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XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1–TAK1 in the BMP signaling pathway

Kyoko Yamaguchi, Shin-ichi Nagai[1], Jun Ninomiya-Tsuji, Michiru Nishita[1], Katsuyuki Tamai[2], Kenji Irie, Naoto Ueno[1], Eisuke Nishida[3], Hiroshi Shibuya[1] and Kunihiro Matsumoto[4]

Department of Molecular Biology, Graduate School of Science, Nagoya University and CREST, Japan Science and Technology Corporation, Chikusa-ku, Nagoya 464-01, [1]Division of Morphogenesis, Department of Developmental Biology, National Institute for Basic Biology, Okazaki 444, [2]Medical and Biological Laboratories Co., Naka-ku, Nagoya 460 and [3]Department of Biophysics, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan

[4]Corresponding author
e-mail: g44177a@nucc.cc.nagoya-u.ac.jp

Signals elicited by transforming growth factor-β (TGF-β) superfamily ligands are generated following the formation of heteromeric receptor complexes consisting of type I and type II receptors. TAK1, a member of the MAP kinase kinase kinase family, and its activator, TAB1, participate in the bone morphogenetic protein (BMP) signaling pathway involved in mesoderm induction and patterning in early *Xenopus* embryos. However, the events leading from receptor activation to TAK1 activation remain to be identified. A yeast interaction screen was used to search for proteins that function in the pathway linking the receptors and TAB1–TAK1. The human X-chromosome-linked inhibitor of apoptosis protein (XIAP) was isolated as a TAB1-binding protein. XIAP associated not only with TAB1 but also with the BMP receptors in mammalian cells. Injection of XIAP mRNA into dorsal blastomeres enhanced the ventralization of *Xenopus* embryos in a TAB1–TAK1-dependent manner. Furthermore, a truncated form of XIAP lacking the TAB1-binding domain partially blocked the expression of ventral mesodermal marker genes induced by a constitutively active BMP type I receptor. These results suggest that XIAP participates in the BMP signaling pathway as a positive regulator linking the BMP receptors and TAB1–TAK1.

Keywords: bone morphogenetic protein/signal transduction/TAB1–TAK1/TGF-β family/XIAP

Introduction

Members of the transforming growth factor β (TGF-β) superfamily, which includes activins and the bone morphogenetic proteins (BMPs), elicit diverse activities in the regulation of cell growth, differentiation and morphogenesis (Roberts and Sporn, 1990). TGF-β family members transmit signals through heteromeric receptor complexes consisting of type I and type II Ser/Thr kinase receptors (Massague and Weis-Garcia, 1996; ten Dijke *et al.*, 1996). Type II receptors are constitutively active kinases capable of binding ligand alone, while type I receptors can only bind ligand in cooperation with type II receptors. Ligand binding induces the formation of a complex in which the type II receptor phosphorylates the type I receptor. Phosphorylation results in the activation of the type I receptor, which in turn mediates downstream signaling events.

BMPs have been identified in a wide variety of organisms ranging from insects to mammals. BMPs play an important role during the early development of both vertebrates and invertebrates (Harland, 1994; Kingsley, 1994). In *Drosophila*, Decapentaplegic (Dpp), which is closely related to mammalian BMP2 and BMP4, is implicated in the regulation of cell fate throughout development (Padgett *et al.*, 1987). In *Xenopus*, BMP2 and BMP4 are capable of inducing ventral mesoderm to differentiate into ventral tissues and act as negative regulators of neuralization (Harland, 1994; Sasai *et al.*, 1994; Wilson and Hemmati-Brivanlou, 1995). Similar to TGF-β and activins, BMPs also form heteromeric complexes with type II and type I receptors. A truncated form of the type I receptor lacking its intracellular Ser/Thr kinase domain acts as a dominant-negative inhibitor of the cellular responses to BMP2/4 in *Xenopus* embryos (Graff *et al.*, 1994; Suzuki *et al.*, 1994). Overexpression of this dominant-negative type I receptor in the ventral side of *Xenopus* embryos causes prospective ventral mesoderm to differentiate into dorsal mesoderm, and induces the expression of a neural marker in the ectoderm.

The intracellular pathways for mediating transmission of the TGF-β family signal from the membrane to the nucleus have recently begun to be elucidated. Smad proteins were identified as mediators of the TGF-β superfamily signal transduction pathways in a variety of species (Heldin *et al.*, 1997; Massague *et al.*, 1997). The first known member of this family, Mad, was identified by genetic analysis of the *Drosophila* Dpp signaling pathway (Sekelsky *et al.*, 1995). While Smad1 mediates BMP2/4 signaling, Smad2 and Smad3 transduce activin and TGF-β signaling (Heldin *et al.*, 1997; Massague *et al.*, 1997). Exposure of cells to TGF-β-related factors activates specific receptors which in turn phosphorylate either Smad1, Smad2 or Smad3, depending on the cell type. Once phosphorylated, Smads heteromerize with Smad4 and translocate to the nucleus. Thus, the various receptor-regulated Smad proteins appear to function as mediators specific to a particular TGF-β superfamily pathway, while Smad4 serves as a common partner of these proteins. Recently, Smad2 has been identified in a DNA-binding complex containing FAST1, which belongs to the winged helix transcription factor family. FAST1 functions in this complex as the DNA-binding component and interacts

with Smad2 and Smad4 to activate the activin response gene (Chen *et al.*, 1996, 1997; Liu *et al.*, 1997). These results suggest that Smads act as co-activators, associating with particular transcription factors in response to specific signals.

We have previously identified two proteins, TAK1 and TAB1, which function in the TGF-β superfamily signaling pathway (Yamaguchi *et al.*, 1995; Shibuya *et al.*, 1996). TAK1 is a member of the MAP kinase kinase kinase family, whose kinase activity is stimulated in response to TGF-β1 or BMP4. TAB1 functions as an activator of TAK1. We recently showed that ectopic co-expression of *Xenopus* TAB1 and TAK1 (xTAB1 and xTAK1) can induce ventral mesoderm formation and suppress neural differentiation (Shibuya *et al.*, 1998). This suggests that overexpression of TAB1 and TAK1 mimics the BMP signals involved in mesoderm induction. Consistent with this possibility, a dominant-negative mutant of xTAK1 (xTAK1-KN) can partially revert the ventralization caused by ectopic expression of a constitutively active BMP type I receptor. Thus, TAB1 and TAK1 participate in the BMP signaling pathway to induce ventral mesoderm. It is likely therefore that a MAP kinase activation cascade mediated by TAB1–TAK1 might be involved in signaling by TGF-β/BMP. Smad1 and Smad5 have been shown to be specifically involved in BMP signaling (Kretzschmar *et al.*, 1997; Suzuki *et al.*, 1997; Wilson *et al.*, 1997). Although xTAK1-KN can block the expression of ventral mesoderm marker genes induced by Smad1 or Smad5 (Shibuya *et al.*, 1998), it is unclear at present precisely how the Smads and TAB1–TAK1 cooperate in BMP signaling.

