movements in response to activin.







Dominant Inhibitory FGF receptor + Activin receptor

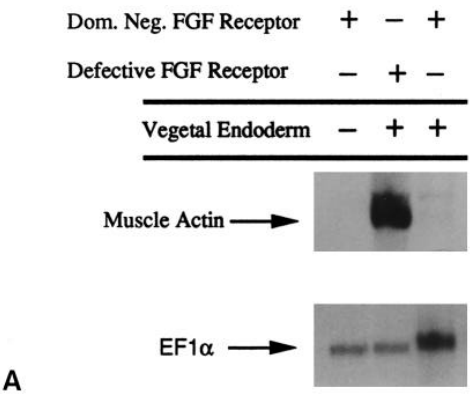
Dominant Inhibitory FGF receptor

Dominant Inhibitory FGF receptor + FGF receptor

(1991) on the dominant inhibitory FGF receptor they did not observe an effect on activin induction, the inhibition of FGF induction achieved in their animal cap assays was incomplete. Indeed, when individual animal caps expressing the dominant inhibitory FGF receptor were examined for induction of muscle-specific actin in response to FGF, the extent of inhibition was found to be extremely variable. This was most likely due to the variable and mosaic expression levels of the dominant inhibitory receptor that were achieved in these experiments (Amaya et al., 1991, 1993). In our hands, at doses of dominant inhibitory receptor where the block to FGF induction is incomplete, we do not see a block to activin signaling (not shown).

The dominant negative FGF Receptor is specific for FGF signaling

Previous work with the dominant inhibitory FGF receptor demonstrated that coinjection of wild-type FGF receptor could rescue the response to FGF. To confirm the specificity of the dominant inhibitory FGF receptor's block to activin-mediated mesoderm induction, we investigated whether wild-type FGF receptor or activin receptor could rescue activin induction. As



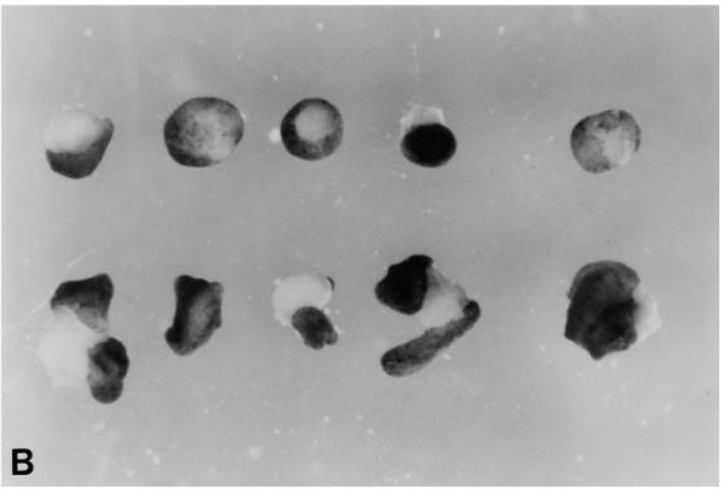


Fig. 3. Dominant inhibitory FGF receptor inhibits muscle actin induction in Nieuwkoop recombinants. Stage 8 animal caps expressing the dominant inhibitory FGF receptor or non-functional control receptor were recombined with vegetal endoderm from uninjected embryos. Mesoderm induction was assessed by examination of gross morphology or by muscle actin northern. (A) Recombinants expressing the dominant inhibitory FGF receptor express greatly reduced levels of muscle actin (lane 3) as compared with control recombinants (lane 2). (B) Recombinants expressing the control FGF receptor underwent significant morphogenetic movements while those expressing the dominant inhibitory FGF receptor failed to elongate.

shown in Fig. 2A co-expressed FGF receptor, but not activin receptor, rescued the induction of the immediate early genes *Xbra* and *Mix1*. Levels of *EF1α* remained unchanged, indicating that template levels were equal across these samples. Co-expressed FGF receptor was also sufficient to rescue induction of muscle-specific actin, a marker of differentiated mesoderm, as well as the morphogenetic movements associated with activin induction (Fig. 2B,C). This demonstrates the specificity of the dominant negative mutant, and underscores that FGF signaling is required for activin induction of *Xbra* and *Mix1*, even in the presence of overexpressed activin receptor.

