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Short Communication

The N-terminus zinc finger domain of *Xenopus* SIP1 is important for neural induction, but not for suppression of *Xbra* expression

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ABSTRACT Smad-interacting protein-1 (SIP1), also known as δEF2, ZEB2 and zfhx1b, is essential for the formation of the neural tube and the somites. Overexpression of *Xenopus* SIP1 causes ectopic neural induction via inhibition of bone morphogenetic protein (BMP) signaling and inhibition of *Xbra* expression. Here, we report the functional analyses of 4 domain-deletion mutants of XSIP1. Deletion of the N-terminus zinc finger domain suppressed neural induction and BMP inhibition, but these were not affected by deletion of the other domains (the Smad binding domain, the DNA-binding homeodomain together with the CtBP binding site and the C-terminus zinc finger). Therefore SIP1 does not inhibit BMP signaling by binding to Smad proteins. In contrast, all of the deletion constructs inhibited *Xbra* expression. These results suggest that the N-terminus zinc finger domain of XSIP1 has an important role in neural induction and that *Xbra* suppression occurs via a mechanism separate from the neural inducing activity.

KEY WORDS: *Smad-interacting protein-1, Brachyury (T), ZEB, δEF, Zfhx1, homeodomain*

Introduction

The δEF1 family proteins, δEF1/ZEB1/Zfhx1a and Smad-interacting protein-1 (SIP1)/δEF2/ZEB2/Zfhx1b, were originally identified as transcriptional repressors (van Grunsven *et al.*, 2001). Proteins in this family have multiple conserved domains: a homeodomain (HD), a C-terminal binding protein (CtBP) binding site (CBS) and two two-handed zinc finger domains (one at the N-terminus, NZf; and one at the C-terminus, CZf). SIP1 also has a Smad binding domain (SBD), which interacts with regulatory Smads, the mediators of TGF-β superfamily signaling (Funahashi *et al.*, 1993, Verschueren *et al.*, 1999, Yoshimoto *et al.*, 2005).

Loss-of-function studies have shown that δEF1/ZEB1 plays an essential role in skeletal formation and T-cell development (Higashi *et al.*, 1997, Takagi *et al.*, 1998). Biochemical studies have shown that δEF1/ZEB1 represses transcription by a mechanism that involves binding of the CBS to CtBP, which recruits histone deacetylases (Chinnadurai, 2002, Furusawa *et al.*, 1999, Postigo and Dean, 1999b). The zinc finger domains also play a role in transcriptional repression, with NZfs functioning in T lymphocytes and CZfs in muscle (Postigo and Dean, 1999a). In addition, the NR domain, which is closest to the N-terminus, is required for complete repression of *beta1-crystallin* enhancer (Sekido *et al.*, 1994). On the other hand, δEF1 has been shown to function as a transcriptional activator for bone morphogenetic protein (BMP) signaling, by associating with p300 and p/CAF (Postigo *et al.*, 2003). These findings suggest that δEF1/ZEB1 has both repressor and activator functions and that the multiple domains enable it to play different roles depending upon context.

In comparison to δEF1/ZEB1, the properties of SIP1/δEF2/ZEB2 have been less extensively investigated. SIP1 was originally identified as a protein binding to Smad1 by screening using the yeast two-hybrid system (Verschueren *et al.*, 1999). SIP1 represses transcription on the *E-cadherin* promoter, independently of CtBP binding (van Grunsven *et al.*, 2003). Deletion of the

Abbreviations used in this paper: BMP, bone morphogenetic protein; CtBP, C-terminal binding protein; CZf, C-terminus zinc finger; HD, homeodomain; NZf, N-terminus zinc finger; SBD, smad binding domain; SIP, smad-interacting protein.

