The EMBO Journal Vol.17 No.4 pp.1019–1028, 1998

Role of TAK1 and TAB1 in BMP signaling in early *Xenopus* development

Hiroshi Shibuya[1,2,3], Hiroshi Iwata[4], Norihisa Masuyama[5,6], Yukiko Gotoh[5], Kyoko Yamaguchi[7], Kenji Irie[7], Kunihiro Matsumoto[7], Eisuke Nishida[5,6] and Naoto Ueno[1]

[1]Division of Morphogenesis, Department of Developmental Biology, National Institute for Basic Biology, Okazaki 444, [2]Precursory Research for Embryonic Science and Technology, Japan Science and Technology Corporation, Hikari-dai, Kyoto 619-02, [4]Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, [5]Department of Genetics and Molecular Biology, Institute for Virus Research and [6]Department of Biophysics, Graduate School of Science, Kyoto University, Kitashirakawa-Oiwake, Sakyo-ku, Kyoto 606-01 and [7]Department of Molecular Biology, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan

[3]Corresponding author
e-mail: shibuya@nibb.ac.jp

Transforming growth factor-β (TGF-β) superfamily members elicit signals through stimulation of serine/threonine kinase receptors. Recent studies of this signaling pathway have identified two types of novel mediating molecules, the Smads and TGF-β activated kinase 1 (TAK1). Smads were shown to mimic the effects of bone morphogenetic protein (BMP), activin and TGF-β. TAK1 and TAB1 were identified as a MAPKKK and its activator, respectively, which might be involved in the up-regulation of TGF-β superfamily-induced gene expression, but their biological role is poorly understood. Here, we have examined the role of TAK1 and TAB1 in the dorsoventral patterning of early *Xenopus* embryos. Ectopic expression of *Xenopus* TAK1 (xTAK1) in early embryos induced cell death. Interestingly, however, concomitant overexpression of *bcl-2* with the activated form of xTAK1 or both xTAK1 and xTAB1 in dorsal blastomeres not only rescued the cells but also caused the ventralization of the embryos. In addition, a kinase-negative form of xTAK1 (xTAK1KN) which is known to inhibit endogenous signaling could partially rescue phenotypes generated by the expression of a constitutively active BMP-2/4 type IA receptor (BMPR-IA). Moreover, xTAK1KN could block the expression of ventral mesoderm marker genes induced by Smad1 or 5. These results thus suggest that xTAK1 and xTAB1 function in the BMP signal transduction pathway in *Xenopus* embryos in a cooperative manner.

Keywords: bone morphogenetic protein/pattern formation/signal transduction/TAB1/TAK1

Introduction

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily, which have been implicated in the patterning of the mesoderm and ectoderm of *Xenopus* embryos. Both BMP-2 and BMP-4 are capable of inducing ventral mesoderm and respecifying prospective dorsal mesoderm to differentiate into ventral tissues in *Xenopus* embryos (Dale *et al.*, 1992; Jones *et al.*, 1992; Clement *et al.*, 1995). TGF-β elicits signals through a heteromeric complex consisting of the transmembrane type I and type II TGF-β receptors, which contain a cytoplasmic ser/thr-specific kinase domain (Wrana *et al.*, 1994). Constitutively active type I receptors can signal biological responses in the absence of either ligand, type II receptors or both (Wieser *et al.*, 1995). Three structurally related type I receptors, BMPR-IA [also known as ALK3 (ten Dijke *et al.*, 1993)], BMPR-IB [also known as ALK6 (ten Dijke *et al.*, 1994)] and ActRIA [also known as ALK2 (ten Dijke *et al.*, 1993)], and one type II receptor, BMPR-II (Liu *et al.*, 1995; Nohno *et al.*, 1995), have been identified as the receptors for BMPs. It is known that a truncated form of BMPR-IA lacking its intracellular ser/thr kinase domain acts as a dominant-negative inhibitor of the cellular responses to BMP-2 and BMP-4 ligands in *Xenopus* embryos (Graff *et al.*, 1994; Suzuki *et al.*, 1994). Overexpression of this dominant-negative BMPR-IA in the ventral side of *Xenopus* embryos causes prospective ventral mesoderm to differentiate into dorsal mesoderm, and induces the expression of a neural marker, neural cell adhesion molecule (N-CAM), in the ectoderm.

Recently, several molecules which are associated with TGF-β family receptors have been cloned. Several groups have reported that the immunophilin FKBP12 interacts with TGF-β family type I receptors (Wang *et al.*, 1994, 1996b). FKBP12 binding to type I receptors may function to suppress spurious signaling resulting from the innate tendency of TβR-I and TβR-II to interact with one another (Chen *et al.*, 1997). The α subunit of farnesyl-protein transferase (FT-α) was also isolated as a TβR-I-associated molecule (Kawabata *et al.*, 1995; Wang *et al.*, 1996a). Farnesyltransferase is known to play a critical role in the activation of $p21^{RAS}$ by attaching a farnesyl group, and aiding in its membrane association. Although FT-α was shown to be phosphorylated by TβR-I, the functional role of this modification is not clear.

More recently, a group of proteins, the Smads, have been identified as important components of the TGF-β superfamily signal transduction pathway in a variety of species (Baker and Harland, 1996; Eppert *et al.*, 1996; Graff *et al.*, 1996; Hoodless *et al.*, 1996; F.Liu *et al.*, 1996; Thomsen, 1996). Smad1 has been shown to mimic the effects of BMP-2/4 in *Xenopus* as well as in mammalian cells. Smad2 and its close isoform Smad3 mediate signaling elicited by TGF-β or activin. Smad4/DPC4, originally isolated as a tumor-suppressor gene on chromosome 18q21 (Hahn *et al.*, 1996), cooperates with Smad1, Smad2

and Smad3 to act as a common mediator of signaling by members of the TGF-β superfamily (Lagna *et al.*, 1996). Smad5 is a closely related gene product of Smad1 (Riggins *et al.*, 1996) and mediates a BMP-like signal in *Xenopus* (Suzuki *et al.*, 1997). Although Smads have been shown to be critical for TGF-β superfamily signaling, it is not clear whether the involvement of other factors is required for signal transduction.

Previously, we identified a novel mouse protein kinase designated TAK1 (TGF-β activated kinase 1) that functions as a MAPKKK (Yamaguchi *et al.*, 1995). TAK1 was found to be involved in at least one TGF-β-induced gene response, and its kinase activity was stimulated in response to TGF-β1 or BMP-4. Furthermore, we identified a human protein (TAB1) that interacts with TAK1 using a yeast interaction screen, and provided data indicating that TAB1 functions as an activator for TAK1 in TGF-β signal transduction (Shibuya *et al.*, 1996).

We report here that *Xenopus* TAK1 (xTAK1) and xTAB1 induce ventral mesoderm. A kinase-negative form of xTAK1 (xTAK1KN) reverts the ventralization caused by constitutively active BMPR-IA and Smad1/5. These results suggest that xTAK1 and xTAB1 are important for the BMP signaling pathways involved in mesoderm induction and patterning in early *Xenopus* embryo development.

Results

Isolation of the Xenopus TAK1 and TAB1 cDNAs and their expression patterns in Xenopus embryos

To analyze the function of TAK1 and TAB1 in embryonic development, cDNAs encoding *Xenopus* TAK1 and TAB1 (xTAK1 and xTAB1) were isolated from an oocyte cDNA library under low stringency conditions using the mouse TAK1 (mTAK1) and human TAB1 (hTAB1) cDNAs as probes, respectively. The longest xTAK1 and xTAB1 cDNAs recovered were ~2.3 and 2.5 kb, respectively. Sequence analysis of the longest cDNAs for xTAK1 and xTAB1 each revealed a single open reading frame. The nucleotide sequences were predicted to encode a protein of 616 and 498 amino acids with a molecular mass of 68 and 54 kDa for TAK1 and TAB1, respectively. The amino acid sequence comparison between xTAK1 and mTAK1, or xTAB1 and hTAB1, shown in Figure 1, reveals that they are 76 or 70% identical, respectively.