Whereas receptor-activated Smads are known to directly associate with the type I receptors (Heldin *et al.*, 1997; Massague *et al.*, 1997), the molecular mechanism linking the receptors to the activation of TAK1 is still obscure and is likely to involve additional signaling molecules. One means of understanding the role of TAB1 and TAK1 in the pathway is to identify proteins that function between the receptors and TAB1–TAK1. The C-terminal 68 amino acid portion of TAB1 [TAB1(437–504)] is sufficient for binding to and activation of TAK1 (Shibuya *et al.*, 1996). This suggests that the N-terminal region of TAB1 may play a regulatory role, interacting with other components required to execute the signal. Consistent with this possibility, a truncated form of TAB1 [TAB1(1–418)] lacking the C-terminal TAK1-binding domain has been shown to function as a dominant-negative inhibitor of TGF-β responses, inhibiting TGF-β-induced gene expression and TAK1 activation (Shibuya *et al.*, 1996; Shirakabe *et al.*, 1997). Presumably, TAB1(1–418) blocks the TGF-β signaling pathway at a receptor-proximal step that precedes TAK1 activation by sequestering other components required to execute the signal. It is likely therefore that the N-terminal region of TAB1 transmits TGF-β signals through protein–protein interactions.

In this study, we identified one cellular member of the inhibitor of apoptosis protein (IAP) family, XIAP, as a TAB1-binding protein. XIAP interacts not only with TAB1 but also with BMP type I and type II receptor complexes in mammalian cells. Our results suggest that XIAP serves as an adaptor protein linking the receptors and TAB1–TAK1.

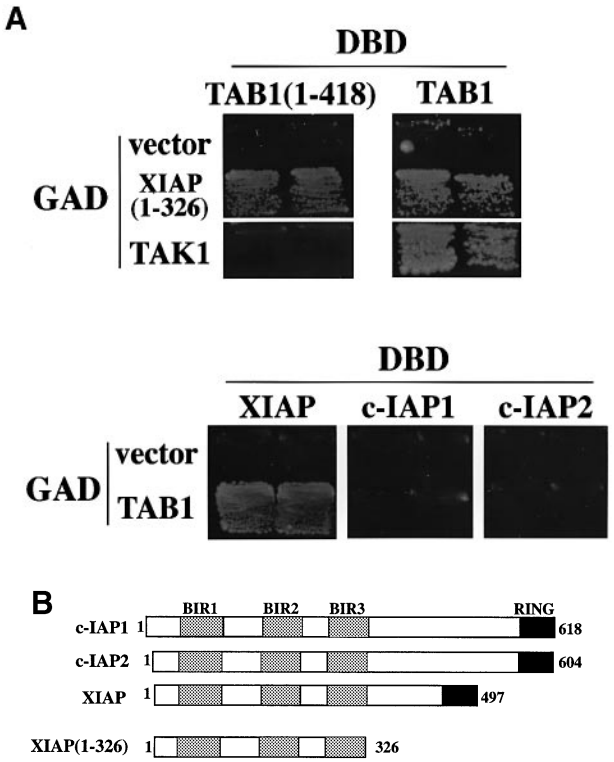

Fig. 1. Interaction between TAB1 and cellular members of the IAP family in yeast. (**A**) Protein interactions in the two-hybrid system. Yeast L40 or HF7c cells were transformed with expression vectors encoding the indicated DNA-binding domain (DBD) and GAL4 transcription activation domain (GAD) fusion proteins. Each transformant was tested for growth on medium lacking histidine. GAD-XIAP(1–326) was the original clone isolated as a TAB1(1–418) interacting protein. (**B**) Diagrammatic representation of cellular IAPs. The locations of the BIR motifs (grey boxes) and RING zinc-finger domains (black boxes) are shown in the block diagram of each protein.

Results

Identification of XIAP as a TAB1-associating protein

The C-terminal 68 amino acid portion of TAB1 [TAB1(437–504)] is able to associate with TAK1 (Shibuya *et al.*, 1996), whereas the N-terminal region of TAB1 [TAB1(1–418)] failed to do so (Figure 1A). This raises the possibility that the TAB1(1–418) domain interacts with other proteins which may link TAB1 to the receptors. To identify potential components functioning upstream of TAB1–TAK1 in the signaling pathway, we screened for proteins that associate directly with TAB1 by yeast two-hybrid protein interaction cloning. An expression vector that encodes TAB1(1–418) fused to the LexA DNA-binding domain was used as bait in a two-hybrid screen of a human brain cDNA library. From the ~1×10^6 transformants screened, 15 positive clones were obtained, as determined by activation of a *HIS3* reporter gene. Five of these clones encoded one cellular member of the IAP family, designated XIAP (Liston *et al.*, 1996) [also known as MIHA (Uren *et al.*, 1996) or ILP (Duckett *et al.*, 1996)] (Figure 1A).

The IAP proteins, originally identified in baculoviruses, are evolutionarily conserved among eukaryotic cells. Among the mammalian cellular IAPs (c-IAP1, c-IAP2 and XIAP), c-IAP1 and c-IAP2 are the most closely