Dominant negative FGF receptor blocks responsiveness to the endogenous mesoderm-inducing signal

The above experiments demonstrate that a functional FGF-signaling pathway is necessary for animal caps to respond to exogenous activin. We next asked if this requirement extends to the endogenous mesoderm inducer. Animal caps expressing the inhibitory FGF receptor or the non-functional FGF receptor were combined with vegetal endoderm, the source of the natural inducer (Nieuwkoop, 1969). These Nieuwkoop recombinants were cultured for 48 hours and then assayed for induction using morphogenetic movements and muscle actin expression as criteria. Fig. 3 demonstrates that recombinants made with control animal caps elongated extensively and strongly expressed muscle-specific actin, while recombinants expressing the dominant inhibitory FGF receptor showed little morphogenetic movement or muscle-specific gene expression. Recombinants overexpressing a dominant inhibitory FGF receptor also failed to express *Xbra*, an immediate early marker of mesoderm induction (not shown). Previous work has demonstrated that a dominant inhibitory ras allele also blocks mesoderm induction by vegetal endoderm in Nieuwkoop recombinants (Whitman and Melton, 1992). Together these results suggest that the endogenous mesoderm inducer, like activin, requires a functional FGF-signaling pathway in order to mediate mesoderm induction in animal caps.

MAP/ERK kinase and c-ras are activated by FGF but not by activin

The MAP/ERK kinases are serine/threonine-specific cytosolic kinases which, in cell culture, become phosphorylated on threonine and tyrosine in response to a wide variety of growth factors that signal through c-ras (reviewed in Pelech and Sanghera, 1992). At least one member of this family has been identified in *Xenopus*, and becomes activated during ras-dependent maturation of oocytes (Zaitsevskaya and Cooper, 1992). Since previous work in *Xenopus* suggested that c-ras was a necessary component of mesoderm induction by both activin and FGF (Whitman and Melton, 1992), we examined the ability of these growth factors to activate MAP/ERK kinase. Animal caps were isolated from stage 8 embryos and dissociated to increase the number of cells accessible to inducing factor (Green and Smith, 1990). Cells were treated with FGF or activin for various time points, lysed and analyzed