The xTAK1 and xTAB1 transcripts are 3.0 and 3.2 kb in size, respectively. Northern blot analysis of mRNA obtained during early embryogenesis revealed that these transcripts are stored maternally and expressed throughout development (Figure 2A). The xTAK1 and xTAB1 mRNAs are not localized to any specific region in the early gastrula stages, as determined by whole-mount *in situ* hybridization (Figure 2B), and this ubiquitous expression was confirmed by RT–PCR amplification of RNA from dissected embryonic regions (data not shown). However, whole-mount *in situ* hybridization of embryos showed that xTAK1 and xTAB1 transcripts become localized to the nervous system at the end of neurulation, and are restricted to the central nervous system, eye and head neural crest cell populations by the early tadpole stages (Figure 2C). xTAK1 and xTAB1 transcripts are roughly co-localized, supporting the previous data indicating that these molecules function together in TGF-β family signaling.

xTAK1 induces ventral mesoderm in Xenopus embryos

To investigate the possible role of xTAK1 in embryonic development, xTAK1 mRNA was injected into the ventral or dorsal sides of *Xenopus* four-cell stage embryos. Injection of 0.2–1 ng of xTAK1 mRNA caused almost all of embryos to die at stage 10.5 as revealed by staining with acridine orange (Figure 3A). To confirm whether the cell death observed is programed cell death, we utilized the TUNEL method (Gavrieli *et al.*, 1992), which revealed that cells were dying by apoptosis, as shown in Figure 3B. Apoptosis induced by xTAK1 was significantly inhibited by co-injection of human *bcl-2* mRNA, resulting in normal embryos or those with only minor defects in head formation (Figure 4A). These results suggest that xTAK1, when overexpressed, can induce apoptosis.

It has been shown previously that expression of an activated form of mammalian TAK1 (TAK1ΔN), a truncation lacking the NH_2-terminal 20 amino acids, is able to enhance transcription from a reporter gene driven by the plasminogen activator inhibitor-1 (PAI-1) gene promoter (Yamaguchi *et al.*, 1995). We therefore generated an mRNA encoding a similar truncated form of xTAK1 (xTAK1ΔN) and examined its effects in embryos. The injection of the xTAK1ΔN mRNA also induced cell death, but at doses lower than that required for the intact xTAK1 mRNA (data not shown). When xTAK1ΔN mRNA and human *bcl-2* mRNA were co-injected into the dorsal marginal zone of four-cell embryos, cell death was inhibited and the embryos were significantly ventralized (Figure 4A, bottom). On the other hand, injection of both xTAK1ΔN and *bcl-2* mRNAs into the ventral side had no effect on the development of the embryos (data not shown). Furthermore, injection of xTAK1ΔN and *bcl-2* mRNA induced ventral posterior mesoderm in animal caps. As shown in Figure 4B, ventral (Xwnt-8 and globin) and posterior (Xhox-3) mesoderm markers, but not a dorsal mesoderm marker (α-actin), were induced in embryos co-injected with xTAK1ΔN and *bcl-2*. No mesodermal marker gene was induced by *bcl-2* alone. Moreover, the injection of the cDNA encoding the activated form of another MAPKKK, MEKK1 (an activator of MKK3 and 4), along with *bcl-2* did not induce any mesodermal marker genes (Figure 4B, lane 5), although injection of the activated form of MEKK1 mRNA alone caused apoptosis (data not shown). These results indicate that the induction of ventral mesoderm appears to be an event specifically induced by TAK1ΔN. The pattern of mesoderm induction by xTAK1 is similar to that induced by BMP-4, suggesting that TAK1 may function in the BMP-4 signaling pathway.

xTAB1 enhances xTAK1-inducing mesodermal patterning

It has been shown previously that expression of mammalian TAK1 with TAB1 is able to enhance transcription from a reporter gene driven by the PAI-1 gene promoter (Shibuya *et al.*, 1996). We next examined the effects of expression of both xTAK1 and xTAB1 in embryos. At lower doses of xTAK1 (50 pg) and xTAB1 (1 ng), the injection of both mRNAs with or without *bcl-2* mRNA into the dorsal marginal zone resulted in no change from the normal phenotypes (Figure 5A, data not shown). At higher doses of xTAK1 (100 pg) and xTAB1 (1 ng), the

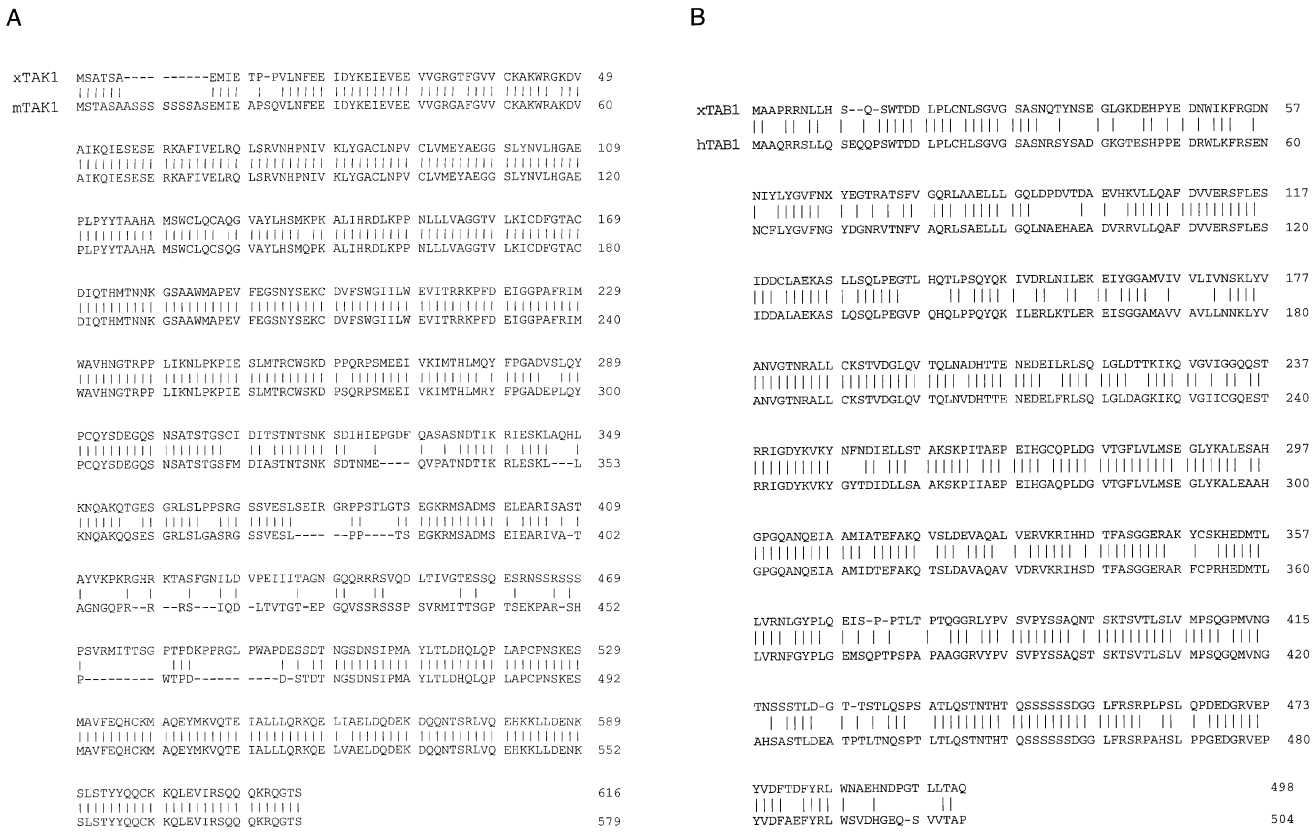

Fig. 1. Protein sequence comparison of *Xenopus* TAK1 and TAB1 with mammalian homologs. (**A**) xTAK1 sequence and comparison with mTAK1. Alignment of the predicted amino acid sequences of *Xenopus* and mouse TAK1. The xTAK1 cDNA encodes a predicted protein of 616 amino acids. The xTAK1 protein is 76% identical to mTAK1 at the amino acid level. Residues conserved in both sequences are indicated by vertical bars, and the amino acid residues are numbered on the right. Note the high degree of conservation in the catalytic (98%) and C-terminal (99%) portions of the proteins. The GenBank accession No. for xTAK1 is U92030. (**B**) xTAB1 sequences and comparison with human TAB1. The xTAB1 protein is 70% identical to hTAB1 at the amino acid level. The GenBank accession No. for xTAB1 is U92031.