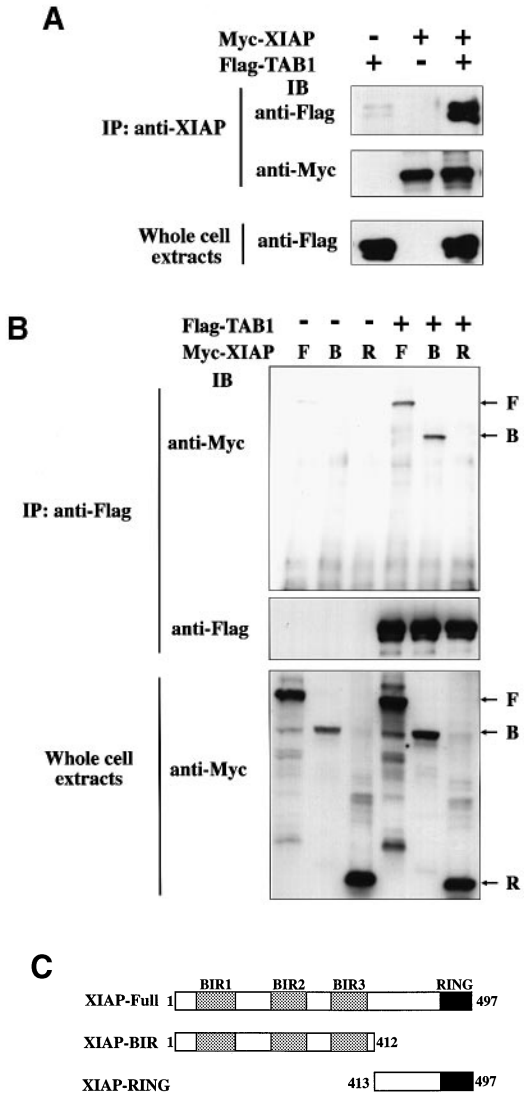

Fig. 2. Interaction of XIAP with TAB1 in mammalian cells. (**A**) Interaction of XIAP with TAB1. COS7 cells were transiently co-transfected with expression vectors encoding Myc-XIAP and Flag-TAB1 as indicated. Cell extracts were subjected to immunoprecipitation with a mouse monoclonal antibody against XIAP (anti-XIAP). The immune complexes were washed, resolved by SDS–PAGE, transferred to PVDF membranes, and subjected to immunoblot analysis with mouse monoclonal antibody M2 (anti-Flag), recognizing the Flag epitope, or 9E10 (anti-Myc), recognizing Myc. Cell extracts were also directly subjected to immunoblot analysis. (**B**) Interaction of TAB1 with XIAP deletion mutants. 293 cells were transiently co-transfected with expression vectors encoding Myc-XIAP-full length (F), Myc-XIAP-BIR (B) or Myc-XIAP-RING (R) and Flag-TAB1 as indicated. Cell extracts were subjected to immunoprecipitation with anti-Flag. Precipitated Myc-XIAP was detected with a rabbit polyclonal antibody against Myc, A-14. Cell extracts were also directly subjected to immunoblot analysis. (**C**) Structure of XIAP deletion mutants.

related to one another, sharing 73% amino acid identity, whereas XIAP exhibits only 43% conserved identity with c-IAP1 and c-IAP2. c-IAP1 and c-IAP2 have been shown to associate with tumor necrosis factor (TNF) receptor-associated factor 1 (TRAF1) and 2 (TRAF2) (Rothe *et al*., 1995), whereas XIAP itself is unable to interact with either (Uren *et al.*, 1996). Next, we examined the specificity of the interaction between TAB1 and cellular IAPs in the two-hybrid system. Direct association could be detected between TAB1 and XIAP, but not between TAB1 and c-IAP1 or c-IAP2 (Figure 1A). Thus, XIAP interacts specifically with TAB1. The IAP proteins contain characteristic N-terminal baculovirus IAP repeats (BIR) and a C-terminal RING zinc-finger domain. The mammalian cellular IAPs are characterized further by the presence of three BIR motifs and a single RING finger motif (Figure 1B). The original cDNAs of XIAP isolated in our two-hybrid screen are truncated and lack the C-terminal RING-finger domain sequences (Figure 1). Thus, the N-terminal region of XIAP mediates its interaction with TAB1, probably via the BIR motifs.

To determine whether XIAP can interact with TAB1 in mammalian cells, we carried out co-immunoprecipitation assays. An expression vector encoding Myc epitope-tagged XIAP (Myc-XIAP) was transfected alone or with an expression vector encoding Flag epitope-tagged TAB1 (Flag-TAB1) into COS7 cells. Cell lysates were immunoprecipitated using an anti-XIAP monoclonal antibody, and co-precipitating TAB1 was detected by immunoblot analysis with a monoclonal antibody against the Flag epitope. In this assay, XIAP was able to co-precipitate TAB1 (Figure 2A), confirming the interaction detected by yeast two-hybrid analysis.

The interaction between XIAP and TAB1 was also observed in human embryonic kidney 293 cells (Figure 2B). To confirm that the N-terminal region of XIAP mediates its interaction with TAB1, the interaction of TAB1 with truncation mutants of XIAP was determined by co-immunoprecipitation assays. For this purpose, we constructed Myc epitope-tagged XIAP mutants, XIAP-BIR (Myc-XIAP-BIR) and XIAP-RING (Myc-XIAP-RING), which contain either the BIR motifs or the RING finger domain, respectively (Figure 2C). These mutants of XIAP were co-expressed with Flag-TAB1 in 293 cells. In co-precipitation assay, TAB1 associated with the C-terminal deletion mutant of XIAP expressing the BIR domain, but failed to associate with the N-terminal deletion mutant of XIAP expressing the RING finger domain (Figure 2B). This supports the possibility that the BIR domain of XIAP mediates the interaction with TAB1.

Interaction of XIAP with BMP receptors

To examine whether XIAP can interact with the BMP type I receptor BMPR-IA in mammalian cells, we used a chimeric protein consisting of the BMPR-IA cytoplasmic domain (BMPR-IA-C) fused to glutathione *S*-transferase (GST). Vectors encoding GST–BMPR-IA-C or GST were co-transfected into COS7 cells along with an expression vector encoding Myc-XIAP. Cell lysates were precipitated with glutathione–Sepharose beads, and analyzed by Western blot analysis with an anti-Myc antibody. Myc-XIAP was found to co-precipitate with GST–BMPR-IA-C but not with GST alone (Figure 3A), demonstrating that XIAP binds to the cytoplasmic domain of BMPR-IA. Mutational analysis of XIAP was performed to investigate the structural requirements for its association with the BMPR-IA cytoplasmic domain. For this purpose, we used Myc-XIAP-BIR and Myc-XIAP-RING, which contain the BIR motifs and the RING finger domain, respectively (Figure 2C). These mutants of XIAP were co-expressed with GST–BMPR-IA-C or GST alone in COS7 cells. In co-precipitation assay, the mutant XIAP protein