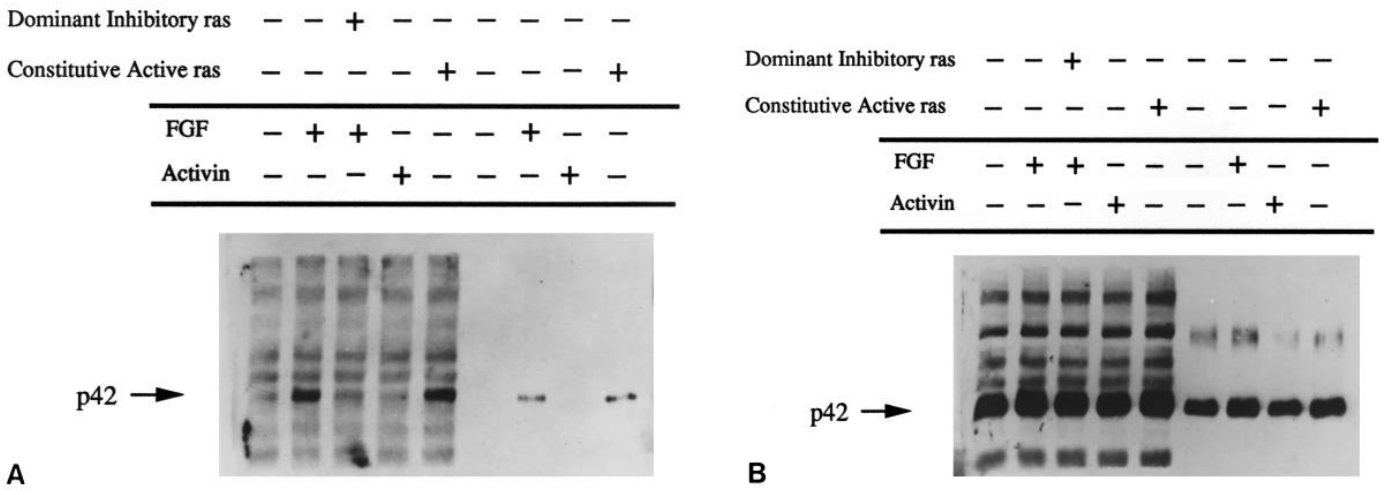


Fig. 4. MAP kinase is rapidly activated in response to FGF but not activin in a ras-dependent manner. Stage 8.5 animal caps were treated with FGF or activin for 5 minutes, analyzed directly by Laemmli gel electrophoresis (left five lanes) or immunoprecipitated with an anti-MAP kinase antibody (right four lanes) and immunoblotted with anti-phosphotyrosine (A) or anti-MAP kinase (B). For analysis of the effects of constitutively activated $p21^{ras}$ and dominant inhibitory $p21^{ras}$, mRNAs encoding these mutants were injected into both cells at the 2-cell stage, caps were explanted at stage 8, incubated for 5 minutes with or without FGF and processed as above. Migration of MAP kinase is indicated by an arrow.

by Laemmli gel electrophoresis/western blotting using either anti-phosphotyrosine (Fig. 4A, lanes 1-5) or anti-MAP kinase antibody (panel B, lanes 1-5). To confirm the identification of p42 as MAP/ERK kinase and to reduce background, lysates were also immunoprecipitated with anti-MAP kinase antibody before western blotting as above (Fig 4A,B lanes 5-9). While MAP/ERK kinase was phosphorylated on tyrosine within minutes of FGF treatment, no significant phosphorylation of MAP/ERK kinase was seen in response to activin either in whole cell lysates or in anti-MAP kinase immunoprecipitates (Fig. 4). No activin stimulated phosphorylation was seen over treatment times of 1 minute to 1 hour when examined by anti-phosphotyrosine blot (not shown). Animal caps expressing an activated ras allele displayed FGF-independent phosphorylation of MAP/ERK kinase, while a dominant inhibitory ras, $p21^{(Asn17)ras}$, blocks FGF-mediated phosphorylation of MAP/ERK kinase (Fig. 4). We have confirmed that phosphorylation of MAP/ERK kinase in these experiments correlates with activation of kinase activity (not shown).

These results indicate that FGF activates MAP/ERK kinase in a ras-dependent fashion, and this has been independently confirmed by Graves and colleagues (unpublished data). Although a dominant inhibitory ras blocks activin induction of mesoderm, activin does not appear significantly to activate MAP/ERK kinase, suggesting that activin may not directly stimulate ras. Direct measurements of the GDP/GTP-bound state of ras following a 10-minute stimulation with FGF or activin demonstrated that FGF but not activin triggered activation of p21 ras (M Whitman, unpublished). These experiments indicate that, while FGF generated signals are required for activin induction, this signaling pathway is not activated to detectable levels by activin.

A dominant inhibitory raf blocks mesoderm induction by both FGF and activin

To investigate further the requirement for FGF-generated signals during activin induction, we examined the role of Raf-1 during mesoderm induction. Raf-1 is a cytoplasmic serine/threonine kinase, which is activated downstream of c-ras and upstream of MAP/ERK kinase in other systems where tyrosine kinase-dependent signaling has been studied (Dickson et al., 1992; Hann et al., 1993; Kolch et al., 1991; Wood et al., 1992). Indeed, recent reports have shown that Raf-1 binds directly to $p21^{ras}$ and that this interaction may be important for its activation (Van Aelst et al., 1993; Vojtek et al., 1993). We have obtained a mutant Raf-1 kinase that acts as a potent dominant inhibitory mutant in other systems (Fabian et al., 1993; Morrison et al., 1993). Synthetic mRNAs encoding this dominant inhibitory raf were injected into *Xenopus* embryos at the 2-cell stage. Animal caps explanted from these embryos at stage 8 were treated with FGF or activin, and the degree of induction was assessed by both an examination of gross morphology and muscle-specific actin induction. Animal caps from embryos injected with a control RNA elongated when treated with activin or FGF. Animal caps injected with 4 ng of the dominant inhibitory raf mRNA showed no elongation in response to activin or FGF (not shown). When these animal caps were assayed for muscle-specific actin expression, cardiac actin expression of the dominant inhibitory raf prevented induction of this marker by either FGF or activin (Fig. 5) The muscle actin probe cross hybridizes with cytoskeletal actin, which serves as an internal control for RNA loading. When lower doses (2 ng) of the dominant inhibitory receptor were injected, cardiac actin expression was still completely blocked in response to FGF, but only partially blocked in response to activin. When the level of injected mRNA was further lowered (1 ng), the block to FGF remained significant although not complete, while little effect was seen on the levels of muscle actin mRNA induced by activin. Fig. 5 also shows that the ability of the dominant inhibitory raf to block mesoderm induction can be rescued by coinjection with c-raf, confirming the specificity of this effect.

