Development 124, 827-837 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV3585

A *Xenopus* type I activin receptor mediates mesodermal but not neural specification during embryogenesis

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SUMMARY

Activins and other ligands in the TGFβ superfamily signal through a heteromeric complex of receptors. Disruption of signaling by a truncated type II activin receptor, XActRIIB (previously called XAR1), blocks mesoderm induction and promotes neuralization in *Xenopus* embryos. We report the cloning and characterization of a type I activin receptor, XALK4. Like truncated XActRIIB, a truncated mutant (tXALK4) blocks mesoderm formation both in vitro and in vivo; moreover, an active form of the receptor induces mesoderm in a ligand-independent manner. Unlike truncated XActRIIB, however, tXALK4 does not induce neural tissue. This difference is explained by the finding that tXALK4 does not block BMP4-mediated epidermal specification, while truncated XActRIIB inhibits all BMP4 responses in embryonic explants. Thus, the type I and type II activin receptors are involved in overlapping but distinct sets of embryonic signaling events.

Key words: mesoderm induction, neural induction, activin, BMP4, type I activin receptor, *Xenopus*, TGFβ

INTRODUCTION

In *Xenopus*, as in other vertebrates, the earliest events in the establishment of the body axis involve the formation of the three germ layers: ectoderm, mesoderm and endoderm. The subsequent movements of these layers during gastrulation generate new tissue interactions which lead to the final determination of the body axis (Gilbert, 1992). Many growth factors have been identified that mediate one or more of the steps in this process. Notably, the fibroblast growth factor (FGF) family and members of the transforming growth factor β (TGFβ) superfamily including activins, Vg1 and BMPs have been shown to induce a variety of embryonic tissue types (for reviews, see Klein and Melton, 1994; Harland, 1994; Kessler and Melton, 1994). Activins, Vg1 and several BMPs are expressed maternally as RNA or protein. Activin can induce several types of mesoderm as well as endoderm in ectodermal explants (animal caps) in a concentration-dependent manner (Green and Smith, 1990; Green et al., 1992; Symes et al., 1994; Gamer and Wright, 1995; Henry et al., 1996). It is proposed that activin may act as a morphogen in *Xenopus* embryos, forming a gradient to determine different tissue types along the dorsal-ventral axis (Green and Smith, 1991; Gurdon et al., 1994). Vg1 is a vegetally localized maternal RNA (Weeks and Melton, 1987) that has the ability to rescue a complete axis in UV-ventralized embryos (Thomsen and Melton, 1993). While both activin and Vg1 can induce a whole repertory of embryonic tissues in embryonic explants, BMPs have only been found to mediate the induction of ventral types of mesoderm (Koster et al., 1991; Dale et al., 1992; Jones et al., 1992; Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995). Recent studies have also suggested that BMP4 is involved in epidermal specification and neural inhibition (Wilson and Hemmati-Brivanlou, 1995; Sasai et al., 1995; Hawley et al., 1995; Xu et al., 1995).

While FGF signals through ligand-induced homodimerization of receptors with intrinsic protein tyrosine kinase activity, activin and BMP4 utilize a heteromeric receptor complex containing at least one molecule from each of the two subfamilies of receptor serine/threonine (ser/thr) kinases (reviewed in Mathews, 1994). Type II receptors (ActRII, TβRII, BMPRII) have been identified either by ligand binding or by association with type I receptors (Mathews and Vale, 1991; Lin et al., 1992; Liu et al., 1995). The type I receptors (also called ALKs – activin receptor like kinase) were originally characterized based on sequence similarity to the type II receptors (ten Dijke et al., 1993; Ebner et al., 1993; ten Dijke et al., 1994a). Both classes of receptors have been implicated in signal transduction. For activin (Attisano et al., 1996; Willis et al., 1996) and TGFβ (Wrana et al., 1992; Wrana et al., 1994), a model for receptor activation has been suggested in which type I receptors become phosphorylated, in a type II-dependent manner, at a conserved glycine/serine-rich sequence in the juxtamembrane region; it is likely that BMP receptors become activated in a similar fashion (Liu et al., 1995). Although activin and TGFβ display no apparent binding or functional interactions with each other's receptors, it appears that there may be significant sharing of receptors between activin and BMPs. As evidence that a particular type II receptor can interact with multiple ligands, the activin type II receptor, ActRII, has been reported to bind to, and potentially signal in response to, BMP7 as well as activin (Yamashita et al., 1995);

similarly, the type II BMP receptor, BMPRII, binds both BMP2 and BMP7 (ten Dijke et al., 1994b; Liu et al., 1995). Comparable data indicate that both activins and BMPs may signal through receptor complexes containing a single type I receptor ALK2 (ActRI), together with ActRII (Yamashita et al., 1995). Finally, it has been proposed that individual type I receptors may associate with different type II receptors; ALK2, ALK3 (BMPR-IA) and ALK6 (BMPR-IB) have been found to interact with both ActRII and BMPR-II (ten Dijke et al., 1994a; Liu et al., 1995; Yamashita et al., 1995). To date, neither the relative binding affinities nor the signaling capacities of the various ligand-receptor complexes have been accurately determined.

Several receptor ser/thr kinases have been cloned from *Xenopus*, including type II activin and type I BMP4 receptors (Mathews et al., 1992; Hemmati-Brivanlou et al., 1992; Graff et al., 1994). Expression of truncated forms of these receptors has revealed that deletion of the intracellular domains yields molecules that can inhibit signaling in a dominant negative manner, and the results have also implicated these signaling pathways in mesoderm induction and patterning (Hemmati-Brivanlou and Melton, 1992; Graff et al., 1994; Suzuki et al., 1994). Consistent with the ventralizing activity of BMP4 ligand, truncated type I BMP4 receptor converts ventral mesoderm to dorsal mesoderm, implying that the wild-type receptor is involved in ventral mesoderm specification (Graff et al., 1994; Suzuki et al., 1994). In the case of type II activin receptor, the truncated mutant blocks all mesoderm formation, suggesting a requirement for activin. However, this truncated receptor may block more than one ligand and the role of activin in mesoderm induction remains controversial (Schulte-Merker et al., 1994; Hemmati-Brivanlou and Thomsen, 1995). In addition to mesoderm induction, experiments with these receptors have uncovered previously unsuspected roles for their ligands in early development. Truncated versions of both type II activin receptor and type I BMP4 receptor can divert the ectoderm from an epidermal to a neural fate (Hemmati-Brivanlou and Melton, 1992, 1994; Xu et al., 1995). These studies have shown that members of this growth factor family are not only important in mesoderm formation, but also play an inhibitory role in the formation of the neural tissues. Since studies in cell culture indicate that each type II receptor may interact with several type I receptors to bind different ligands, determining the function of each ligand in frog embryos will require the cloning and functional characterization of the complete family of related ligands and receptors.

In this paper, we report the cloning and functional study of an activin type I receptor, which we have named XALK4 (for *Xenopus* ALK4). Consistent with a role in early embryonic development, this receptor is expressed maternally and is distributed widely in early embryos. The type II activin receptor, previously called XAR1 and renamed here XActRIIB (to remain consistent with the original nomenclature used for these receptors), is also expressed in most of the cells of the early embryo. As was the case with the type II activin receptor (Hemmati-Brivanlou et al., 1992; Mathews et al., 1992), ectopic expression of wild-type XALK4 induces mesoderm in embryonic explants, and truncated XALK4 (tXALK4) blocks mesoderm formation both in explants and in the embryo. Surprisingly, however, in contrast to truncated XActRIIB (previously referred to as Δ1XAR1 and here, because of the new nomenclature, tXActRIIB), tXALK4 inhibits the mesoderm-inducing activity of BMP4 without concurrent neuralization of animal cap explants. In addition, truncated XALK4 does not block epidermal induction by BMP4 in the context of dissociated animal cap cells. Moreover, while wild-type XALK4 rescues mesoderm induction by activin in the presence of tXALK4, it cannot rescue mesoderm induction by BMP4. These results uncover for the first time distinct functions of the two types of activin receptors in the formation of different tissue types in *Xenopus*. They also point to a possible difference between the mechanisms of mesodermal and epidermal induction by BMP4 ligand.