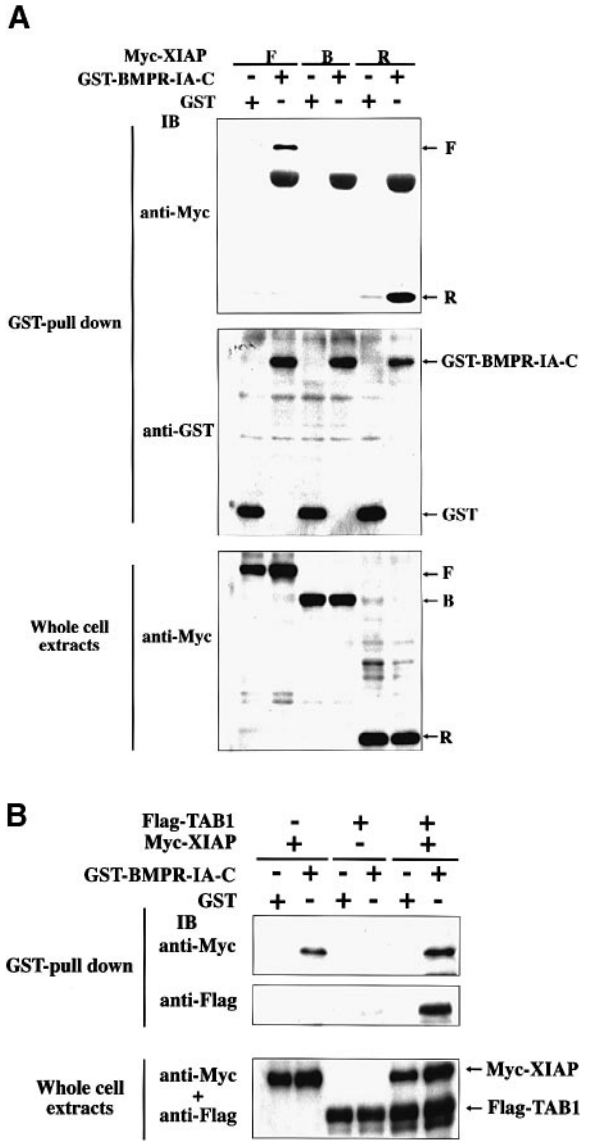

Fig. 3. Interaction of XIAP with the cytoplasmic region of BMPR-IA in mammalian cells. (**A**) Interaction of the cytoplasmic region of BMPR-IA (BMPR-IA-C) with XIAP. COS7 cells were transiently co-transfected with expression vectors encoding GST or GST–BMPR-IA-C and Myc-XIAP-full length (F), Myc-XIAP-BIR (B) or Myc-XIAP-RING (R) as indicated. Cell lysates were subjected to the GST pull-down procedure and then immunoblotted with anti-Myc or a monoclonal antibody against GST (anti-GST). Expression of XIAP was measured by anti-Myc immunoblotting of aliquots from cell lysates. Anti-Myc cross-reacted with precipitated GST–BMPR-IA-C. (**B**) Interaction of BMPR-IA-C with XIAP and TAB1. COS7 cells were transiently transfected with vectors encoding Myc-XIAP, Flag-TAB1, GST or GST–BMPR-IA-C as indicated. Cell lysates were subjected to the GST pull-down procedure and then immunoblotted with anti-Myc and anti-Flag. Expression of XIAP and TAB1 was measured by anti-Myc and anti-Flag immunoblotting, respectively, of aliquots from cell lysates. Comparable amounts of GST or GST–BMPR-IA-C were precipitated between samples (data not shown).

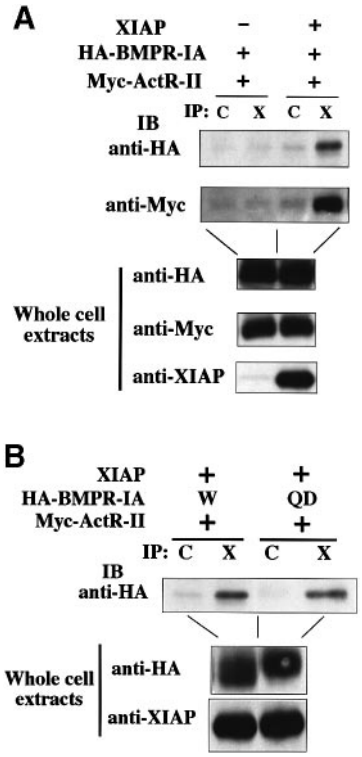

Fig. 4. Association of XIAP with BMP receptor complex. (**A**) Interaction of XIAP with BMP receptor complex. 293 cells were transiently transfected with the indicated expression vectors encoding HA-BMPR-IA and Myc-ActR-II in the absence (–) or presence (+) of co-transfection with XIAP. The cell lysates were immunoprecipitated with anti-XIAP (X) or mouse IgG control (C). Co-precipitated HA-BMPR-IA and Myc-ActR-II were detected by immunoblotting with the polyclonal antibody Y-11 (anti-HA), recognizing the HA epitope, and anti-Myc, respectively. Cell extracts were also directly subjected to immunoblot analysis. (**B**) Interaction of XIAP with a constitutively active BMP type I receptor (BMPR-IA-QD). 293 cells were transiently transfected with expression vectors encoding Myc-ActR-II, XIAP and HA-BMPR-IA (W) or HA-BMPR-IA-QD (QD) as indicated. The cell lysates were immunoprecipitated with anti-XIAP (X) or mouse IgG control (C). Co-precipitated BMPR-IA was detected by immunoblotting with anti-HA. Cell extracts were also directly subjected to immunoblot analysis.

comprising the RING finger domain of the molecule associated with GST–BMPR-IA-C (Figure 3A).

The interaction of XIAP with BMPR-IA-C and TAB1 occurs via its C-terminal RING finger domain and N-terminal BIR domain, respectively. Therefore, XIAP might be able to bind simultaneously both BMPR-IA-C and TAB1, thereby recruiting TAB1 to the receptor. To explore this possibility, we co-transfected COS7 cells with vectors encoding GST–BMPR-IA-C, Myc-XIAP and Flag-TAB1. In COS7 cells expressing GST–BMPR-IA-C and Flag-TAB1, little TAB1 associated with GST–BMPR-IA-C (Figure 3B), suggesting that no direct interaction occurs between the receptor and TAB1. However, when the same experiments were performed on lysates from cells that also expressed Myc-XIAP, TAB1 was co-precipitated with GST–BMPR-IA-C (Figure 3B). These results provide evidence that XIAP can serve as an adaptor protein and recruit TAB1 to the receptor.