injection of both mRNAs also induced cell death (data not shown). When xTAK1 (100 pg) and xTAB1 (1 ng) mRNAs and human *bcl-2* mRNA were co-injected into the dorsal marginal zone of four-cell embryos, cell death was inhibited and the embryos were significantly ventralized (Figure 5A). At the same doses, the injection of xTAK1 (100 pg) and *bcl-2* mRNAs induced only minor defects in head formation as indicated above, suggesting that TAB1 enhances TAK1 activity. Injection of these mRNAs into the ventral side had no effect on the development of the embryos. Injection of xTAK1, xTAB1 and *bcl-2* mRNAs also induced ventral posterior mesoderm in animal caps. As shown in Figure 5B, ventral (Xwnt-8 and globin), posterior (Xhox-3) and pan- (Xbra) mesoderm markers, but not dorsal mesoderm markers (goosecoid and α-actin), were induced in embryos injected with xTAK1, TAB1 and *bcl-2*. Interestingly, the injection of xTAK1 with *bcl-2* mRNAs also induced only the Xwnt-8 and globin marker genes, consistent with the observed weak defect in phenotype. These results suggest that both TAK1 and TAB1 may function in the BMP-4 signaling pathway.

xTAK1 functions downstream of the BMP-2/4 receptor

A mutant of BMPR-IA, in which glutamine is replaced by aspartic acid at 233, is constitutively active, independent of binding by BMP-2/4 ligands (Hoodless *et al.*, 1996). We found that overexpression of this constitutively active BMP receptor in the dorsal side of *Xenopus* embryos caused ventralization (Figure 6A), mimicking the effect of BMP-2/4. If xTAK1 functions downstream of the BMP type I receptor, inhibition of xTAK1 function is expected to rescue the phenotypes generated by expression of the constitutively active BMP type I receptor. Overexpression of TAK1KN has been shown previously to inhibit TGF-β-stimulated transcription from a PAI-1 gene promoter (Yamaguchi *et al.*, 1995). We thus examined the effect of overexpressing this mutant, in which Lys52 of the ATP-binding site of the xTAK1 catalytic domain has been mutated to Arg, on the ventralization of *Xenopus* embryos caused by the expression of the constitutively active form of the BMP-2/4 type I receptor. We co-injected xTAK1KN mRNA along with an mRNA encoding the constitutively active BMPR-IA mRNA. Expression of xTAK1KN partially reverted the ventralization (Figure 6A) and reversed the inhibition of anterior neural (Xotx-2) and ectodermal (XAG-1) marker expression (Figure 6B) caused by dorsal expression of the constitutively active BMPR-IA.

We next examined whether the dominant-negative effect of xTAK1KN is specific to ventral or dorsal signaling. We found that expression of xTAK1KN inhibited the induction of ventral mesoderm markers otherwise caused by expression of xTAK1 and TAB1, BMP-4 or Smad1. In contrast, expression of xTAK1KN did not markedly reverse the induction of dorsalization markers by expression of activin, the constitutively active activin type IB

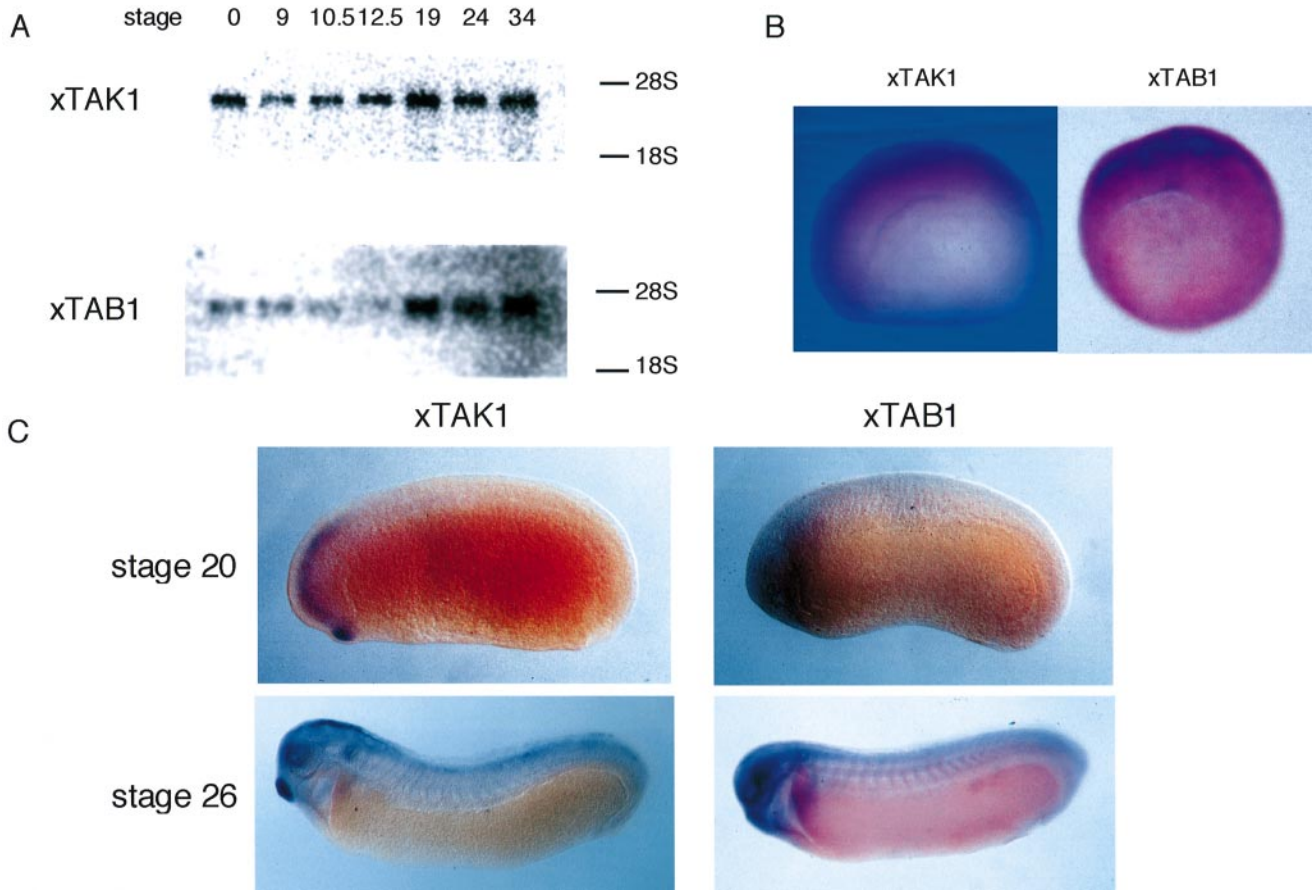

Fig. 2. Developmental expression of *Xenopus* TAK1 and TAB1. (**A**) Northern blot of total embryonic RNAs shows that xTAK1 and xTAB1 are maternal and expressed at all stages of early development. The mRNAs for xTAK1 and xTAB1 are 3.0 and 3.2 kb long, respectively. Lane numbers correspond to developmental stages: 0, egg; 9, blastula; 10.5, 12.5, gastrula; 19, neurula; 24, tailbud tadpole; 34, swimming tadpole. (**B**) Expression of xTAK1 and xTAB1 genes in the early gastrula stages. Whole-mount *in situ* hybridization shows that the xTAK1 and xTAB1 transcripts are uniformly distributed in the early gastrula stages. Views of the vegetal pole are shown and dorsal is up. (**C**) Whole-mount *in situ* hybridization revealed the localization of the xTAK1 and xTAB1 transcripts. At the tailbud tadpole stage, xTAK1 and xTAB1 expression is high in the central nervous system and head.