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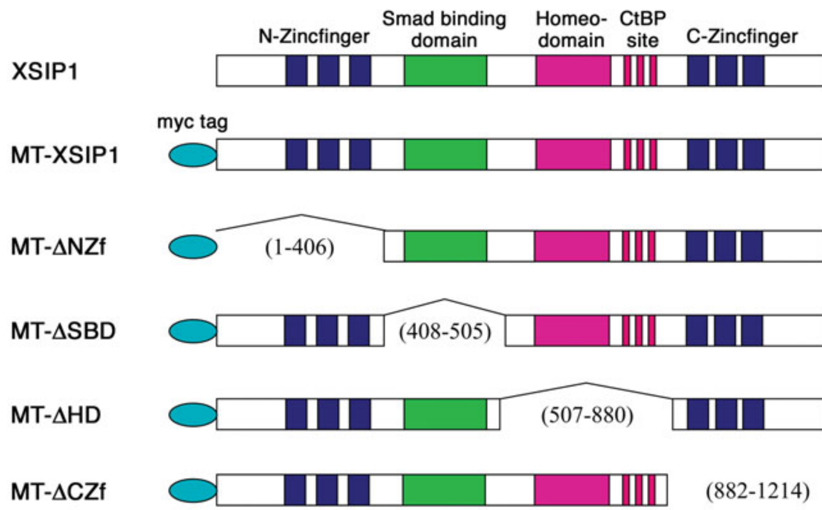
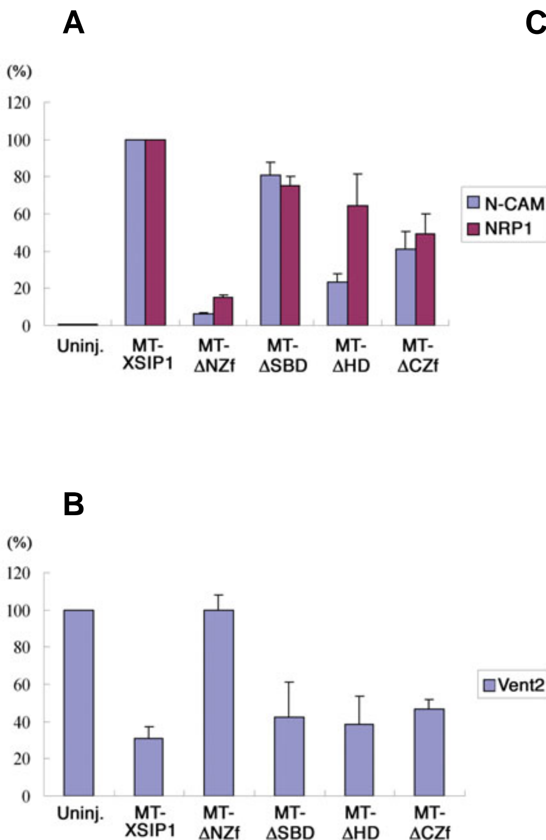


Fig. 1. *XSIP1* deletion constructs. *Numbers indicate amino acid positions for deleted sequences. The stop codon of the pCS2-MT vector was used for* MT-XSIP1-ΔCZf.

SBD decreases repressor activity on the *E-cadherin* promoter (Comijn *et al.*, 2001). *Xenopus* SIP1 (XSIP1) inhibits BMP signaling and drives the putative epidermis towards a neural fate (Eisaki *et al.*, 2000, Nitta *et al.*, 2004, van Grunsven *et al.*, 2006). Overexpression of XSIP1 suppresses the transcription of BMP and genes downstream of BMP signaling (Nitta *et al.*, 2004, Postigo, 2003, van Grunsven *et al.*, 2006). One of the genes downstream of BMP signaling is *Xenopus Vent2*. The promoter for *Xenopus Vent2* contains an E2 box (the binding site for the δEF1 family) and is negatively regulated by SIP1 (Postigo *et al.*, 2003). In addition, XSIP1 directly inhibits expression of the pan-mesodermal gene, *Xbrachyury* (*Xbra*) (Papin *et al.*, 2002, van Grunsven *et al.*, 2006). It has also been suggested that SIP1 can function as a transcription activator, based on its action on the *Foxe3* promoter (Yoshimoto *et al.*, 2005). A recent study has revealed that XSIP1 associates with p300 and p/CAF (van Grunsven *et al.*, 2006), suggesting that SIP1, like δEF1, can act as both an activator and a repressor in a context-dependent manner.

Here we analyzed which domain of SIP1 is required for neural formation, using 4 domain-deletion mutants of XSIP1. Deletion of NZf markedly reduced the neural inducing activity of SIP1. The other mutations, including the SBD deletion, did not affect the neural inducing activity or BMP inhibitory activity. In addition, all deletion mutants retained inhibitory activity against *Xbra* expression. These results suggest that the inhibitory activity of XSIP1 on BMP signaling is dependent upon the N-terminal domain and that suppression of *Xbra* expression caused by SIP1 is regulated by a different mechanism.