Since TGF-β/BMP signaling is mediated by heteromeric complex formation between the type I and type II receptors (Massague and Weis-Garcia, 1996; ten Dijke *et al.*, 1996), we investigated whether XIAP can associate with the BMP receptor complex using the BMP type I receptor BMPR-IA and the shared BMP/activin type II receptor ActR-II. 293 cells were transiently transfected with expression vectors encoding hemagglutinin (HA) epitope-tagged BMPR-IA (HA-BMPR-IA), Myc epitope-tagged ActR-II (Myc-ActR-II) and XIAP. Cell extracts were immunoprecipitated with an antibody to XIAP, followed by immuno-

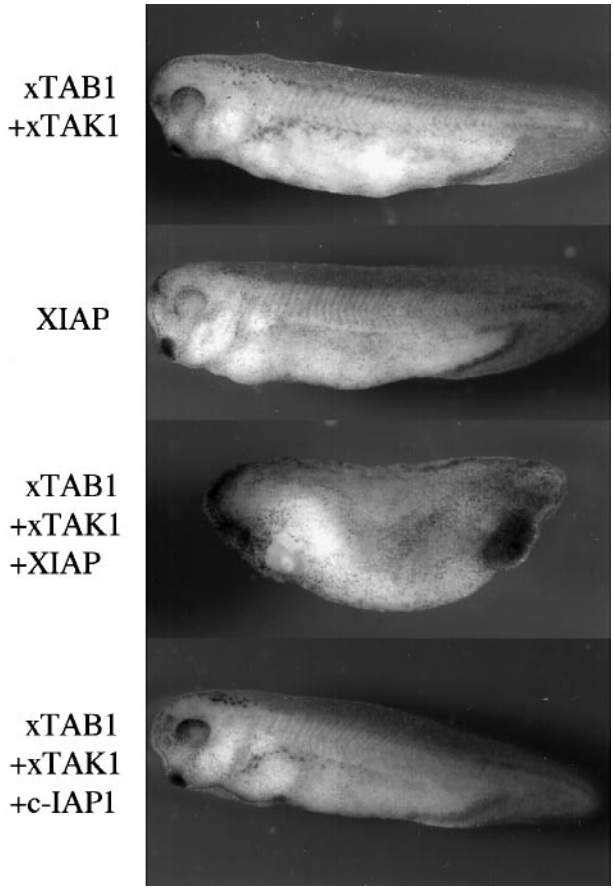

Fig. 5. Dorsal injection of xTAB1, xTAK1 and XIAP mRNAs causes ventralization. Synthetic mRNAs encoding the indicated DNA sequences were injected into the equatorial regions of two dorsal or two ventral blastomeres at the 4-cell stage, and phenotypes were scored at tadpole stage 36. Row 1, xTAB1 (1 ng) and xTAK1 (50 pg) mRNAs; row 2, XIAP (500 pg) mRNA; row 3, xTAB1 (1 ng), xTAK1 (50 pg) and XIAP (500 pg) mRNAs; row 4, xTAB1 (1 ng), xTAK1 (50 pg) and c-IAP1 (500 pg) mRNAs. 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: xTAB1 and xTAK1, average DAI = 4.9 (n = 31); XIAP, average DAI = 5.0 (n = 30); xTAB1, xTAK1 and XIAP, average DAI = 3.2 (n = 25); xTAB1, xTAK1 and c-IAP1, average DAI = 4.9 (n = 27).

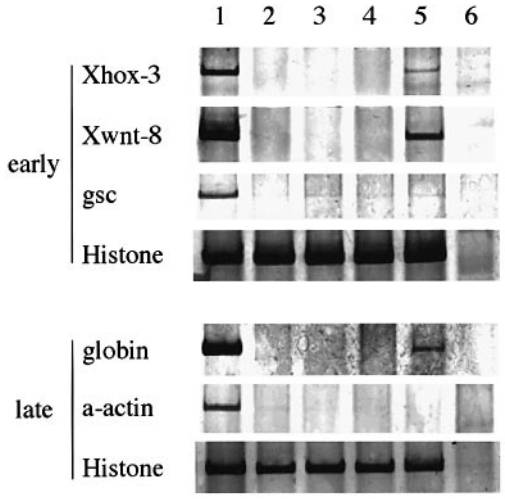

Fig. 6. RT–PCR analysis of mesodermal marker gene expression in animal caps. Synthetic mRNAs encoding the indicated DNA sequences were injected into the equatorial region blastomeres at the 2-cell stage. Animal caps injected with the indicated mRNAs were cultured until gastrula stage 11 (early) or tadpole stage 38 (late), and total RNA was harvested. RNA was analyzed by RT–PCR for the presence of the indicated transcripts: 1, whole embryo; 2, uninjected; 3, xTAB1 (1 ng) and xTAK1 (50 pg); 4, XIAP (500 pg); 5, xTAB1 (1 ng), xTAK1 (50 pg) and XIAP (500 pg); 6, –RT. 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. Globin is a definitive ventral marker. Goosecoid (gsc) and α-actin are markers of dorsal mesoderm.

blotting with anti-HA and -Myc antibodies. In the absence of XIAP transfection, we detected no interaction between XIAP and the receptors. In cells transfected with the XIAP expression vector, BMPR-IA and ActR-II could clearly be detected co-precipitating with XIAP (Figure 4A). To test whether activation of the receptor affects its interaction with XIAP, we compared interactions between XIAP and either wild-type BMPR-IA or constitutively active BMPR-IA-QD, in which glutamine is replaced by aspartic acid at amino acid position 233 (Hoodless *et al.*, 1996). The level of XIAP binding to BMPR-IA-QD was similar to that of wild-type BMPR-IA (Figure 4B). Furthermore, the interaction of XIAP with TAB1 was not affected in the presence of the constitutively active type I receptor (data not shown). These data suggest that the complexes consisting of the receptor, XIAP and TAB1 are present constitutively.