receptor [CA-ActRIB; ALK4 (ten Dijke *et al.*, 1993)] or Smad2. In addition, xTAK1KN barely inhibited the induction of a pan-mesoderm marker (Xbra) induced by basic fibroblast growth factor (FGF) (Figure 7). Injection of activin, CA-ActRIB or Smad2 mRNAs is known to induce dorsal mesoderm in animal caps (Baker and Harland, 1996; Graff *et al.*, 1996; Chang *et al.*, 1997), and we observed that each induced expression of the mesoderm-specific genes, goosecoid and Xbra (Figure 7). We further observed that co-injection of xTAK1KN did not reverse the induction of these markers by activin, CA-ActRIB or Smad2 (Figure 7B). These results suggest that xTAK1 may function in early *Xenopus* embryos in a pathway downstream of the BMP-2/4 receptor, but not downstream of the activin receptor.

Cooperation of xTAK1 and Smad

Smad1 mediates signaling by BMP-2/4 in mammalian cells or *Xenopus* embryos (Graff *et al.*, 1996; Hoodless *et al.*, 1996; F.Liu *et al.*, 1996; Thomsen, 1996). Smad5, which is closely related to Smad1 (Riggins *et al.*, 1996), also mediates a BMP-like signal in *Xenopus* (Suzuki *et al.*, 1997). Injection of Smad1 or Smad5 mRNA into the dorsal marginal zone of four-cell embryos resulted in their significant ventralization (data not shown), suggesting that Smad5 also induces ventral mesoderm in *Xenopus* embryo. To examine the relationship between TAK1 and Smad1 or Smad5, we co-injected xTAK1KN mRNA together with an mRNA encoding either Smad1 or Smad5. Co-injection of xTAK1KN mRNA reversed the expression of ventral mesoderm markers (Xwnt-8, Xvent-1 and globin) induced by Smad1 or Smad5 (Figure 8), whereas it had no effect on dorsal markers (goosecoid, Xbra and actin). These results suggest that normal regulation of the BMP signaling pathway may require the cooperation of TAK1 with Smad1 or Smad5.

xTAK1 inhibits neural differentiation

To elucidate the role of xTAK1 in the inhibition of neural differentiation by BMP-2/4, the mRNA encoding the dominant-negative BMPR-IA described above, or noggin, a factor which induces neural differentiation by binding to BMP-4, was injected with or without the xTAK1, xTAB1 and/or *bcl-2* mRNAs. Figure 9A shows that xTAK1 and xTAB1 can suppress neural differentiation in animal caps normally triggered by the expression of dominant-negative BMPR-IA or noggin. Expression of dominant-negative BMPR-IA or noggin alone induced N-CAM, whereas expression of xTAK1 and xTAB1 together with *bcl-2* suppressed this induction. Moreover, expression of

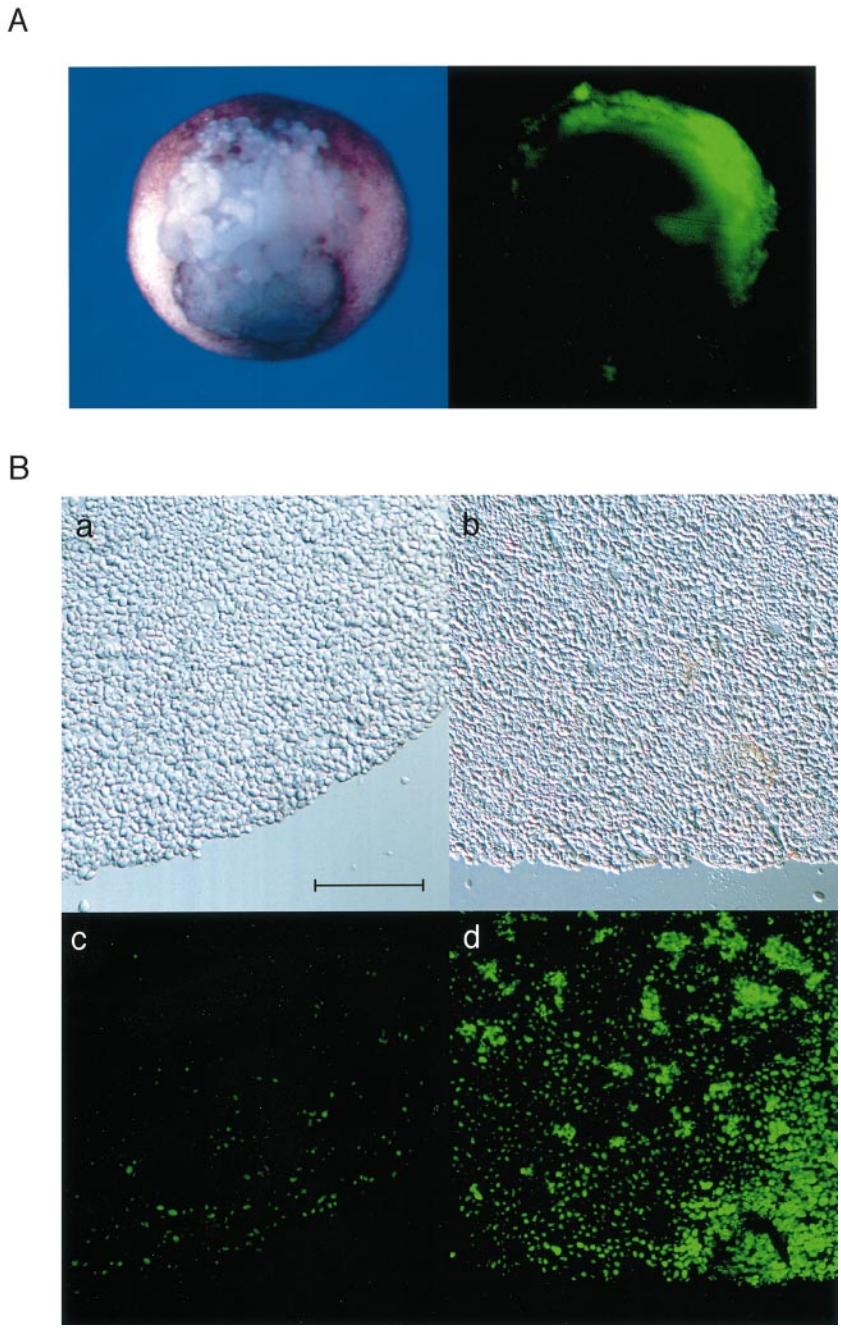

Fig. 3. xTAK1 induces apoptosis. (**A**) Detection of xTAK1-induced cell death in early gastrula stages. Dorsal injection of xTAK1 mRNA (500 pg) in the four-cell stage caused the cell death at early gastrula stage. Left side: whole embryo without staining (vegetal view with dorsal on the top). Right side: whole embryo with acridine orange staining (lateral view with dorsal on the top). At least 10 embryos were examined and the data are representative of three separate experiments. (**B**) Detection of apoptotic cells by the TUNEL method in *Xenopus* embryos. Synthetic mRNAs containing xTAK1 (500 pg) were injected into the equatorial region of blastomeres at the two-cell stage. Paraffin sections at stage 9 were prepared and the TUNEL method for detecting fragmented DNAs diagnostic of apoptosis was performed. (a and b) Phase contrast micrographs; (c and d) fluorescence micrographs. Yellow–green signal indicates labeled apoptotic cells in sections of the control embryo (c) and the xTAK1 mRNA-injected embryo (d) in the early gastrula stage. The signal in the control embryo shows the background level. The scale bar indicates 10 μm. At least 10 embryos were examined in an experiment and the data are representative of three separate experiments.

the kinase-negative xTAK1 alone induced N-CAM (Figure 9B), while neither the expression of xTAK1 with *bcl-2*, of xTAB1 with *bcl-2* nor *bcl-2* alone induced neural tissue (data not shown). Furthermore, expression of xTAK1KN suppressed epidermal differentiation (Figure 9B). Expression of dominant-negative BMPR-IA or noggin alone suppressed the induction of keratin (Wilson *et al.*, 1997), as did expression of xTAK1KN. Taken together, these results show that xTAK1 can induce ventral mesoderm formation and suppress neural differentiation, and support the idea that xTAK1 functions downstream of the BMP-2/4 receptor.