MATERIALS AND METHODS

Cloning and sequencing of XALK4 type I activin receptor and construction of mutants

Two degenerate oligos, CCGGAATTCA(C/T)(A/C)G(G/A/C)-GA(T/C)(A/T/C)TNAA(A/G)(A/T)C and CCGAAGCTTA(C/T)-(C/T)TCNGGNGCCAT(A/G)TA, coding for peptide sequences HRD(L/F/I)KS and YMAPEV conserved within serine/threonine kinase domain, were used for PCR cloning of type I activin receptor. A fragment of 180 bp sequence bearing the highest homology to kinase domain of type I activin receptor was used to screen 0.6×10^6 plaques of a *Xenopus* oocyte library (gift of Dr P. Klein, University of Pennsylvania). Four independent clones were isolated, one encoding full-length type I activin receptor XALK4. The sequence was obtained with dideoxynucleotide sequencing method (Sanger et al., 1977) and sequence alignment of ALK4 with other receptors was made with DNA Star, MegAlign, program. Truncated XALK4 was constructed by PCR using the following two primers: GGAGATC-TACCATGGCGGAGCTACCGGCC and GGAGATCTTCA-CATTTCACATGATGGATCC. The PCR fragment encoded N-terminal 164 amino acids of XALK4, including the extracellular and transmembrane domains of the receptor. The *Bgl*II-digested fragment was inserted into the *Bgl*II site of pSP64T vector (Krieg and Melton, 1984). ALK4 and ALK-T206E were constructed as described elsewhere (Willis et al., 1996) and cloned into the *Bgl*II site of pSP64T.

Embryos, RNA preparation, microinjection and animal cap explants

Xenopus embryos, both pigmented and albino, were obtained as previously described (Hemmati-Brivanlou and Harland, 1989). Embryonic stages were determined as described in Nieuwkoop and Faber (1967). The dorsal side of embryos was determined according to animal pole pigmentation and blastomere size at the 4- to 8-cell stage. (Dorsal cells are lighter and smaller than ventral cells.) RNAs encoding wild-type and mutant receptors were synthesized with linearized templates derived from pSP64T vector, using SP6 polymerase (Ambion mMessage mMachine kit). For tXActRIIB and tBR, the linearization of templates was done as described previously (Hemmati-Brivanlou and Melton, 1992; Graff et al., 1994). For tXALK4, as well as for ALK4 and ALK4-T206E, the templates were linearized with *Xba*I. BMP4 RNA was obtained as previously described (Hemmati-Brivanlou and Thomsen, 1995). The RNAs were then injected into the animal poles or marginal zones of early stage embryos. Amount of injected in vitro synthesized RNAs and sites of injection are as described in the Results section. Animal cap explants were removed with hair knives at late blastula stages and allowed to grow until control sibling embryos reached either gastrula or neurula stages. Total RNA was then extracted and analyzed with RT-PCR. In experiments with activin induction, activin RNA was injected at 30 ng into mature oocytes and the oocyte-conditioned medium was collected 3

days later (Kessler and Melton, 1995). A 1:500 dilution of this conditioned medium was used.

RT-PCR assay

RT-PCR assay was performed as previously described (Wilson and Melton, 1994), with the modification that random hexamers rather than oligo(dT) were used to prime reverse transcription. Primers for EF1-α, muscle actin, Xbra and NCAM were described in Hemmati-Brivanlou and Melton (1994). Primers for Xhox-3, globin (Hemmati-Brivanlou and Thomsen, 1995), epidermal keratin (Wilson and Hemmati-Brivanlou, 1995) and NRP-1 (Lamb and Harland, 1995) were as previously described. For XALK4, the following primers were used: XALK4-U: 5′-GCGGAGCTACCGGCCTTCTTC-3′ and XALK4-D: 5′-TGGGATTGCAATAACAGCTAC-3′ and the PCR conditions were: 94°C, 30 seconds; 55°C, 1 minute; 72°C, 30 seconds; for 25 cycles.

Whole-mount in situ hybridization and immunochemistry

Whole-mount in situ hybridization was performed as described (Hemmati-Brivanlou et al., 1990; Harland, 1991). Antisense digoxigenin-labeled Xbra was obtained as previously described (Hemmati-Brivanlou and Melton, 1992). XALK4 antisense probe was synthesized with T7 RNA polymerase with *Eco*RI linearized pBluescript template (Stratagene) containing entire XALK4 coding sequence. Whole-mount antibody staining was performed as described by Hemmati-Brivanlou and Harland (1989). Three antibodies were used: Tor70.1 for notochord staining (Bolce et al., 1992), 12/101 for muscle (Kintner and Brockes, 1984) and 6F11, which is a neural antigen-specific antibody (A gift from Dr W. Harris, UCSD). Tor70.1 and 12/101 were monoclonal antibodies used at 1:500 dilution. 6F11 was from the hybridoma-conditioned medium and was used at 1:1 dilution. The secondary antibody was a goat anti-mouse IgG coupled to horseradish peroxidase (Jackson Laboratories) and was used at 1:250 dilution.

Cell dissociation and reaggregation

The animal pole cells were dissociated in Ca^{2+}/Mg^{2+}-free medium at stage 9-10 as described before (Grunz and Tacke, 1989; Wilson and Melton, 1994). Dissociated cells were reaggregated immediately or after 4 hours and then incubated until control sibling embryos reached late neurula stages. Purified recombinant human BMP4 protein (Gift of Genetic Institute) was used on dissociated cells at 50 ng/ml.

RESULTS

Isolation of a *Xenopus* XALK4 cDNA

To isolate *Xenopus* TGF-β receptors, we performed a PCR amplification of first strand cDNA made from oocyte RNA. The primers were designed to hybridize to the conserved region of all receptor serine/threonine kinases. A fragment of 180 bp whose sequence showed the highest homology to the mammalian type I activin receptor, ALK4 (activin receptor-like kinase, also called ActRIB), was used to screen a maternal oocyte library. A screen of 0.6×10^6 plaques yielded eleven positives containing four different clones, of which one contained a 3 kb insert encoding the full-length receptor. The predicted protein sequence of this clone is shown in Fig. 1A. There are seven conserved cysteine residues in the extracellular domain at intervals characteristic of all type I receptors and a single ser/thr kinase domain following the putative transmembrane region. The sequence shows greater than 85% identity to all ALK4 mammalian, with highest conservation in the cytoplasmic kinase domain (Fig. 1A). Notably, the glycine/serine-rich domain (GS domain) is conserved between the frog gene and all other type I receptor genes; this region has been proposed to play an important role in signal transduction by type I activin receptors. Based on this homology, we named the frog gene *XALK4*. RT-PCR and in situ analysis indicates that *XALK4* is present maternally and expressed widely throughout early embryogenesis, such as in mesodermal and ectodermal tissues (Fig. 1B and data not shown). Thus both the type I and type II activin receptors are expressed from the first cell cycle onward.

To perform functional studies with ALK4, we have utilized mutant forms of both the human *ALK4* and frog *XALK4* genes in early *Xenopus* embryogenesis. Fig. 1C shows a schematic drawing of the receptors used in this study. In addition to wild-type *Xenopus* and human genes, we examined a mutant generated by truncation of most of the intracellular domain (tXALK4), and a point mutant in the GS domain (ALK4-T206E), which can induce expression of activin-dependent genes in the absence of ligand in cell culture (Willis et al., 1996). Because tXALK4 retains intact extracellular ligand-binding sequences, but is impaired in its capacity to transduce signals, it can presumably interfere with the wild-type receptors in a dominant fashion by competing for ligands and type II receptors. Because ALK4-T206E signals without ligand, it represents a constitutively active receptor.