Effects of XIAP on BMP signaling pathway

BMP plays a central role in embryonic patterning. In *Xenopus*, BMP2 and BMP4 are potent ventralizing molecules and act as negative regulators of neuralization (Harland, 1994; Sasai *et al.*, 1994; Wilson and Hemmati-Brivanlou, 1995). Recently we have shown that injection of greater amounts of *Xenopus* TAB1 (xTAB1) and xTAK1 mRNAs in early embryos induces cell death (Shibuya *et al.*, 1998). However, concomitant overexpression of human *bcl-2* with both xTAK1 and xTAB1 in dorsal blastomeres rescues cell death, induces ventral mesoderm formation and suppresses neural differentiation (Shibuya *et al.*, 1998). Thus, TAB1 and TAK1 participate in the BMP signaling pathway to induce ventral mesoderm in *Xenopus* early development. The ability of XIAP to associate with TAB1 allows us to address the potential negative or positive functional role of XIAP in TAB1–TAK1-mediated signaling events. We investigated the effect of XIAP on ventralization induced by co-expression of xTAB1 and xTAK1. If XIAP plays a positive role upstream of TAB1–TAK1 in BMP signaling, XIAP overexpression would be expected to promote ventralization in a manner dependent on the presence of TAB1 and TAK1. At lower doses of xTAK1 (50 pg) and xTAB1 (1 ng), injection of both mRNAs into the dorsal marginal zone resulted in no change from the normal phenotypes (Figure 5). However, when XIAP mRNA (500 pg) was co-injected with xTAK1 (50 pg) and xTAB1 (1 ng) mRNAs into the dorsal marginal zone of 4-cell embryos, the embryos were significantly ventralized, whereas injection of XIAP mRNA (500 pg) alone had little effect on the development of the embryos (Figure 5). These results suggest that the effect of XIAP on ventral patterning is dependent on the presence of TAB1 and TAK1. To examine whether this effect was an event specifically induced by XIAP, we tested the action of c-IAP1 on ventralization induced by co-expression of xTAK1 and xTAB1. In

contrast to XIAP, injection of c-IAP1 mRNA (500 pg) together with xTAK1 (50 pg) and xTAB1 (1 ng) mRNAs had no effect on the development of the embryos (Figure 5). This is consistent with the result showing that TAB1 interacts with XIAP but not with c-IAP1 (Figure 1).

The effect of XIAP on development was also analyzed in animal pole explants. In animal caps, co-injection of XIAP (500 pg) with xTAK1 (50 pg) and xTAB1 (1 ng) led to expression of ventral (Xwnt-8 and globin) and posterior (Xhox-3) mesodermal makers, but not of dorsal mesodermal markers (goosecoid and α-actin) (Figure 6). No mesodermal markers were induced by XIAP alone. Thus, XIAP enhances the ventralization mediated by the TAB1–TAK1 pathway. Taken together, these results demonstrate that XIAP functions as a positive regulator of the TAB1–TAK1 pathway which induces mesodermal patterning.

Overexpression of a constitutively active BMP type I receptor BMPR-IA-QD in the dorsal side of *Xenopus* embryos causes ventralization, mimicking the effect of BMP2/4 (Shibuya *et al*., 1998). Consistent with this, injection of an mRNA encoding BMPR-IA-QD induced ventral mesodermal markers such as Xwnt-8, Xhox3 and Xvent-1 in animal caps (Figure 7). To demonstrate the involvement of XIAP in the BMP2/4 signaling pathway, we examined the effects of XIAP mutants, XIAP-BIR and XIAP-RING (see Figure 2C), on induction of ventral mesodermal markers in the animal cap assay. As shown in Figure 7, the N-terminal deletion mutant of XIAP expressing the RING finger domain, which retains its BMP receptor-binding activity, partially reversed the expression of ventral mesodermal markers induced by the constitutively active BMPR-IA. In contrast, the full-length XIAP or the C-terminal deletion mutant expressing the BIR motifs had no such effect. These results suggest that the truncated form of XIAP lacking the BIR motifs act as a dominant-negative inhibitor of the BMP2/4 signaling pathway by interacting with the BMP receptors through the RING finger domain. This provides evidence that XIAP plays an important role in BMP signaling.

We have shown previously that injection of greater amounts of xTAK1 (100 pg) and xTAB1 (1 ng) mRNAs in early embryos induces cell death (Shibuya *et al*., 1998). However, concomitant overexpression of human *bcl-2* with both xTAK1 and xTAB1 in dorsal blastomeres not only rescues cell death but also causes the ventralization of the embryos. On the other hand, BMP2/4 or the constitutively active BMPR-IA induce only ventral mesoderm formation in early *Xenopus* embryos. These results raise the possibility that an anti-apoptotic molecule functions in the BMP signaling pathway between the BMP receptors and TAB1–TAK1. It is likely that XIAP is a candidate for such a molecule, since overexpression of XIAP can inhibit apoptosis induced by a variety of stimuli (Duckett *et al.*, 1996; Liston *et al.*, 1996; Uren *et al.*, 1996). We tested the ability of XIAP to prevent TAB1–TAK1-induced cell death. When XIAP mRNA (500 pg) was co-injected with xTAK1 (100 pg) and xTAB1 (1 ng) mRNAs into the dorsal marginal zone of 4-cell embryos, cell death was inhibited and the embryos were ventralized more significantly than those obtained by injection of *bcl-2* with both xTAK1 and xTAB1 (Table I). Thus, XIAP blocks apoptosis induced by TAB1–TAK1 and enhances ventral mesoderm formation in early *Xenopus* embryos.

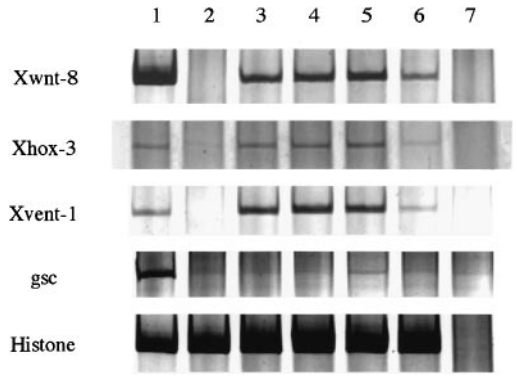

Fig. 7. Effect of XIAP mutants on mesoderm induction caused by a constitutively active BMPR-IA-QD. Synthetic mRNAs encoding the indicated DNA sequences were injected into the equatorial regions of two dorsal blastomeres at the 4-cell stage, and total RNAs were prepared at gastrula stage 11. RNA was analyzed by RT–PCR for the presence of the indicated transcripts: 1, whole embryo; 2, uninjected; 3, BMPR-IA-QD; 4, BMPR-IA-QD and XIAP; 5, BMPR-IA-QD and XIAP-BIR; 6, BMPR-IA-QD and XIAP-RING; 7, –RT. Xvent-1 is a marker of ventral mesoderm.