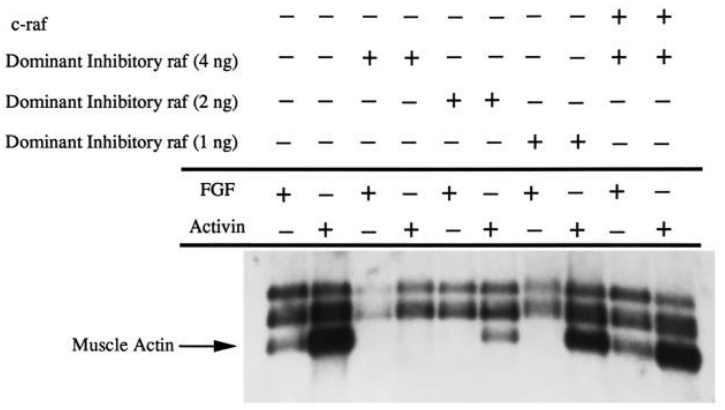


Fig. 5. A dominant inhibitory raf inhibits induction of muscle actin by both FGF and activin. Embryos were injected in both cells at the 2-cell stage with 4 ng, 2 ng or 1 ng of dominant inhibitory raf RNA. Animal caps were explanted at stage 8, treated with FGF or activin, cultured for 36 hours, and analyzed for muscle actin expression by northern blot. Co-injection with c-raf is sufficient to rescue muscle actin induction.

MacNicol et al. (1993) have previously reported that expression of dominant inhibitory raf in *Xenopus* embryos can block FGF signaling without blocking activin signaling. Our observations are consistent with this report at low concentrations of RNA injection (1-2 ng) but, at higher concentrations, we observe a block to induction by both activin and FGF. The inhibitory mutant used by MacNicol et al. (1993) has a mutation in a conserved lysine in the ATP-binding site (Lys 375) that destroys kinase activity. We have used a mutant raf containing a point mutation in a regulatory phosphorylation site, Ser 621, that is reported to be a more potent inhibitor of c-raf function than the lysine mutant (D. Morrison, personal communication). A difference in potency between these mutants may account for the difference between our observations and those of MacNicol et al. (1993) concerning the inhibition of activin by dominant inhibitory raf.

Co-expression of V-ras or V-raf restores responsiveness to activin

Since c-ras and c-raf appear to act downstream of FGF during mesoderm induction, and since activated mutants of these proto-oncogenes are sufficient to induce ventrolateral mesoderm when expressed at high doses (MacNicol et al., 1993; Whitman and Melton, 1992; C.L and M.W., unpublished data), we examined the ability of these activated oncogenes to rescue activin responsiveness in animal caps expressing the dominant inhibitory FGF receptor. We found that when mRNA encoding activated raf or ras was coinjected with the inhibitory receptor at levels that alone induce mesoderm poorly, animal caps responded strongly to exogenously added activin (Fig. 6A), which induced cardiac actin to levels seen in control caps. Coinjection of the dominant inhibitory FGF receptor and a control mRNA that does not induce mesoderm did not restore activin-mediated muscle-specific actin induction.

In these experiments, the activated raf and ras alleles rescued the induction of cardiac actin to levels significantly greater than they themselves can induce. However, we wished to ascertain whether these mutants could rescue the induction of a marker