Discussion

Although previous studies of TAK1 and TAB1 in mammalian cells have suggested their possible role in the regulation of a TGF-β-responsive gene (Yamaguchi *et al.*, 1995;

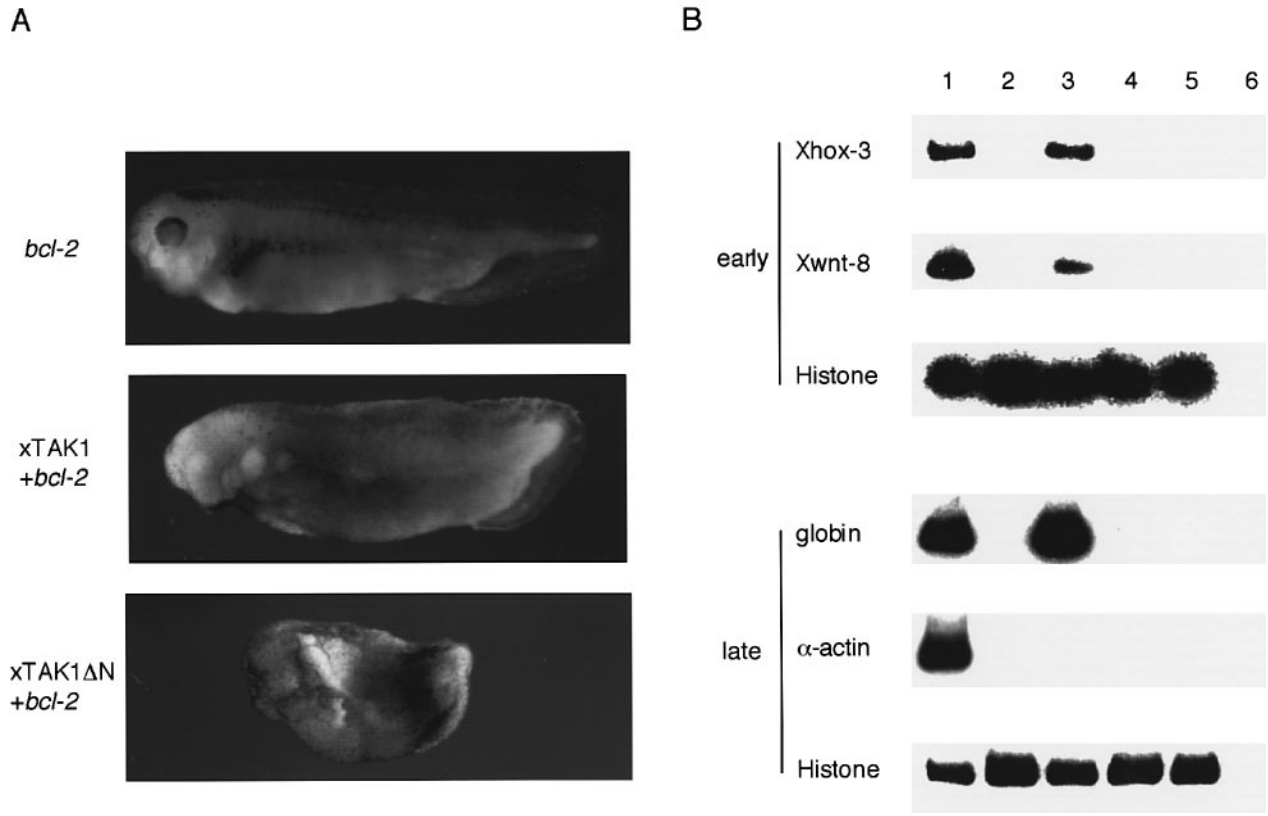

Fig. 4. Effect of xTAK1 on *Xenopus* embryonic development. (**A**) Dorsal injection of *bcl-2* mRNA (250 pg) resulted in the development of normal embryos (top). Dorsal injection of both xTAK1 (100 pg) and *bcl-2* mRNAs caused weak ventralization (middle). Dorsal injection of xTAK1ΔN (100 pg) and *bcl-2* mRNAs caused severe ventralization (bottom). Synthetic mRNAs containing xTAK1 or *bcl*-2 sequences were injected into the equatorial region of two dorsal or two ventral blastomeres at the four-cell stage, and phenotypes were scored at tadpole stage 36. The average dorso-anterior index (DAI; Kao and Elinson, 1989), a measure of the degree of dorsal and anterior mesodermal patterning, for each group was: *bcl-2*, average DAI = 5.0 (n = 21); xTAK1, average DAI = 4.1 (n = 21); xTAK1ΔN, average DAI = 2.6 (n = 22). (**B**) RT–PCR analysis of mesodermal marker gene expression in animal caps. Synthetic mRNAs containing xTAK1 or *bcl*-2 sequences were injected into the equatorial region of blastomeres at the two-cell stage. Animal caps injected with xTAK1ΔN (100 pg) and *bcl-2* (250 pg), or *bcl-2* mRNA, were cultured until gastrula stage 11 (early) or tadpole stage 38 (late), and total RNA was harvested. RNA was analyzed by RT–PCR for the presence of the indicated transcripts: lane 1, whole embryo; 2, uninjected; 3, xTAK1ΔN and *bcl-2*; 4, *bcl-2*, 5, constitutively active MEKK, 6, –RT. xTAK1 induces the expression of the ventral mesodermal marker globin but not the dorsal marker α-actin. Histone, ubiquitously expressed, was used as a loading control. Xhox-3 is a marker of ventral and posterior mesoderm. Xwnt-8 is a marker of ventral and lateral mesoderm. The globin is a definitive ventral marker. The α-actin is a marker of dorsal mesoderm.

Shibuya *et al.*, 1996), the involvement of TAK1 and TAB1 in biological responses elicited by the TGF-β superfamily *in vivo* had not been demonstrated. In the present studies, we have provided evidence showing that xTAK1 and xTAB1 are expressed in regions overlapping those of BMP-2 and BMP-4 expression, and can induce ventral mesoderm in early *Xenopus* embryo. Furthermore, we show that TAK1 and TAB1 are essential for BMP signaling elicited by the BMP type I receptor. Additional evidence suggests that TAK1 may cooperate with Smad1 or 5 in the BMP signaling pathway in early development. Collectively, these results support the idea that TAK1 and TAB1 function downstream of the ligands and receptors in the BMP signal transduction cascade in early development.

The sequence conservation between xTAK1 and mTAK1 is most striking within their catalytic and C-terminal regions. Within the catalytic portion of xTAK1 (23–280), 252 of 258 residues (98%) are identical, and in the C-terminus (498–616), 118 out of the last 119 amino acids (99%) are identical. These regions are the binding sites for TAB1 and TAB2 (Shibuya *et al.*, 1996), respectively, and might be important in the regulation of the function of TAK1. The localization of xTAK1 transcripts coincides roughly with that of xTAB1 and overlaps with the location of BMP-2 and BMP-4 transcripts in *Xenopus* embryos (Fainsod *et al.*, 1994; Hemmati-Brivanlou and Thomsen, 1995). The co-localization of xTAK1 and xTAB1 transcripts is consistent with the idea that these molecules function together in early development.