Table I. Effect of XIAP on TAB1–TAK1-induced apoptosis in *Xenopus* embryonic development

mRNAs	Percent survival (number of embryos)[a]	Average DAI[b]
xTAK1 + xTAB1	0 (39)	–
XIAP	94 (32)	5.0
xTAK1 + xTAB1 + XIAP	89 (28)	2.7
xTAK1 + xTAB1 + *bcl-2*	100 (27)	3.4

[a]Dorsal injection of higher doses of xTAK1 (100 pg) and xTAB1 (1 ng) mRNAs in the 4-cell stage caused the cell death at the early gastrula stage. At stage 36, survival embryos were scored. Dorsal injection of *bcl-2* or XIAP together with xTAK1 (100 pg) and xTAB1 (1 ng) blocked apoptosis and caused ventralization.
[b]Synthetic mRNAs containing xTAK1, xTAB1 and *bcl*-2 or XIAP sequences were injected into the equatorial regions of two dorsal or two ventral blastomeres at the 4-cell stage and phenotypes were scored at tadpole stage 36. The average DAI (Kao and Elinson, 1989) is a measure of the degree of dorsal and anterior mesodermal patterning for each group.

Discussion

Previously we have identified TAK1 and its activator TAB1 as components functioning in the TGF-β family signal transduction pathway (Yamaguchi *et al*., 1995; Shibuya *et al*., 1996). We failed to detect association of TAB1 with wild-type, kinase-negative or catalytically active BMP type I receptor by co-transfection and co-immunoprecipitation methods (data not shown). This suggests that TAB1 may function several steps downstream from the receptors. In the present study we identified XIAP as a candidate mediator of BMP signaling which links the receptors and TAB1–TAK1. We found that XIAP associated not only with TAB1 but also with the BMP receptors, prompting us to examine whether it played any role, either positive or negative, in the transmission of the BMP signal. For example, if XIAP is responsible for transducing the activation signal from the receptors to

TAB1–TAK1, XIAP should help to activate the downstream events. Indeed, we found that overexpression of XIAP in dorsal blastomeres stimulated the ventralization of *Xenopus* embryos in a TAB1–TAK1-dependent manner. This suggests that XIAP is a positive regulator functioning between the receptors and TAB1–TAK1 in the BMP signaling pathway. However, it is unclear exactly how XIAP regulates BMP signaling upstream of TAB1–TAK1 in the pathway. One possibility is that XIAP may serve to stabilize the TAB1–TAK1 complex. Alternatively, XIAP may be recruited to the receptors where it then functions as an adaptor or scaffolding protein for recruitment of TAB1–TAK1. Other mammalian IAP members, c-IAP1 and c-IAP2, are recruited to TNF receptor II by the TRAF1 and TRAF2 complex (Rothe *et al.*, 1995). In addition, c-IAP1 and TRAF2 are recruited to TNF receptor I by TRADD (Shu *et al*., 1996). Although these findings suggest that c-IAP1 and c-IAP2 act to mediate or modulate TNF-α signaling, their functional role in the TNF-α signal transduction pathway is still unresolved. Understanding how the interaction of XIAP with the receptors and TAB1 contributes to BMP signaling will help to elucidate the role of c-IAP1/c-IAP2 proteins as mediators of TNF-α signaling.

Given the degree of functional conservation in the TGF-β superfamily signaling pathways observed between the mammals and *Drosophila*, it is tempting to speculate that an IAP-like protein may also play a role in *Drosophila* TGF-β family Dpp signal transduction. This possibility is supported by a recent finding that the *Drosophila* IAP homologs DIAP1 and DIAP2 (Hay *et al*., 1995) interact with the Dpp type I receptor, Thick veins (Tkv) (Oeda *et al*., 1998). Therefore, the discovery that IAP proteins are involved in the process of TGF-β family signal transduction may provide additional insights into the TGF-β family signaling.

A common structural feature of all IAP family members are tandem repeat units, termed BIR motifs. Our two-hybrid and co-immunoprecipitation analyses provide evidence that it is the BIR motif-containing domain of XIAP that mediates its interaction with TAB1. Likewise, c-IAP1 and c-IAP2 have been shown to associate with TRAF1 and TRAF2 through their N-terminal BIR motifs (Rothe *et al.*, 1995), confirming the role of these motifs in mediating protein–protein interaction. Thus, the BIR motif probably plays a critical role in the ability of IAP proteins to modulate signal transduction by facilitating protein–protein interaction. However, TAB1 and TRAF1/TRAF2 have no apparent amino acid similarity, suggesting that the structures recognized by the c-IAP1/c-IAP2 BIRs and XIAP BIRs differ. Accordingly, c-IAP1 and c-IAP2 do not interact with TAB1, and XIAP does not associate with TRAF1 or TRAF2 (Uren *et al.*, 1996).

In addition to the BIR motifs, all known IAP family members contain RING finger domains. This sequence motif has been observed in a wide variety of nuclear and cytoplasmic proteins and is thought to be involved in protein–DNA and protein–protein interactions. The RING finger domains of IAPs may represent effector or regulatory domains that are involved in IAP function. The RING finger motif of XIAP was involved in association with BMP type I receptor. Consistent with this result, *Drosophila* DIAP1 interacts with the Dpp type I receptor via the RING finger domain (Oeda *et al*., 1998). Thus, the interaction of IAP proteins with the receptors is conserved between mammals and *Drosophila*. This raises an interesting possibility that *Drosophila* DIAP protein might interact with the *Drosophila* homolog of TAB1 through its BIR motifs. In the case of *Drosophila*, DIAP1 can interact with Tkv receptor in the yeast two-hybrid assay (Oeda *et al*., 1998), indicating that DIAP1 can directly associate with Tkv. On the other hand, we failed to detect an interaction between XIAP and the BMP type I receptor in a yeast two-hybrid assay (unpublished data). Although c-IAP1 and c-IAP2 were identified as proteins which co-purify with the TNF receptor II, they were shown not to interact directly with the receptor, but rather to be recruited to the receptor via their interaction with the TRAF1/TRAF2 complex (Rothe *et al*., 1995). Therefore, the possibility remains that another factor(s) may mediate the interaction between XIAP and the receptors.