Effects on mesoderm induction of wild-type and mutant ALK4

Because ALK4-T206E has been characterized by gain-of-function studies (Willis et al., 1996) in mammalian cells, we began the functional characterization of ALK4 by testing the effects of this mutant in early embryos. Although this set of experiments was performed using human wild-type and mutant ALK4, the wild-type *Xenopus* clone had identical function in all assays (not shown). We injected 2 ng of in vitro synthesized ALK4 or ALK4-T206E RNA into animal poles of 2-cell embryos. Animal caps were explanted at mid-blastula, incubated in the presence or absence of activin and assayed at late neurula stages for both morphology and tissue-specific molecular markers (Fig. 2). In the presence of activin uninjected explants, as well as explants expressing ALK4 and ALK4-T206E, elongated and expressed the mesodermal markers Xbra and muscle actin. In the absence of activin, ALK4-injected caps showed weak signs of elongation and induction of only the general mesoderm marker Xbra. This weak induction without ligand presumably reflects a low level of basal kinase activity; a similar effect has been observed with other serine/threonine kinase receptors (e.g. Mathews et al., 1992; Hemmati-Brivanlou et al., 1992). Caps expressing ALK4-T206E elongated strongly and expressed both Xbra and muscle actin, suggesting that it acted as a constitutively active receptor that could signal in a ligand-independent manner.

Inhibition of mesoderm induction by activin and BMP4 using truncated XALK4

In *Xenopus*, at least two other receptor ser/thr kinases have been cloned: the type II activin receptor XActRIIB and a type I BMP4 receptor (BMPRI, Hemmati-Brivanlou et al., 1992; Graff et al., 1994). Because receptors in this class act as heteromers, truncation mutants can act as dominant inhibitors of signaling, and this strategy has been successfully used to study

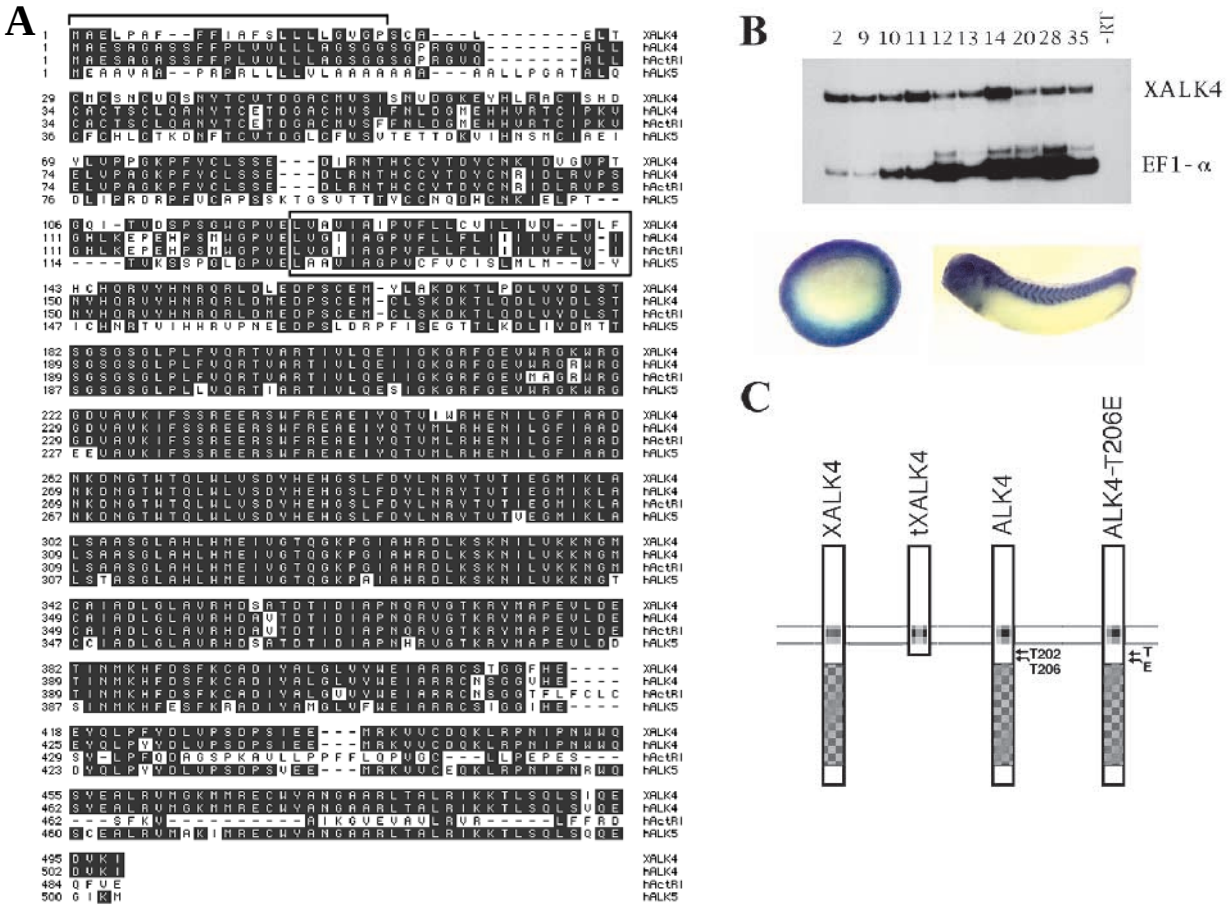

Fig. 1. Wild-type and mutant type I activin receptors. (A) Protein sequence alignment between *Xenopus* ALK4 (XALK4) and three human type I receptors: ALK4, ActRI and ALK5. The signal sequence is overlined and the transmembrane domain is boxed. The cytoplasmic domain starts with amino acid (aa) 143, the GS domain contains the GS core sequence at aa 182-187. (B) XALK4 expression pattern. Top, RT-PCR analysis of XALK4 at different developmental stages; stage 2, 2-cell stage; stages 9 to 11, gastrula stages; stages 12 to 20, neurula stages; stage 28 tailbud stage; stage 35, tadpole. XALK4 is expressed maternally and persists during development. Bottom: in situ hybridization of XALK4 at gastrulation (left, vegetal view with dorsal lip at the top) and tailbud (right, anterior at the left) stages, showing uniform expression in dorsal and ventral sides at early stage and expression in many tissues in late embryos. (C) Schematic presentation of the wild-type and mutant receptors used in this study. tXALK4 contains the first 164 aa of XALK4, which includes the extracellular and transmembrane domains, but excludes GS and kinase domains. GS domain of ALK4 contains Thr at positions 202 and 206, which have been shown to be important for its function in cell culture assays. ALK4-T206E mutant induces transcriptional responses in a ligand-independent manner in cell culture (Willis et al., 1996).

the functions of XActRIIB and BMPRI in embryogenesis (Hemmati-Brivanlou and Melton, 1992; Hemmati-Brivanlou and Melton, 1994; Graff et al., 1994; Suzuki et al., 1994; Xu et al., 1995). To compare the functions of these receptors with type I activin receptor, we generated a truncated *XALK4* gene with a deletion of its kinase domain (tXALK4, see Fig. 1B) and studied the role of this receptor in mesoderm induction by activin and the related factor BMP4.