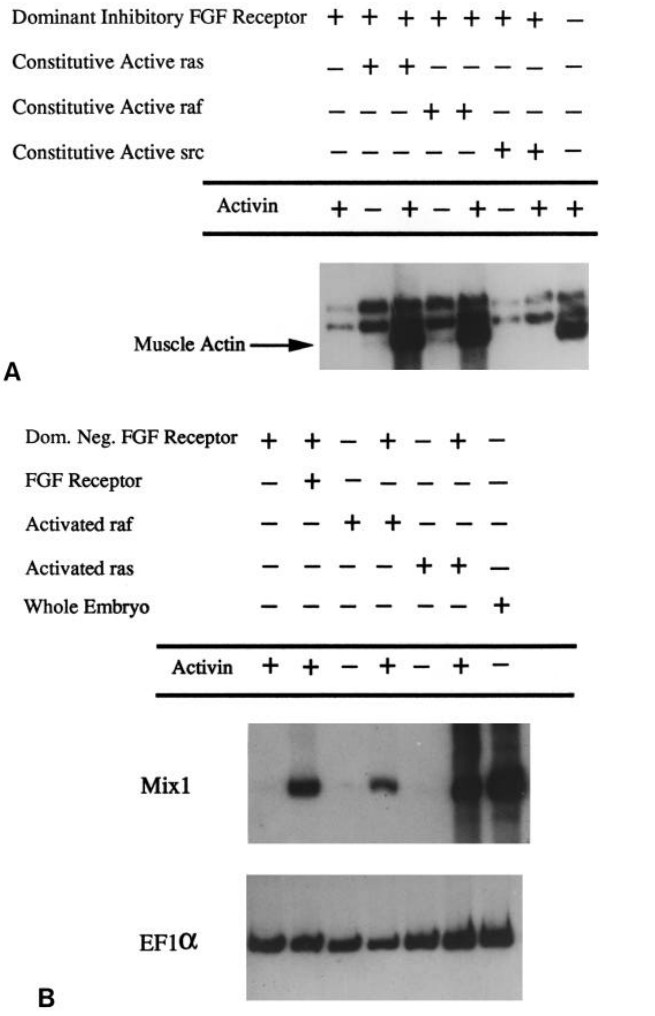


Fig. 6. Co-expression of activated ras or raf mutants is sufficient to rescue the block to activin induction by the dominant inhibitory FGF receptor. (A) Muscle actin Northern of animal caps co-expressing the dominant inhibitory FGF receptor and constitutively active ras (lanes 2,3), raf (lanes 3,4) or src (lanes 5,6). raf and ras rescue activin-mediated muscle actin induction to control levels (lane 8). (B) RT-PCR analysis of activated raf and ras rescue of immediate early response gene *Mix-1*. *EF1-α* expression provides a control for template levels across PCR reactions.

that they themselves cannot induce at all. *Mix1*, an activin responsive immediate early gene, is such a marker. As shown in Fig. 6b no *Mix1* expression was found in untreated animal caps explanted from embryos injected with activated ras or activated raf mRNA, or in activin-treated animal caps expressing the dominant inhibitory FGF receptor. However, co-injection of mRNA encoding either of these activated oncogenes together with the dominant inhibitory FGF receptor rescues the activin-dependent induction of Mix1 (Fig. 6), demonstrating that activation of components downstream of the FGF receptor is sufficient to rescue activin induction.

DISCUSSION

In this report, we partially define an FGF signal transduction

pathway that functions during early *Xenopus* development, and show that it is required for many aspects of activin-dependent mesoderm induction. This result, together with those of Cornell and Kimelman (1994), helps redefine the role of FGF during early inductive signaling.

It has been known for some time that both FGF and activin are capable of inducing mesoderm in explanted animal pole cells. FGF, however, has been found to induce only a subset of activin-inducible genes. Until recently, most of the known FGF-inducible genes were found to be expressed in ventral and lateral regions. This led to the hypothesis that FGF was a ventral mesoderm inducer and activin was a dorsal mesoderm inducer. More recently, however, a novel FGF-inducible gene, *Xnot*, was identified and found to be expressed at the organizer, the dorsalmost region of early gastrulae (von Dassow et al., 1993). Overexpression of the dominant inhibitory FGF receptor in the dorsal marginal zone was shown to abolish this expression. This indicated that FGF was not solely a ventral mesoderm inducer, and further highlighted an apparent functional overlap between FGF and activin induction. Indeed, it has recently been demonstrated that overexpression of a dominant inhibitory FGF receptor in the marginal zone inhibits formation of dorsal tissues such as notochord in addition to ventral and lateral mesoderm, and that patterning in these embryos is most consistently and severely perturbed when the injected mRNA is targeted to the dorsal rather than the ventral side (Amaya et al., 1993).