Injection of xTAK1 mRNA caused cell death in almost all embryos at stage 10.5. We confirmed that the cell death observed is apoptosis by staining with acridine orange and by the TUNEL method. Cell death induced by xTAK1 was inhibited by co-injection of human *bcl-2* mRNA. These results suggest that xTAK1 has the potential for inducing apoptosis, an activity that may be related to the activation levels of MKK3, MKK4 or MKK6. Recently, Moriguchi *et al.* (1996) provided data indicating the existence of a novel kinase cascade consisting of TAK1, MKK6/MKK3 and p38/MPK2, and another report has indicated that the MKK4-JNK or MKK3-p38 pathway induces apoptosis (Xia *et al.*, 1995). MEKK1 is also known to induce apoptosis through activation of JNK or p38. Although both TAK1 and MEKK1 overexpression

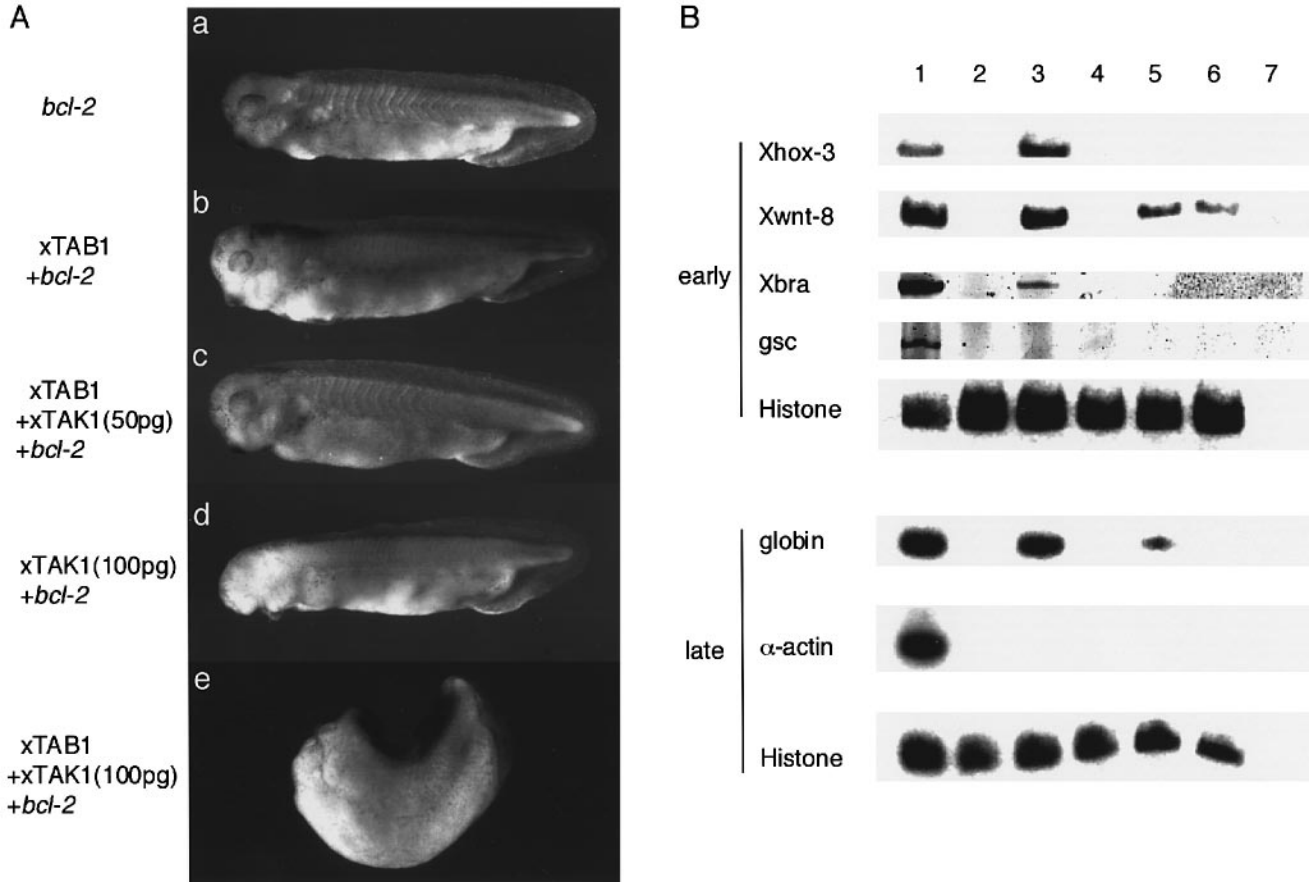

Fig. 5. Effect of xTAK1 and xTAB1 on *Xenopus* embryonic development. (**A**) Dorsal injection of *bcl-2* (250 pg), xTAB1 (1 ng) with *bcl-2* or xTAB1 and xTAK1 (50 pg) with *bcl-2* mRNAs resulted in the development of normal embryos (a–c). Dorsal injection of both xTAK1 (100 pg) and *bcl-2* mRNAs caused weak ventralization (d). Dorsal injection of xTAK1 (100 pg), xTAB1 (1 ng) and *bcl-2* mRNAs caused severe ventralization (e). Synthetic mRNAs containing xTAK1, xTAB1 or *bcl*-2 sequences were injected into the equatorial regions of two dorsal or two ventral blastomeres at the four-cell stage, and phenotypes were scored at tadpole stage 36. The average DAI (Kao and Elinson, 1989) for each group was: *bcl-2*, average DAI = 5.0 (n = 20); xTAK1 and *bcl-2*, average DAI = 4.1 (n = 20); xTAK1, xTAB1 and *bcl-2*, average DAI = 2.9 (n = 20). (**B**) RT–PCR analysis of mesodermal marker gene expression in animal caps. Synthetic mRNAs containing xTAK1, xTAB1 or *bcl*-2 sequences were injected into the equatorial regions of blastomeres at the two-cell stage. Animal caps injected with xTAK1 (100 pg), xTAB1 (1 ng) and *bcl-2* (250 pg), xTAK1 and *bcl-2*, xTAB1 and *bcl-2*, or *bcl-2* mRNA were cultured until gastrula stage 11 (early) or tadpole stage 38 (late), and total RNA was harvested. RNA was analyzed by RT–PCR for the presence of the indicated transcripts: lane 1, whole embryo; 2, uninjected; 3, xTAK1, xTAB1 and *bcl-2*; 4, *bcl-2*; 5, xTAK1 and *bcl-2*; 6, xTAB1 and *bcl-2*; 7, –RT. Xbra is a marker of pan-mesoderm. Goosecoid is a marker of dorsal mesoderm.

lead to the induction of apoptosis, the effect of TAK1 on development appears to be more specific. We observed that expression of activated MEKK1, activated xTAK1 or normal xTAK1 plus xTAB1 all caused apoptosis which could be inhibited by the additional expression of *bcl-2* (Figures 4 and 5). However, inhibition of apoptosis by *bcl-2* further revealed that expression of activated xTAK1, or normal xTAK1 plus xTAB1, could induce ventralization of embryos and of mesodermal marker genes whereas expression of activated MEKK1 did not. These results indicate that the induction of ventral mesoderm appears to be a specific event caused by TAK1, and not by any effects of *bcl-2*.