Embryos were injected with 1 ng of in vitro synthesized receptor RNAs into the animal pole of each blastomere at the 2-cell stage. At blastula stages, animal pole explants were removed and cultured with or without inducing factors. Total RNA was harvested at either gastrula or neurula stage, and RT-PCR was used to assay for expression of mesodermal markers (Fig. 3). As previously demonstrated, activin induced dorsal mesoderm in animal cap explants, as revealed by the expression of muscle actin as well as Xbra (compare lane 1 with lane 5 in Fig. 3A). Injection of tXALK4 blocked mesoderm induction by this ligand (compare lanes 5 and 6 in Fig. 3A). This result, in agreement with observations made in cell culture systems (Wrana et al., 1994), shows that activin signal transduction requires an intact type I receptor with an active cytoplasmic kinase domain. The truncated type II receptor, tXActRIIB, also completely blocked activin induction of these markers, while truncated BMPRI (tBR) did not (Fig. 3A and Graff et al., 1994). There was, however, a somewhat lowered level of expression of Xbra in the presence of tBR. This partial effect has been observed previously (Schmidt et al., 1995; Graff et al., 1994) and could be due to dorsalization of induced mesoderm.

Because tXActRIIB could block both activin and BMP4 signaling (Hemmati-Brivanlou and Melton, 1992; Hemmati-Brivanlou and Thomsen, 1995), we asked whether signaling by BMP4 could also be inhibited by truncated XALK4. Previously it has been shown that BMP4 can induce ventral mesoderm in animal cap explants and can ventralize the dorsal marginal zone (Koster et al., 1991; Dale et al., 1992; Jones et al., 1992; Fainsod et al., 1994; Suzuki et al., 1994; Hemmati-

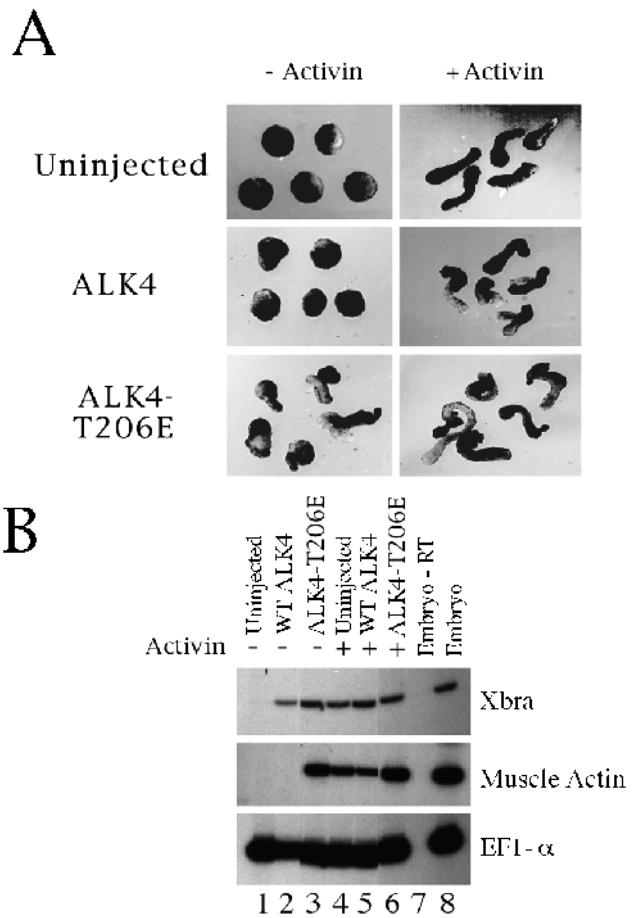

Fig. 2. Mesoderm induction assay by wild-type and mutant ALK4 in ectodermal explants. (A) Morphology of animal caps expressing the different forms of ALK4. Embryos were injected with RNAs encoding wild-type ALK4 or a constitutively active point mutant ALK4-T206E. Caps dissected at blastula stages 8 to 9 were allowed to grow in the absence (left panels) or presence (right panels) of activin until sibling control embryos reached neurula stage 18. (B) Expression of mesoderm-specific markers in animal caps injected with different receptor RNAs. Xbra is an early pan-mesodermal marker, muscle actin is a marker of paraxial mesoderm. Lanes 1-3, caps incubated in buffer alone; lanes 4-6, caps incubated in the presence of activin. Lanes 1 and 4, uninjected control caps; lanes 2 and 5, caps injected with wild-type ALK4 RNA; lanes 3 and 6, caps injected with ALK4-T206E RNA; lanes 7 and 8, whole-embryo controls, in the absence (lane 7) or presence (lane 8) of reverse transcriptase in the RT-PCR reaction. While wild-type ALK4 is a weak mesoderm inducer, ALK4-T206E induces mesoderm in animal caps in the absence of added activin.

Brivanlou and Thomsen, 1995). We injected 0.5 ng in vitro synthesized BMP4 RNA into 2-cell-stage embryos, either alone or together with different receptor RNAs. RT-PCR analysis of animal cap explants expressing BMP4 at gastrula stages revealed that mesodermal markers Xbra, Xwnt8 and Xhox3 were all induced. At later tadpole stages, the ventral mesoderm marker αT1-globin was also expressed (Fig. 3B, lane 5). In contrast, when BMP4 was coinjected with tXALK4 RNA, the expression of all these markers was blocked (compare lane 5 with 6). As a control, we showed that both tBR and tXActRIIB blocked mesoderm induction by BMP4 (Fig. 3B, lanes 7 and 8).

Because ALK4 has not been observed to mediate BMP4-induced transcriptional responses in mammalian cells (L. S. M., unpublished data), we asked whether the block to activin and BMP signaling was specifically due to inhibition of ALK4 activity. We performed rescue experiments by coinjecting wild-type XALK4 or XActRIIB along with tXALK4 (Fig. 4). Coexpression of XALK4 with tXALK4 rescued mesoderm induction by activin, as measured by expression of muscle actin. In contrast, mesoderm induction by injected BMP4 RNA could not be rescued by XALK4. Expression of both early and late markers remained unresponsive to BMP4 in embryos injected with tXALK4, even in the presence of wild-type XALK4. Surprisingly coexpression of wild-type activin type II receptor XActRIIB did reverse the suppressive effect of tXALK4 on mesoderm induction by BMP4, though it did not rescue activin-induced mesoderm formation. The different effect of rescue by wild-type receptors suggests that XALK4 and XActRIIB are differentially involved in mesoderm induction by activin and BMP4. The inhibition of BMP4-mediated mesoderm induction by tXALK4 may result from its titration of a type II activin receptor employed in both pathways.

To see if mutant ALK4 can non-specifically inhibit mesoderm induction by other growth factors, we examined the effect of tXALK4 on FGF-induced mesoderm formation. As described previously, bFGF induced expression of the mesodermal markers Xbra and Xwnt8, but only weak expression of muscle actin and weak elongation (Fig. 3C and data not shown). None of the truncated receptor ser/thr kinases affected bFGF induction of Xbra and muscle actin, demonstrating that inhibition by tXALK4 is specific to members of the TGFβ family such as BMP4 and activin, and that mesoderm induction by bFGF is unaffected.

Involvement of XALK4 in mesoderm formation in vivo

To determine whether XALK4 is involved in mesoderm formation in the context of the embryo, we injected 2 ng of either tXALK4 or ALK4-T206E RNA into the marginal region of one blastomere of 2-cell-stage albino embryos. The embryos were allowed to develop to early gastrula stages before they were fixed and assayed for Xbra expression by whole-mount in situ hybridization (Fig. 5A-F). In the control uninjected early gastrula, Xbra is expressed in a ring around the equator (Smith et al., 1991, Fig. 5A,D). Injection of tXALK4 RNA blocked Xbra expression in half the circumference of the embryo, resulting in a half ring expression pattern (Fig. 5B,E). This result parallels the observations made with tXActRIIB and demonstrates that truncated form of both activin receptors can block endogenous mesoderm formation. In contrast, the constitutively active receptor, ALK4-T206E, enhanced Xbra expression, expanding the pattern of staining into the animal pole in the injected half (Fig. 5C,F). Together, these results implicate XALK4 in mesoderm induction in vivo.