We have previously shown that TAK1 activation induces not only cell death but also ventral mesoderm formation (Shibuya *et al*., 1998). On the other hand, BMP2/4 or the constitutively active BMP type I receptor induce only ventral mesoderm formation in early *Xenopus* embryos. Cell death induced by TAK1 is apoptotic in nature and is blocked by co-expression of the anti-apoptotic protein Bcl-2 (Shibuya *et al*., 1998). Therefore, it can be expected that an anti-apoptotic protein functions between the receptors and TAB1–TAK1 in the BMP signaling pathway. Our discovery that XIAP functions in this manner raises the possibility that XIAP acts to regulate cellular apoptosis in early *Xenopus* embryos. In support of this notion, overexpression of XIAP was able to block TAK1-induced apoptosis in *Xenopus* embryos. This may be due to the ability of XIAP to directly inhibit caspase activity (Deveraux *et al*., 1997). XIAP death-preventing activity localizes to the N-terminal BIR motifs. Taken together these results suggest that activation of the BMP receptors by ligand binding elicits a signal via interaction with XIAP. XIAP in turn inhibits apoptosis and activates TAK1 by interacting with caspases and TAB1, respectively. Activated TAK1 probably induces apoptosis through activation of the JNK or p38 MAP kinase cascade. Thus, the BMP2/4 ligands may activate both apoptotic and anti-apoptotic pathways, enabling them to induce ventral mesoderm formation without apoptosis in early *Xenopus* embryos. In some systems, BMP4 can induce apoptosis, 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 the TNF-α signaling pathway, the TNF-α signal activates both the apoptotic and anti-apoptotic pathways, which are mediated by caspase activation and NF-κB induction, respectively (Boldin *et al*., 1996; Liu *et al*., 1996; Muzio *et al*., 1996). Thus, there appears to be a delicate balance between the opposing activities of proteins that promote and those that inhibit cells death.

The recently identified Smad family of proteins, which are related to *Drosophila* Mad, have been shown to play a pivotal role downstream in the signal transduction pathways mediating the effects of various TGF-β superfamily members (Heldin *et al.*, 1997; Massague *et al.*, 1997). Smads function as the physiological substrates of type I receptors and transmit signals directly from the

receptor to the nucleus. However, functional links between the Smads and the TAB1–TAK1 pathways remain unknown. Co-transfection of Smad4, TAB1 and TAK1 together resulted in the strong activation of TGF-β-dependent gene expression (unpublished data). This demonstrates that the Smad and TAK1 pathways functionally co-operate, suggesting that TGF-β signals mediated by each pathway are integrated in the nucleus to activate gene expression. Recently, a novel winged-helix transcription factor termed FAST1 was identified as a sequence-specific DNA-binding protein that interacts with Smad2 and Smad4 to activate the activin response gene (Chen *et al*., 1996, 1997; Liu *et al*., 1997). Thus, the Smad proteins form a transcriptional complex in co-operation with particular transcription factors in response to specific signals. One likely mechanism by which the TAK1-mediated MAP kinase pathway regulates TGF-β-induced gene expression is the phosphorylation of a transcription factor(s). We imagine that TGF-β activates the Smad and TAK1 pathways, resulting in the formation of an active transcription complex composed of Smads and other DNA-binding transcription factors.

Materials and methods

Two-hybrid screening

pLexA-TAB1(1–418) was constructed by fusing the TAB1(1–418) coding sequences (Shibuya *et al*., 1996) in-frame to the LexA DNA-binding domain encoded in the vector pBTM116. This construct and a human brain cDNA library fused to the GAL4 activation domain were expressed in the *Saccharomyces cerevisiae* strain L40, which contains an integrated *HIS3* gene driven by a promoter containing LexA binding sites.

Antibodies

The anti-HA antibody Y-11, the anti-Myc antibodies 9E10 and A-14, and the anti-GST antibody 12 were purchased from Santa Cruz Biotechnology. The anti-Flag antibody M2 was purchased from Kodak. Monoclonal antibodies to XIAP were generated in mice immunized with a GST fusion protein containing full-length XIAP.

Plasmids

A cDNA encoding TAB1 tagged at the N-terminus by the Flag epitope (MDYKDDDDK; recognized by the monoclonal antibody M2) was constructed by inserting the full-length TAB1 cDNA into the vector pFLAG-CMV-2 (Kodak). cDNAs encoding XIAP, XIAP-BIR and XIAP-RING tagged at the N-terminus by the Myc epitope (LEQKLISEEDLN; recognized by the monoclonal antibodies 9E10 and A-14) were constructed by inserting cDNAs of the full-length XIAP, XIAP(1–412) and XIAP(413–497), respectively, into the vector pCS3MT. The cytoplasmic domain of BMPR-IA (amino acid 171–532) was inserted into the mammalian expression vector pEBgs which produces fusion proteins joined to GST at the N-terminus under the control of the EF-1α gene promoter. HA-BMPR-IA (Hoodless *et al.*, 1996) and Myc-ActR-II (Chen *et al*., 1995) have been described previously. Both receptors are tagged at the C-terminus with HA or Myc epitope.

Animal cap assay

The XIAP and c-IAP1 cDNAs were subcloned into the CS2+ vector (Rupp *et al*., 1994). Capped mRNA was synthesized from linearized vectors using the mMessage Machine kit (Ambion). The 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 as 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 gastrula or tadpole stages. Total RNA was then extracted and analyzed with RT–PCR. RT–PCR assays and primer sequences were as published previously (Wilson and Melton, 1994; Wilson and Hemmati-Brivanlou, 1995).

Immunoprecipitation and GST pull-down assay

COS7 or 293 cells were transiently transfected with the indicated constructs by the calcium phosphate method. After 24 h, cells were washed once with phosphate-buffered saline (PBS) and lysed in 0.3 ml of 0.5% Triton X-100 buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM $MgCl_2$, 2 mM EGTA, 10 mM NaF, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF and 20 μM aprotinin. Cellular debris was removed by centrifugation at 10 000 *g* for 5 min. For co-immunoprecipitation, 300 μl aliquots of lysates were incubated with 10 μl of protein G–Sepharose beads to preclear, and then incubated with appropriate antibodies coupled to protein G–Sepharose beads at 4°C for 2 h. Immunoprecipitates were washed five times with 600 μl of washing buffer (20 mM HEPES pH 7.4, 500 mM NaCl 10 mM $MgCl_2$) and analyzed by Western blot analysis with the appropriate antibodies. For the GST pull-down assay, preclearing of lysates was omitted and gluthathione–Sepharose beads were used instead of protein G–Sepharose beads. The immunoprecipitates and aliquots of total lysates were resolved on SDS–PAGE, and transferred to PVDF membranes (Hybond-P, Amersham). The membranes were immunoblotted with various antibodies, and bound antibodies were visualized with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies using the enhanced chemiluminescence (ECL) Western blotting system (Amersham) according to the manufacturer's instructions.

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