Another question is why xTAK1 induces both ventral mesoderm and cell death, while BMP-2/4, the constitutively active BMPR-IA or Smad1 (or Smad5) induce only ventral mesoderm formation in early *Xenopus* embryos. In fact, BMP-4 can induce apoptosis in some systems, for example in rhombomere 3 and 5 (Graham *et al.*, 1994) and the interdigit field of the limb (Yokouchi *et al.*, 1996; Zou and Niswander, 1996) in developing chickens. In early *Xenopus* development, the TAK1-induced apoptosis signal may be suppressed by a BMP signaling-mediated anti-apoptotic activity. In the tumor necrosis factor (TNF) signaling pathway, TNF receptor signaling complex stimulation leads to activation of at least two effector functions, i.e. induction of apoptosis and NF-κB activation. While TNF-induced apoptosis is most likely mediated via FADD-induced MACH/FLICE recruitment and activation of the proapoptotic protease cascade, NF-κB activation is mediated via TRAF2 and RIP recruitment (Boldin *et al.*, 1996; Z.G.Liu *et al.*, 1996; Muzio *et al.*, 1996). Accordingly, activation of NF-κB inhibits TNF-induced apoptosis. Moreover, a recent study showed that the FAS-binding protein, Daxx, activates JNK and induces apoptosis (Yang *et al.*, 1997). In a similar manner, stimulation of the BMP receptor complex may activate multiple signaling pathways leading to the induction of mesoderm, inhibition of neural differentiation, apoptosis, anti-apoptosis, etc. Thus an anti-apoptotic molecule might be involved upstream of BMP receptor-mediated TAB1–TAK1 activation in early development. However, the significance

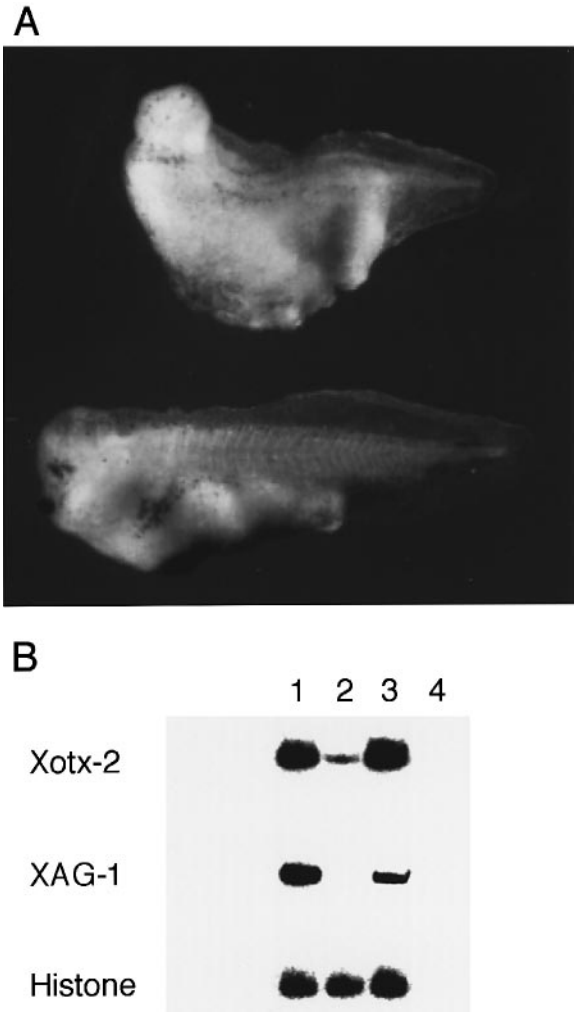

Fig. 6. xTAK1 functions downstream of BMPR-IA. (**A**) Kinase-negative xTAK1 (xTAK1KN) rescues the phenotypes caused by constitutively active BMPR-IA. Synthetic mRNAs were injected into the equatorial regions of two dorsal blastomeres at the four-cell stage, and phenotypes were scored at tadpole stage 36. Injection of constitutively active BMPR-IA mRNA (50 pg) ventralized the embryos with an average DAI of 2.7 (n = 26) (top). Co-expression of xTAK1KN (2 ng) with constitutively active BMPR-IA partially rescued the head structure (average DAI = 3.7, n = 40) (bottom). (**B**) xTAK1KN reverts the expression of anterior marker genes repressed by expression of constitutively active BMPR-IA. Synthetic mRNAs were injected into the equatorial regions of two dorsal blastomeres at the four-cell stage, and total RNAs were prepared at tadpole stage 36. RNA was analyzed by RT–PCR for anterior marker gene expression. Xotx-2 is an anterior neural marker and XAG-1 is an anterior ectodermal marker. Lane 1, uninjected embryo; 2, constitutively active BMPR-IA; 3, constitutively active BMPR-IA and TAK1KN; 4, –RT.

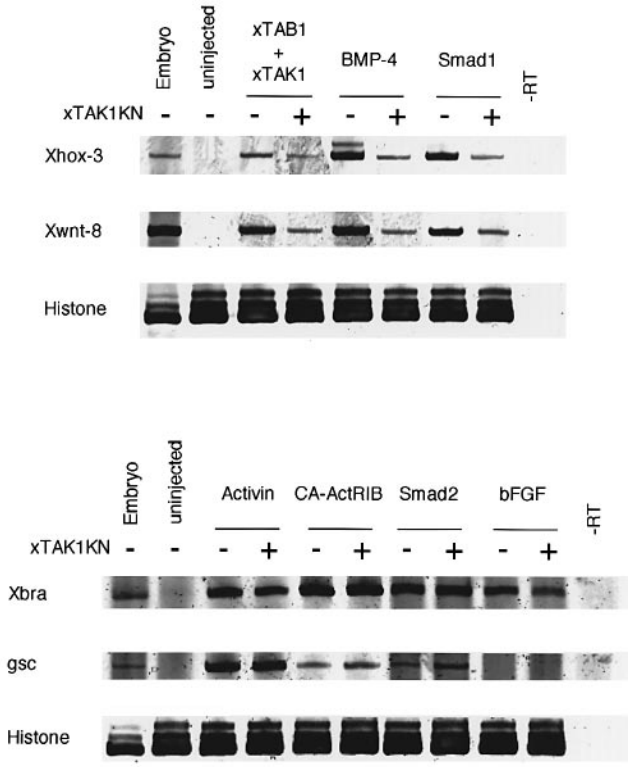

Fig. 7. Kinase-negative xTAK1 inhibits mesoderm induction caused by BMP, but not activin signaling. Embryos were injected with xTAK1KN (2 ng) together with either xTAK1 (500 pg), xTAB1 (1 ng) and *bcl-2* (250 pg), BMP-4 (50 pg), Smad1 (1 ng), activinβB (2 pg), CA-ActRIB (50 pg) or Smad2 (1 ng) mRNAs at the two-cell stage, and animal cap explants were dissected at blastula stages 8 to 9. The caps were either incubated alone or with basic FGF. Total RNA was assayed at gastrula stage 11 by RT–PCR for expression of mesodermal-specific markers.

of our findings demonstrating xTAK1-induced apoptosis needs further investigation.

Our results show that the overexpression of xTAK1 and xTAB1 causes responses in *Xenopus* embryos similar to those caused by the overexpression of BMP-2/4. The expression of xTAK1KN in embryos prevents at least some of the effects induced by the expression of the constitutively active form of BMPR-IA. Moreover, the expression of xTAK1KN induces the neural marker gene, N-CAM, which is similarly induced by the expression of a dominant-negative form of BMPR-IA (Graff *et al.*, 1994; Suzuki *et al.*, 1994). However, the effects of xTAK1 are not as marked as those of BMP-2/4, and it is possible that other genes are required for the full ventralization of the *Xenopus* embryo caused by BMP-2/4. The observation that xTAK1KN is unable to completely reverse the ventralizing effects of the constitutively active form of BMPR-IA is consistent with the above possibility.