Axial defects in embryos overexpressing truncated XALK4

As XALK4 is involved in mesoderm formation both in animal cap explants and in vivo, we wanted to examine the conse-

quence of tXALK4 on the late phenotype of embryos. We injected 1 ng of the truncated XALK4 RNA into the marginal zone of each blastomere at the 4-cell stage. Over 85% of injected embryos displayed defects in axial structures; 50% had no discernible axis (Fig. 6; Table 1). These embryos had neither heads nor tails, and many developed into 'bubble embryos' (compare injected embryos with controls in Fig. 6). These embryos were very similar to those injected with truncated type II receptor (Hemmati-Brivanlou and Melton, 1992). In that case, the embryos had dramatically reduced mesodermal tissues. We therefore examined the effect of tXALK4 on mesodermal tissues by staining with antibodies against muscle (12/101) and notochord (Tor 70.1). As shown in Fig. 6, expression of both muscle (Fig. 6B) and notochord (Fig. 6C) markers was severely reduced in tXALK4-injected embryos. We also found that neural tissue, detected with the antibody against a neural-specific antigen, was reduced, although to a lesser extent (Fig. 6D). Interestingly, the same phenotype can be obtained if only the two dorsal, but not ventral, blastomeres are injected in the marginal zone or vegetal pole (data not shown). This observation parallels the one made for tXActRIIB (A. H. B, unpublished data) and provides, in agreement with previous observation, further evidence that signals responsible for the establishment of the dorsal axis are derived from the dorsal vegetal blastomeres.

Table 1. Axial defect in embryos injected with truncated XALK4 RNA

	No axial structures	Partial axial defects	Normal embryos	Total
No. of embryos	115	87	29	231
Percent	50	37	13	100

Embryos were injected with 1 ng truncated XALK4 RNA in the equatorial region of each blastomere at the 4-cell stage. Embryos were allowed to develop until control siblings reached tailbud stages, when the injected embryos were scored for phenotype and molecular markers. The 'no axial structures' phenotype corresponds to embryos with absent or drastically reduced axial mesodermal marker expression. These embryos were also called 'bubble embryos'. The embryos that showed 'partial axial defects' retained about 20-70% muscle or notochord compared with wild-type uninjected controls and were defective in both head and tail. This division into extreme and partial defects is arbitrary in that a range of defect is observed within each class.

To exclude the possibility that the loss of mesoderm is due to cell death caused by tXALK4 injection, we injected either 100 pg of nuclear-β-Gal alone or nuclear-β-Gal with tXALK4 in a single vegetal blastomere at the 8-cell stage. The comparison of the number of stained nuclei between the control embryos injected with β-Gal alone, versus embryos expressing

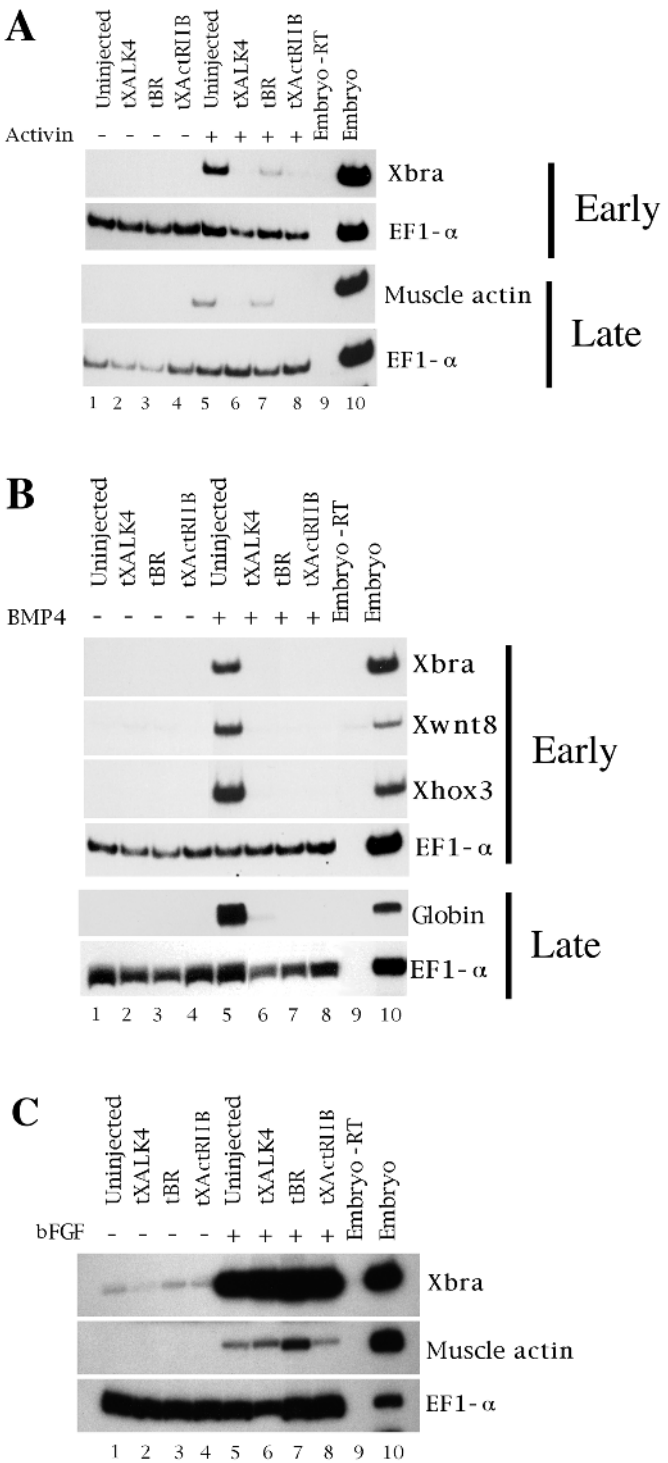

Fig. 3. Truncated XALK4 inhibits mesoderm induction by activin and BMP4. (A) tXALK4 inhibits mesoderm induction by activin. Embryos were injected with tXALK4, tXActRIIB or tBR RNAs at 2-cell stage and animal cap explants were dissected at blastula stages 8 to 9. The caps were either incubated alone (lanes 1 to 4), or with activin (lanes 5 to 8). Total RNA was assayed at either gastrula stage 11 (top panels, 'Early') or tailbud stage 28 (bottom panels, 'Late') by RT-PCR for expression of mesodermal-specific markers. Lanes 1 and 5, uninjected controls; lanes 2 and 6, injected with tXALK4; lanes 3 and 7, injected with tBR; lanes 4 and 8, injected with tXActRIIB. Lane 9 is a negative control without reverse transcriptase in the RT-PCR reaction, and lane 10 is a positive control using RNA extracted from whole embryos at either gastrula (top panel) or tailbud (lower panel) stages. (B) tXALK4 inhibits mesoderm induction by BMP4. BMP4 RNA was injected alone or coinjected with the truncated receptor RNAs. Explants were assayed as described above. Xwnt8 and Xhox3 are early markers of ventroposterior mesoderm. Globin is a marker of ventral mesoderm. (C) tXALK4 does not inhibit mesoderm induction by bFGF. Late blastula explants were incubated in the absence (lanes 1 to 4) or presence (lanes 5 to 8) of 100 ng/ml bFGF protein, and assayed when sibling controls reached late neurula stages as described above.