If FGF and activin are both implicated in the formation of dorsal and ventral mesoderm, what then are their respective roles in early inductive signaling? Although data obtained by overexpressing a dominant inhibitory FGF receptor in whole embryos indicate that FGF signaling is required for the induction of most types of mesoderm (Amaya et al., 1991, 1993), experiments with a dominant inhibitory activin receptor suggest that the endogenous levels of FGF signaling may be insufficient to induce mesoderm in vivo. Embryos expressing high levels of a dominant inhibitory activin receptor contain no discernible mesoderm, despite retaining a hyper-functional FGF-signaling pathway (Hemmati-Brivanlou and Melton, 1992).

If the levels of FGF signaling in the embryo are insufficient to induce mesoderm on their own, and if FGF signaling is required for induction of most if not all types of mesoderm, this would appear to rule out any model that assigned endogenous FGF a direct and sufficient role in inducing mesoderm in a specific region of the developing blastulae. Such data would be more consistent with a model where FGF was only one component of a multi-step mesoderm-inductive response. Under such a model, FGF's ability to induce mesoderm when added ectopically in animal cap assays could be explained as a consequence of overstimulating its signaling pathways beyond levels normally achieved endogenously.

Cooperation between FGFs and activins in inducing mesoderm is not completely unexpected. It has previously been shown that low levels of FGF can lower the concentration of activin needed to induce dorsal mesodermal markers (Green et al., 1992). Indeed, as an increasing number of molecules have been identified that can induce or modify the character of mesoderm, it has become clear that mesoderm induction and axial patterning are the result of complex interactions between many factors. In addition to the FGFs and activins, Vg1, a TGFβ family member, has been shown to have potent mesoderm-inducing activity (Thomsen and Melton, 1993). Noggin and some Wnt-related molecules, while not mesoderm-inducing factors themselves, have a dorsalizing effect on induced mesoderm (Christian et al., 1992; Smith and Harland, 1992; Sokol and Melton, 1992; Thomsen and Melton, 1993), while Xwnt-8 and BMP4, another TGFβ-related factor, appear to attenuate activins ability to induce mesoderm in animal cap assays (Christian and Moon, 1992; Dale et al., 1992; Jones et al., 1992; Sokol and Melton, 1991). It is unlikely that any of these factors acts in isolation. Instead, they are likely to produce a complex signaling network involving signal amplification and synergism as well as negative feedback loops, which work to pattern and refine the axis. In order to dissect this process, it will be necessary to understand how each of these molecules signals, both individually and in conjunction with other factors.

The observation that a functional FGF-signaling pathway is required for activin induction of a variety of mesodermal response genes suggests that FGF may function endogenously as a component of activin-mediated mesoderm induction.. The ability of a dominant negative FGF receptor to block activin signaling is consistent with a model where FGF plays a central role in the competence of a cell to respond to activin induction. Such a role is not only consistent with available data derived from experiments involving the overexpression of dominant inhibitory receptors for these growth factors, but also helps to explain seemingly contradictory results with activated and inhibitory mutants of the small GTP-binding protein $p21^{ras}$. It has recently been shown that overexpression of a constitutively activated mutant of p21ras is sufficient to induce ventrolateral mesoderm in animal caps, at levels quantitatively and qualitatively similar to that seen when FGF is added ectopically (Whitman and Melton, 1992). Consistent with this, the whole embryo phenotype that results from overexpression of a dominant inhibitory ras is similar to that seen in experiments using a dominant inhibitory FGF receptor or a dominant inhibitory raf (C. LaBonne and M. Whitman, unpublished data). Thus, as has been found in other systems, p21ras is an important downstream component of FGF signaling during mesoderm induction. Surprisingly, however, it was found that the dominant inhibitory ras blocked mesoderm induction by both FGF and activin, raising the possibility that activin acts through activation of $p21^{ras}$. An alternative possibility, suggested by the observation that a dominant inhibitory FGF receptor also inhibits activin induction, is that the effects of the dominant inhibitory ras on activin signaling may be mediated through the FGF pathway. Our finding that FGF, but not activin, potently activates MAP/ERK kinase in a ras-dependent fashion indicates that the FGF-dependent signaling pathway, which includes ras, raf and MAP/ERK kinase, is not directly and/or rapidly activated in response to activin. This suggests that activation of the FGF-signaling pathway is a prior or concurrent requirement for the competence of embryonic cells to respond to activin rather than a downstream component of the activin response.