The invertebrate and vertebrate Smad proteins recently have been identified as important mediators of the TGF-β superfamily signal transduction pathway. Smad1 and Smad2 have been shown to mimic the effects of BMP and activin, respectively, in *Xenopus* as well as in mammalian cells (Baker and Harland, 1996; Eppert *et al.*, 1996; Graff *et al.*, 1996; Hoodless *et al.*, 1996; F.Liu *et al.*, 1996; Thomsen, 1996). On the other hand, Smad4 has been suggested to be a regulated partner of the Smads which function in various signaling pathways activated by TGF-β, activin and BMP (Lagna *et al.*, 1996). Smad4 alone can act as a ventral mesoderm inducer in *Xenopus* (Lagna *et al.*, 1996). In our previous study, we showed that the kinase activity of TAK1 was stimulated by both TGF-β1 and BMP-4 in mammalian cells (Yamaguchi *et al.*, 1995). It is thus possible that TAK1 may function commonly to cooperate with the Smads in TGF-β superfamily signal transduction pathways and that it functions critically in the BMP signaling pathway in the early *Xenopus* developmental stages in an as yet unknown manner. The interrelationship between TAK1 and the

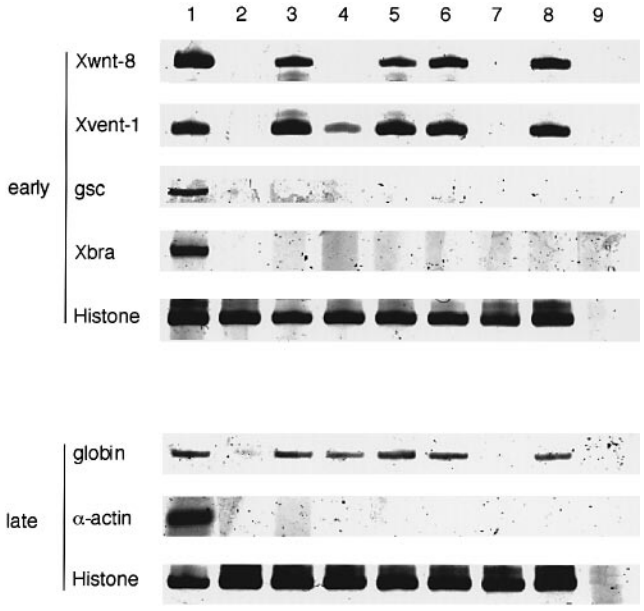

Fig. 8. Cooperation of TAK1 and Smads. xTAK1KN reverts expression of ventral marker genes induced by expression of Smad1 or Smad5. Animal caps co-injected with xTAK1KN (2 ng) and Smad1 (1 ng) or Smad5 (2 ng) mRNA were cultured until gastrula stage 11 (early) or tadpole stage 38 (late), and total RNA was harvested. RNA was analyzed by RT–PCR for the presence of the indicated transcripts: lane 1, whole embryo; 2, uninjected; 3, Smad1; 4, Smad1 and xTAK1KN; 5, Smad1 and β-globin; 6, Smad5; 7, Smad5 and xTAK1KN; 8, Smad5 and β-globin; 9, –RT.

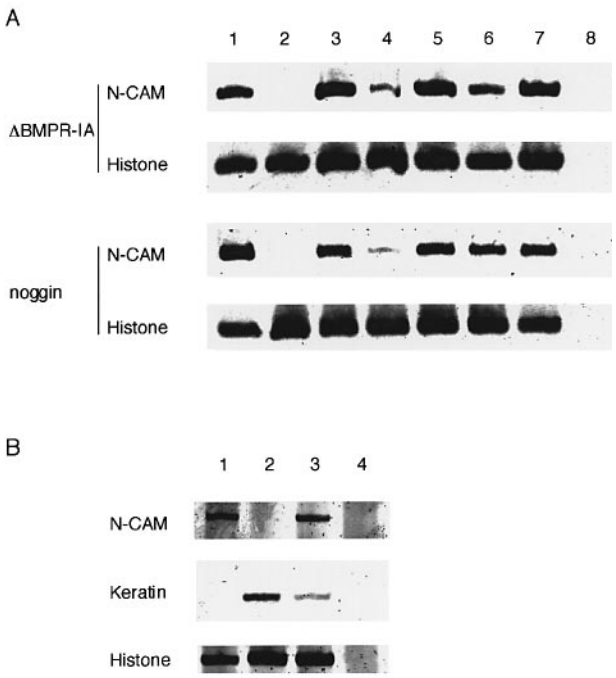

Fig. 9. Involvement of xTAK1 in neural inhibition and epidermal induction. (**A**) xTAK1 reverses neural induction caused by truncated BMPR-IA (ΔBMPR-IA) and noggin expression. Embryos were injected with ΔBMPR-IA (50 pg) or noggin (1 pg) mRNA together with either xTAK1 (500 pg), xTAB1 (1 ng) and *bcl-2* (250 pg), xTAK1 and *bcl-2*; xTAB1 and *bcl-2*, or *bcl-2* mRNAs. At stage 18, animal cap RNA was analyzed by RT–PCR for the neural marker gene, N-CAM: lane 1, embryo; 2, uninjected; 3, alone; 4, xTAK1, xTAB1 and *bcl-2*; 5, *bcl-2*; 6, xTAK1 and *bcl-2*; 7, xTAB1 and *bcl-2*; 8, –RT. (**B**) Kinase-negative xTAK1 inhibits epidermal induction. Embryos were injected as indicated: lane 1, embryo; 2, uninjected; 3, xTAK1KN (2 ng); 4, –RT. At stage 18, animal cap RNA was analyzed by RT–PCR for the epidermis marker gene, keratin.

Smad signaling cascades awaits detailed analyses, as does their precise role in signaling by particular TGF-β receptors.

Materials and methods

cDNA cloning

A *Xenopus* oocyte cDNA library contained in the Lambda Zap vector was screened with random-primed ^{32}P-labeled mouse TAK1 or human TAB1 cDNA as probe. About 1.5×10^6 plaques were hybridized at 60°C on nylon filters, and subsequently washed in 1× SSC, 0.1% SDS, at 60°C. Positive clones were initially isolated and subcloned into the pBluescript vector. Full-length cDNAs were identified and sequenced on both strands by primer walking.

Northern blot analysis

For the developmental Northern blot analysis, 10 μg of total RNA were electrophoresed per lane on 1% agarose gels, and transferred onto nylon membranes according to standard procedures. Probes were labeled with [α-^{32}P]dCTP using the Multiprime labelling kit (Amersham) and hybridized. Specific activity was ~1×10^6 c.p.m./ng for all the probe DNAs.

Whole-mount in situ hybridization

Whole-mount *in situ* hybridization was carried out essentially as described (Harland, 1991). Digoxygenin-labeled riboprobes were prepared from a pBluescript SK(–) plasmid containing the entire xTAK1 and TAB1 cDNAs. Transcripts were detected using the BM-purple substrate (Boehringer).

Detection of cell death

Acridine orange staining was performed as described (Abrams *et al.*, 1993). The TUNEL method (Gavrieli *et al.*, 1992) for detecting fragmented DNAs diagnostic of apoptosis in paraffin sections was performed using an Apoptosis *in situ* detection kit (Biovation) according to the manufacturer's protocol.

Animal cap assay

The xTAK1, xTAK1ΔN, xTAK1KN, xTAB1 and constitutively active activin type IB receptor cDNAs were cloned into the CS2+ vector (Rupp *et al.*, 1994), and constitutively active BMPR-IA cDNA was cloned into pSP64T (Krieg and Melton, 1987). Capped mRNA was synthesized from linearized vectors using the mMessage Machine kit (Ambion). RNAs were then injected into the animal poles or marginal zones of early stage embryos. The amounts of injected *in vitro* synthesized RNAs and sites of injection are described in the text. Animal cap explants were removed with hair knives at late blastula stages and allowed to grow until control sibling embryos reached either the gastrula or neurula stages. Total RNA was then extracted and analyzed with RT–PCR. RT–PCR assays and primer sequences were as published (Wilson and Melton, 1994; Wilson and Hemmati, 1995).

Acknowledgements

We thank Jeffrey Wrana for providing the constitutively active activin type IB receptor cDNA, Norio Tamura and Tomoko Chiba for technical assistance and Marc Lamphier for critical reading of the manuscript. This work was supported by Grants-in-Aid for scientific research from the Ministry of Education, Science, and Culture of Japan and the 'Research for the Future' program of the Japan Society for the Promotion of Science.

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Received July 7, 1997; revised and accepted December 8, 1997