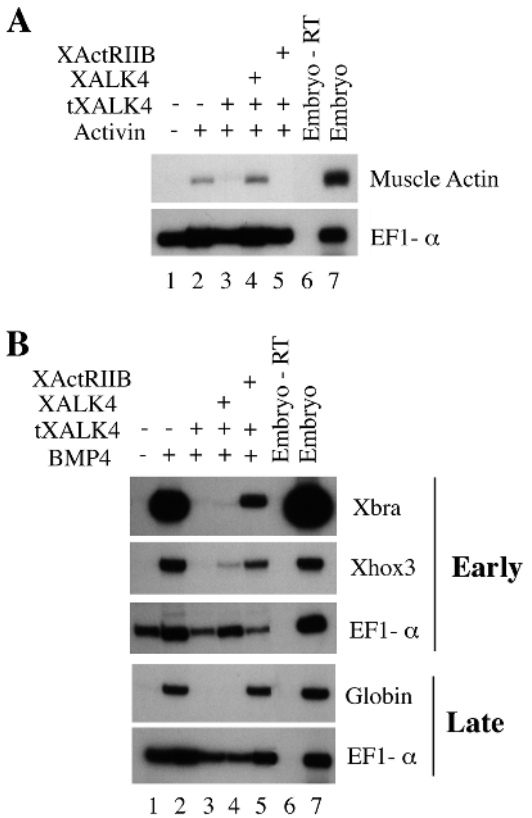

Fig. 4. Wild-type XALK4 receptor rescues tXALK4 blocked activin, but not BMP4, mesoderm induction. Ectodermal explants derived from embryos injected with tXALK4 RNA alone or coinjected with tXALK4 and XALK4 or XActRIIB RNAs were assayed for expression of mesodermal markers. Lane 1, uninjected control explants in buffer alone; lanes 2-5, explants incubated with activin (A) or injected with 0.5 ng BMP4 RNA (B). Lane 3 is animal caps injected with 2 ng tXALK4 RNA, lane 4 is caps coinjected with 2 ng tXALK4 and 0.5 ng XALK4 RNA, and lane 5 is caps coinjected with 2 ng tXALK4 and 0.5 ng XActRIIB RNA. (A) Induction of muscle actin by activin, assayed when control siblings reached tailbud stage 28, was rescued by coinjection of wild-type XALK4 RNA, but not by XActRIIB. (B) Expression of BMP4-induced early mesodermal marker Xbra at control sibling gastrula stage 11 and the late marker globin at stage 28 could not be rescued by co-expression of XALK4, but was rescued by wild-type XActRIIB receptor.

both β-Gal and tXALK4 revealed comparable number of cells and thus suggested that the injected cells did not die (data not shown). To control for the specificity of the tXALK phenotype, we coinjected tXALK4 with wild-type XALK4 RNA in the marginal zone of two dorsal blastomeres of 4-cell embryos. We found that as little as 100 pg of wild-type XALK4 RNA was sufficient to rescue embryos injected with 2 ng tXALK4 RNA and restore the body axis (Fig. 6E), thus demonstrating the specificity of the observed phenotype.

Truncated type I activin receptor does not neuralize ectodermal explants

When signaling was blocked by overexpression of the truncated type II activin receptor in animal cap explants, neural fate was revealed; moreover injection of tXActRIIB into the animal pole resulted in embryos with increased neural structures (cement glands and eyes; Hemmati-Brivanlou and Melton, 1994). These findings suggested that neural fate might be under inhibitory control, and that this inhibitory signaling could be blocked by tXActRIIB. Recent studies indicate that the endogenous neural inhibitor and epidermal inducer is BMP4 or perhaps a related BMP (Wilson and Hemmati-Brivanlou, 1995; Sasai et al., 1995; Hawley et al., 1995; Xu et al., 1995). Because truncated forms of both XALK4 and XActRIIB blocked mesoderm induction by activin and BMP4 (see above), we asked whether tXALK4 could also induce neural tissue in animal cap explants. We injected 2 ng of synthetic RNAs encoding tXALK4, tXActRIIB or tBR, into the animal poles of 2-cell-stage embryos. Expression of neural markers was assayed in animal caps when control sibling embryos reached mid-neurula stage by RT-PCR. Both NCAM (Kintner and Melton, 1987) and NRP-1 (Richter et al., 1990), general neural-specific markers, were induced by tBR or tXActRIIB as previously reported (Xu et al., 1995; Hemmati-Brivanlou and Melton, 1994, Fig. 7A). In contrast, animal caps from embryos injected with the tXALK4 receptor did not express these markers (Fig. 7A), although mesoderm induction by both activin and BMP4 was inhibited at the same dose of RNA (Fig. 3). Increasing the amount of tXALK4 RNA did not neuralize the animal caps (data not shown).

tXALK4 does not block epidermal induction by BMP4

BMP4 is a strong candidate to be the neural inhibitor and epidermal inducer revealed by the neuralizing effects of the truncated type II activin receptor (Wilson and Hemmati-Brivanlou, 1995). When animal caps are dissociated in Ca^{2+}/Mg^{2+}-free medium for 3 to 4 hours before reaggregation, they acquire a neural fate (Grunz and Tacke, 1989); addition of BMP4 to the dissociated cells restores epidermal differentiation (Wilson and Hemmati-Brivanlou, 1995). Expression of tXActRIIB blocks this epidermalizing activity, causing dissociated cells to express neural markers even in the presence of BMP4 (Wilson and Hemmati-Brivanlou, 1995 and Fig. 7B). Since both tXActRIIB and tXALK4 were able to block BMP4-induced mesoderm formation, and yet expression of tXALK4 did not induce neural markers, we asked whether tXALK4 could render ectodermal cells resistant to epidermalization by BMP4. Animal caps were dissociated at blastula stage and reaggregated either immediately or after 4 hours. As observed before, dissociation for 4 hours followed by reaggregation led to expression of the pan-neural marker NCAM and suppression of epidermal keratin (EK; Jonas et al., 1985; Fig. 7B); addition of BMP4 at 50 ng/ml to the dissociated cells eliminated expression of the neural marker and restored EK expression. Injection of 2 ng of tXActRIIB led to neuralization even in the presence of BMP4 (Fig. 7B, lane 9). In contrast, injection of 2 ng of tXALK4 RNA did not change cell response to BMP4: NCAM was repressed and EK induced in BMP4-treated samples from both uninjected and tXALK4-injected embryos. In controls carried out as part of the same experiment, tXALK4 prevented mesoderm induction in intact caps by both activin and BMP4, confirming that the RNA was functional (data not shown). Thus, although tXALK4 blocks induction of mesoderm by BMP4 (Fig. 3), it fails to inhibit its epidermal induction activity.

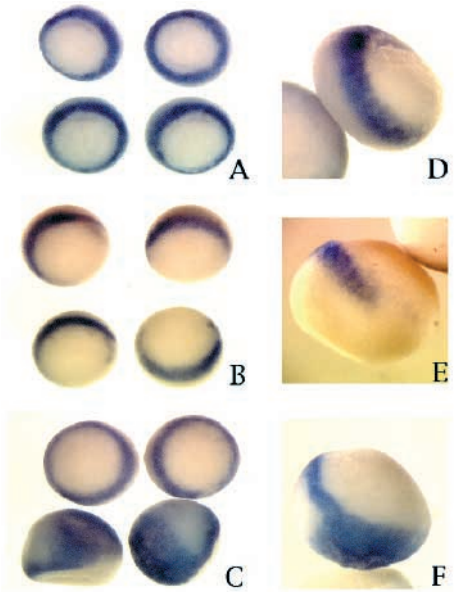

Fig. 5. Truncated XALK4 blocks mesoderm formation in vivo. Albino embryos were injected with 2 ng of tXALK4 or constitutively active ALK4-T206E RNAs in the marginal zone of one blastomere at the 2-cell stage. The embryos were fixed at gastrula stage 10 and whole-mount in situ hybridization was performed using an Xbra antisense probe to assess the effect of these constructs on mesoderm formation in vivo. (A,D) Uninjected control embryos, showing an intact ring of Xbra expression during early gastrula stages. (B,E) Embryos injected with tXALK4 RNA. Consistent with tXALK4 inhibition of mesoderm induction, Xbra expression is only detected in half of the marginal zone. (C,F) Embryos injected with ALK4-T206E RNA. These embryos display an expanded Xbra expression that includes the marginal zone and the animal cap on one side of the embryo.