In other systems where signal transduction by tyrosine kinase receptors has been studied, c-raf activation has been found to be an intermediate step between c-ras and MAP/ERK kinase activation. Indeed, where it has been examined, signaling via c-ras and c-raf have been found to be linked

(Dickson et al., 1992; Hann et al., 1993; Kolch et al., 1991; Wood et al., 1992), and it has recently been found that these proteins physically associate, implicating c-raf as a potential ras effector (Van Aelst et al., 1993; Vojtek et al., 1993). It is therefore not surprising that a dominant negative raf, like the dominant negative ras, blocks mesoderm induction by both FGF and activin when expressed at high levels. We have therefore demonstrated that a block at any one of several steps in this tyrosine kinase signal transduction pathway is sufficient to disrupt activin's ability to induce the expression of several mesodermal markers.

It remains unclear, however, at what step in the activin response an FGF signal is required. Additional components of these signaling pathways will need to be identified before the interactions between the pathways can be understood. In addition, we have shown that even at high doses of dominant negative FGF receptor where induction of dorsal markers such as goosecoid can be significantly reduced, we are unable to block *Xwnt8* induction to a similar degree. Cornell and Kimelman (1994) have identified additional genes, such as *XCad3* and *Xlim-1*, induction of which may have a lower dependency on FGF signaling. This would appear to indicate that there are aspects of activin signaling which are FGF independent. If activin signaling has FGF-dependent and FGF-independent components, one might think that the FGF-dependent response genes would correspond to those genes that are FGF inducible. This appears not to be the case however, as activin induction of *Mix1*, a gene that is not inducible by FGF, can be blocked with the dominant inhibitory FGF receptor, while *Xwnt8*, which is FGF inducible, cannot be completely blocked.

The dominant inhibitory FGF receptor can inhibit activin induction of goosecoid in animal cap assays, although this block is often not complete. Like Amaya et al. (1993), however, we are unable to significantly block goosecoid induction in the marginal zone (C. LaBonne and M. Whitman, unpublished data). This may reflect a fundamental difference in the behavior of cells from the animal pole and the marginal zone, and presents a caveat for the interpretation of animal cap experiments. However, as it appears a more complete block to mesoderm induction can be achieved in animal caps than in the marginal zone, animal cap cells should provide a useful system for assessing the inductive potential of mesoderm-inducing factors in the absence of overriding influences from modifying factors found in the marginal zone. While clearly not naive, animal caps lack high expression of factors such as noggin, and this may be the basis for the differential ability of the dominant negative FGF receptor to inhibit goosecoid expression in animal caps and marginal zones. Because of this, animal cap assays should provide a good system for elucidating where the requirement for FGF signaling in activin induction lies. The experiments reported here explain why the whole embryo phenotype generated by inhibiting ras function closely resembles the reported phenotype for the dominant inhibitory FGF receptor (Amaya et al., 1991) and dominant inhibitory raf (MacNicol et al., 1993), in that all three appear to block a common pathway necessary for activin signaling. It remains unclear, however, why dominant inhibitory FGF receptor, dominant inhibitory raf, or dominant inhibitory ras do not effect a complete block to mesoderm induction in the whole embryo as has been observed for dominant inhibitory activin receptor. It is possible that components of the activin response, which the dominant inhibitory FGF receptor fails to inhibit, or inhibits poorly in the marginal zone, account for the differences in observed phenotype. Alternatively, it is possible that the dominant inhibitory activin receptor inhibits signaling by related TGFβ family members such as Vg1, which may not require a functional FGF-signaling pathway. Final resolution of this question must await further information on how the TGFβ family of receptors transduce signals.

The authors thank Paul Wilson, Sergei Sokol, John Blenis, Adrian Gross and members of the laboratory for helpful discussions. We thank David Kimelman, Ali Hemmati-Brivanlou, Debbie Morrison and Paul Wilson for sharing results prior to publication. We are grateful to Enrique Amaya and Marc Kirschner for FGF receptor constructs, Doug Melton for activin, Debbie Morrison for Raf1 mutants and John Blenis for MAP kinase antibodies. C. L. is supported by an NSF predoctoral fellowship. M. W. is a scholar of the Lucille P. Markey Charitable Trust. This work was also supported by a grant from the NIH.

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(Accepted 18 November 1993)