DISCUSSION

In this paper, we report the characterization of a *Xenopus* type I activin receptor, XALK4, and an analysis of its function in early frog development. We find that, as in the case of the previously described type II activin receptor XActRIIB, a truncated form of XALK4 can block mesoderm induction by activin or related factors. Furthermore, an active form of ALK4 mimics activin-induced mesoderm formation. These results are consistent with cell culture data implicating this protein as a receptor that mediates activin effects. Unlike tXActRIIB, however, truncated XALK4 does not induce neural tissue. This paradox is explained by our finding that truncated XALK4 can block BMP4-induced expression of mesodermal markers, but not BMP4-induced expression of epidermal markers and inhibition of neuralization. In contrast, the dominant negative form of XActRIIB blocks both activities of BMP4. Thus the type I and type II activin receptors are involved in overlapping but distinct sets of embryonic signaling events.

XALK4 and mesoderm induction

Truncated XALK4 blocks mesoderm induction by activin and BMP4 in animal cap explants, as has been shown for the

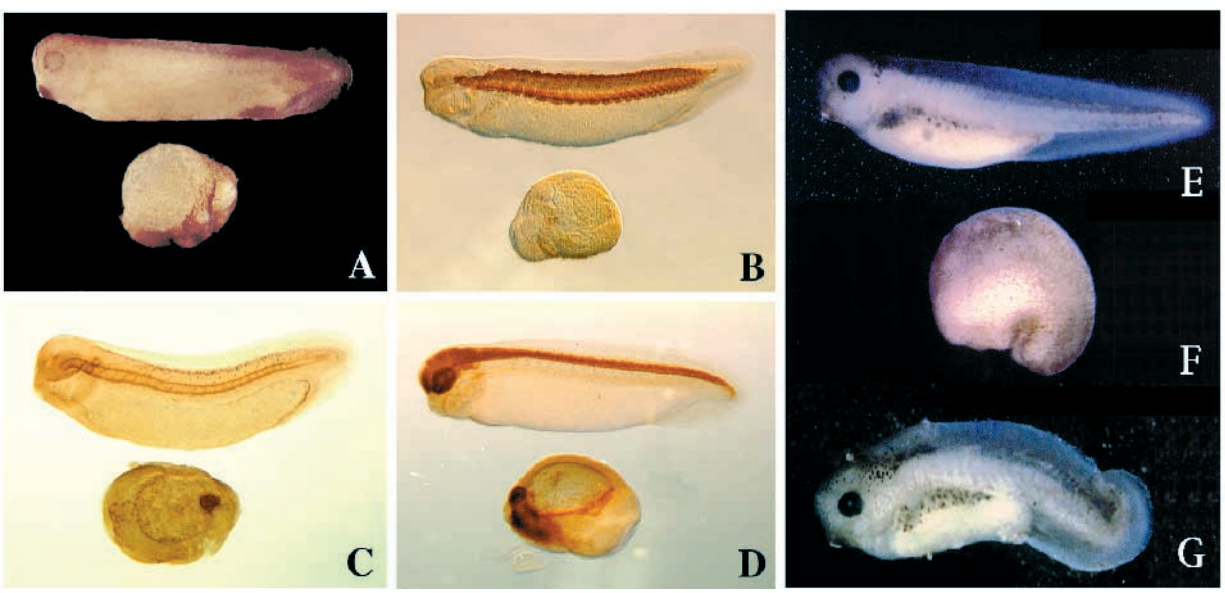

Fig. 6. Expression of tXALK4 in embryos leads to the elimination of the body axis and a severe reduction of mesodermal tissue. (A-D) Embryos at the top of each panels are uninjected controls, while the embryos at the bottom of each panels are injected with tXALK4. (A) Phenotype of embryos injected with 1 ng of tXALK4 RNA in the marginal zone of all four blastomeres at the 4-cell stage, display no obvious axis and resemble the 'bubble' phenotype described for tXActRIIB. (B-D) Analysis of tissue-specific molecular markers by whole-mount immunohistochemistry. (B) Staining with a muscle-specific antibody shows reduced muscle tissue in injected embryo. (C) Staining for a notochord antigen shows the same reduction in the injected embryo as above. (D) Staining for a neural-specific antigen demonstrates that neural tissue, though not reduced as severely, is disorganized. (E-G) The phenotype imposed by tXALK4 in the embryo can be rescued by coinjection of wild-type activin type I receptor. (E) Control uninjected tadpole. (F) Phenotype of embryo injected with 2 ng of tXALK4 into the two dorsal blastomeres in the marginal zone. (G) Coinjection of tXALK4 with 100 pg of the wild-type XALK4 RNA can rescue the phenotype and restore body axis in embryos.

truncated type II activin receptor tXActRIIB (Hemmati-Brivanlou and Melton, 1992; Hemmati-Brivanlou and Thomsen, 1995). Expression of the truncated form of either receptor strongly inhibits mesoderm formation in whole embryos as well, resulting in severely defective embryos which lack both heads and tails. Analysis of molecular markers reveals that expression of early genes, such as *Xbra*, is completely inhibited while development of late mesodermal tissues, such as muscle and notochord, is substantially reduced in these embryos. Moreover, a constitutively active form of ALK4 can induce mesoderm in *Xenopus* animal caps in a ligand-independent manner. These results strongly suggest that XALK4 plays an essential role in endogenous mesoderm induction, perhaps in conjunction with the type II receptor XActRIIB.

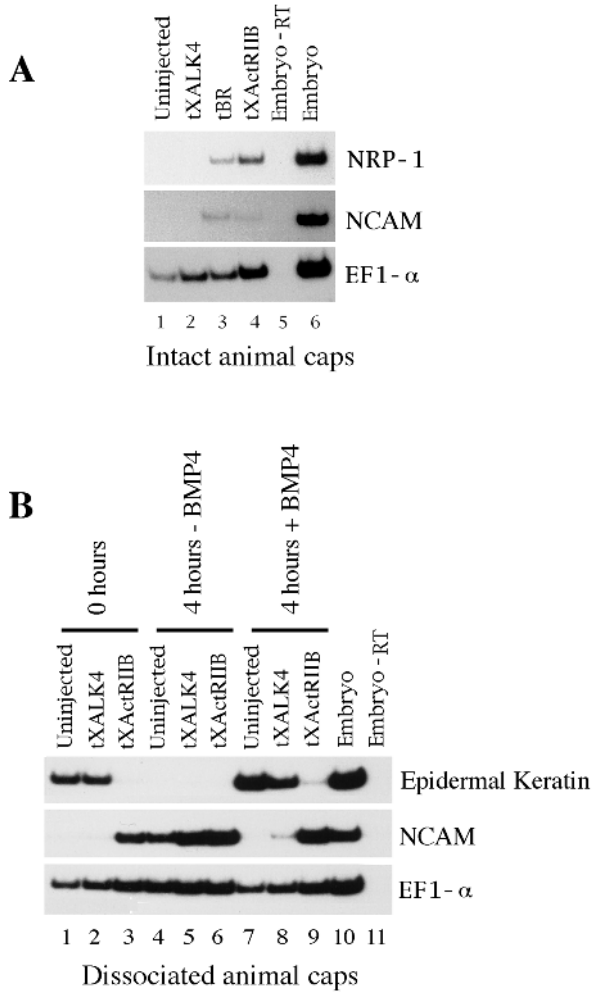

Fig. 7. tXALK4 does not induce neuralization in intact explants and does not inhibit epidermal induction by BMP4. (A) Ectodermal explants derived from embryos injected with tXALK4, tXActRIIB or tBR RNAs were assayed by RT-PCR for the expression of ectodermal-specific markers when sibling stages reached late neurula (stage 20). NRP-1 and NCAM are neural-specific markers. Lane 1 is uninjected animal cap control, lanes 2 to 4 are caps injected with RNA encoding tXALK4, tBR and tXActRIIB, respectively. Lane 5 is a negative control without reverse transcriptase, and lane 6 is a positive control with RNA extracted from whole embryos. tXALK4 does not neuralize intact ectodermal explants. (B) Ectodermal explants derived from embryos injected with tXALK4 or tXActRIIB were dissociated in Ca^{2+}/Mg^{2+}-free medium. They were reaggregated either immediately (lanes 1 to 3) or following 4 hours dissociation (lanes 4 to 9). As shown previously (Wilson and Hemmati-Brivanlou, 1995), 4 hours of dissociation changed animal caps from epidermal to a neural fate (lanes 4-6). In lanes 7 to 9, BMP4 protein was added at 50 ng/ml concentration during dissociated culture. Unlike tXActRIIB, expression of tXALK cannot block BMP4-dependent epidermal induction. Lanes 1, 4 and 7 are uninjected controls; lanes 2, 5 and 8 are embryos injected with tXALK4 RNA; lanes 3, 6 and 9 are injected with tXActRIIB RNA. Total RNA was extracted from explants and assayed by RT-PCR when control siblings reached late neurula stages 17 to 18.

Mesoderm induction by BMP4 protein requires high levels of ligand, approaching 1 μg/ml, or about 40 nM. On the contrary, unpublished data demonstrate that BMP4/BMP7 heterodimers can induce mesoderm at much lower (picomolar) concentrations (A. Suzuki and N. Ueno, personal communication). It is thus unclear whether BMP4 acts alone or with another factor to induce mesoderm in the embryo, or acts instead to ventralize mesoderm induced by other factors such as activin. Since ventralization is a likely role for BMP4 in vivo, it would be interesting to know if this activity could be blocked by tXALK4. We are currently addressing this issue. In animal cap assays, truncated XALK4 blocks mesoderm induction by both activin and BMP4. However, coexpression of wild-type XALK4 with the truncated form does not rescue mesoderm induction by BMP4, although induction by activin is restored (Fig. 4). For this reason, we believe it is unlikely that ALK4 is directly involved in BMP4 induction of mesoderm. The truncated mutant may block BMP4 indirectly, through interaction with another receptor (see below). This notion is also supported by our data that type II activin receptor, when coexpressed with tXALK4, can rescue mesoderm induction by BMP4.

Neural specification and the induction of epidermis: XALK4 and BMP4

Although the type I and type II activin receptors behave very similarly in assays of mesoderm induction, they differ in one crucial aspect. While truncated XActRIIB provokes formation of neural tissue in animal cap explants in the absence of mesoderm, truncated XALK4 does not. Several recent studies suggest that BMP4, rather than activin, is likely to be responsible for neural inhibition and epidermal specification in vertebrate embryos (Wilson and Hemmati-Brivanlou, 1995; Sasai et al., 1995; Hawley et al., 1995; Xu et al., 1995), and suggest that truncated XActRIIB probably neuralizes by blocking BMP4 signaling. We have asked therefore if the difference between type I and type II activin receptors with respect to neuralization is due to a difference in the ability to block BMP4

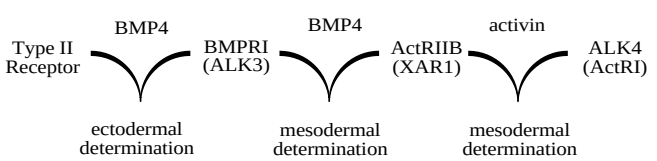

Fig. 8. Working model for differential involvement of XALK4 and XActRIIB in signal transduction by activin and BMP4. Different receptor complexes are involved in mesoderm and epidermis induction by activin and BMP4. For details, see Discussion.

signaling. We have shown that both truncated receptors can inhibit mesoderm induction by BMP4; however, in dissociated animal cap cells only tXActRIIB, and not tXALK4, can block epidermal induction by BMP4. Although the rescue data imply that XALK4 is not involved in either mesoderm or epidermis induction by BMP4, the differential effect of the truncated form of this receptor allows us to infer that different receptor complexes mediate the two activities.

To provide a framework to consider these data, we propose a model in which different ligands can induce distinct cellular responses through the formation of different receptor complexes (Fig. 8). Since we still know so little about the affinities of these receptors for each other and for the various ligands, and since there are a number of BMPs and receptors whose functions remain to be analyzed in the embryo, we limit this discussion to the molecules analyzed above. Under our current thinking, activin may signal through a receptor complex containing a type II receptor (ActRIIB) and a type I receptor (ALK4). A truncated form of either receptor subunit will block signaling and inhibit mesoderm induction by this ligand. On the other hand, BMP4 may mediate mesoderm induction by a BMPRI (ALK3) and ActRIIB complex, while specifying epidermal fate through BMPRI and another type II receptor (possibly a homologue of human BMPRII, Liu et al., 1995). In this case, a truncated BMPRI would block all BMP responses (Graff et al., 1994). A truncated ActRIIB would block BMP4-induced mesoderm formation directly and block epidermal formation indirectly by interacting with BMPRI and titrating it away from a still functional BMP type II receptor. A truncated ALK4, which would not complex with the type II BMP receptor, would not affect BMP4-mediated epidermal induction, although it would block BMP mesoderm induction by competing for ActRIIB. According to this model, coexpression of wild-type ALK4 should rescue mesoderm induction by activin in the presence of truncated ALK4 by restoring the functional receptor complex for activin signaling. However, BMP4-mediated mesoderm formation should not be rescued by expression of ALK4. This is exactly what we have observed (Fig. 4). In addition, mesoderm induction by BMP4 is rescued from tXALK4 by coexpression of XActRIIB, which further supports this model. Clearly the specific interactions will be determined by the relative affinities of the various components for each other. Therefore definitive conclusions must await further data, but we believe our model can serve as a useful starting point for design of experiments to probe the system in greater detail. A feature of the model is consistent with the possibility that a single type I receptor may mediate different intracellular events in combination with different type II receptors. Although a current hypothesis for receptor serine/threonine kinase signaling is that type I receptors are the primary signal transducers (Wrana et al., 1992, 1994; Attisano et al., 1996), a role for type II receptors in determining the nature of the downstream signal has also been proposed (Chen et al., 1993).

In summary, we have cloned a *Xenopus* type I activin receptor, XALK4, and studied its expression and function in early development. Using dominant negative and constitutively active mutants, we show that this receptor, like the type II receptor XActRIIB, is involved in mesoderm induction. In contrast, XALK4 is apparently not involved in the specification of epidermis and thus the control of neural fate. These experiments with dominant-negative receptors, together with the data on rescue with wild-type receptors, allow us to propose a model for how different receptor complexes mediate different biological activities of activin and BMP4.

We would like to thank Dr P. Klein (University of Pennsylvania) for oocyte cDNA library, Dr W. Harris (UC San Diego) for the neural-specific antibody 6F11 and Genetic Institute for purified recombinant BMP4 protein. We also thank Drs A. Suzuki, N. Ueno and J. Graff for communication of unpublished data. We are grateful to Drs C. Altman and W. G. Cox for critical reading of the manuscript, G. Lagna for stimulating discussions and S. Rahman for technical assistance. L. S. M. would like to acknowledge Dr Kohei Miyazono for cDNA encoding human ALK4. This work was supported by a C. H. Li Memorial Fellowship (to C. C.), The Rockefeller University and the Horace W. Goldsmith foundation (to P. W. and A. H. B.), NIH grant 1R01 HD 32105-01 (to A. H. B.), NIH grant GM-50416 (to L. S. M.) and funds from the Searle Scholars Program/The Chicago Community Trust (to both L. S. M. and A. H. B.).

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(Accepted 18 November 1996)