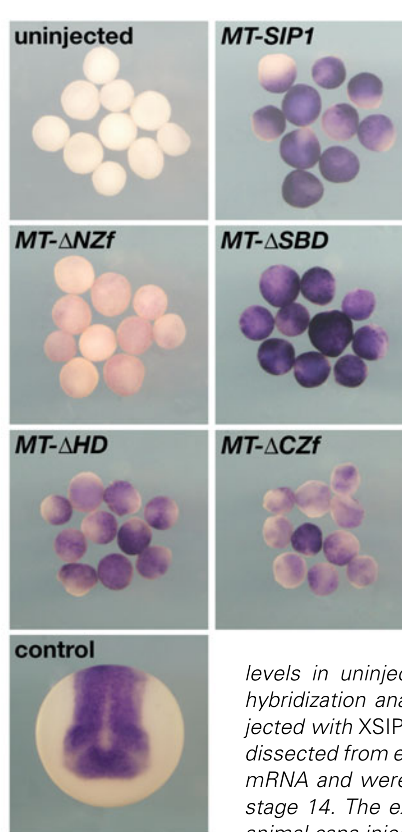


Fig. 2. Real-time RT-PCR and whole-mount *in situ* hybridization analyses of animal caps injected with *XSIP1* deletion mutants. (A) N-CAM *and* NRP1 *expression levels in animal caps injected with* XSIP1 *construct mRNA. Animal caps were dissected from embryos injected with 500 pg of* XSIP1 *construct mRNA and were cultured until the sibling embryos reached stage 32. Expression of* N-CAM *and* NRP1 *mRNA was quantified by real-time RT-PCR. The results are represented as percentages relative to the expression levels in animal caps injected with* MT-XSIP1 *mRNA.* **(B)** Vent2 *expression levels in animal caps injected with* XSIP1 *construct mRNA. Animal caps were dissected from embryos injected with 500 pg of* XSIP1 *deletion mutant mRNA and were cultured until the sibling embryos reached stage 11. Expression of* Vent2 *mRNA was quantified by real-time RT-PCR. The results are represented as percentages relative to the expression levels in uninjected animal caps.* **(C)** *Whole-mount in situ hybridization analysis of* Sox2 *expression in animal caps injected with* XSIP1 *deletion mutant mRNA. Animal caps were dissected from embryos injected with 500 pg* XSIP1 *construct mRNA and were cultured until the sibling embryos reached stage 14. The expression of* Sox2 *was markedly reduced in animal caps injected with* MT-XSIP1-ΔNZf *mRNA.*

Results and Discussion

Deletion of N-terminus containing NZf changes the function of XSIP

To investigate the function of each conserved domain of SIP1, we generated 4 domain-deletion constructs of XSIP1. These corresponded to 4 conserved domains of SIP1: (1) the NZf, (2) the SBD, (3) the HD and CBS together (HD-CBS) and (4) the CZf (Fig. 1). All constructs were tagged with 6 myc epitopes on the N-terminus to confirm the protein expression. We confirmed that the myc-tagged XSIP1 (MT-XSIP1) had neural inducing activity that was equivalent to XSIP1, indicating that the introduced myc-tag did not affect the function of the protein (data not shown).

We first examined the activity of each deletion construct on neural induction. All myc-tagged constructs were overexpressed in animal caps and the expression levels of the neural differentiation markers, *N-CAM* and *NRP1*, were analyzed by real-time RT-PCR (Fig. 2A). While MT-XSIP1 and MT-XSIP1-ΔSBD induced the expression of *N-CAM* and *NRP1*, MT-XSIP1-ΔNZf showed marked loss of activity. Overexpression of MT-XSIP1-ΔHD-CBS and MT-XSIP1-ΔCZf also resulted in weaker expression of these markers, in comparison with MT-XSIP1.

Inhibition of BMP signaling results in neural induction, so we next investigated the expression levels of *Vent2*, a gene downstream of BMP signaling that is directly regulated by XSIP1 (Postigo *et al.,* 2003). When BMP was overexpressed in animal caps, *Vent2* expression was induced (Fig. 2B). MT-XSIP1-ΔNZf did not suppress the expression of *Vent2*, whereas MT-SIP1 and the other 3 deletion constructs did inhibit *Vent2* expression.

We also evaluated the function of NZf on neural induction by *in situ* hybridization of animal caps (Fig. 2C). All 5 constructs were overexpressed in animal caps, followed by *in situ* hybridization for the neural marker gene, *Sox2* (Kondoh *et al.,* 2004, Sasai, 2001). Consistent with the results using real-time RT-PCR, deletion of NZf caused the loss of *Sox2* induction, while activity was retained by the other deletion constructs. These results indicate that NZf is required for both neural induction and suppression of BMP signaling, whereas the other conserved domains of XSIP1 (SBD, HD, CBS and CZf) are not essential for these activities in animal caps. In addition, although SIP1 was originally identified as a protein that interacts with Smad proteins, these findings indicate that binding to Smad1 is not important for these activities of XSIP1. It has been reported that the zinc-finger clusters of the N-terminal and C-terminal regions of δEF1 directly bind to the E2-box sequence and that the NR domain in the N-terminal region acts as an active repressor to silence the enhancer (Postigo and Dean, 1999a, Sekido *et al.,* 1994). The NR domain in the N-terminal region is also conserved in XSIP1, suggesting that XSIP1 works as an active repressor of genes downstream of BMP signaling.

Inhibition of Xbra *expression is not affected by deletion of any single domain*

In addition to inhibition of BMP signaling and neural induction, XSIP1 directly represses endogenous *Xbra* expression (Papin *et al.,* 2002, van Grunsven *et al.,* 2006). *Xbra* expression is induced by Nodal/Smad2 signaling and FGF signaling (Agius *et al.,* 2000, Amaya *et al.,* 1991, Eimon and Harland, 1999, Onuma *et al.,* 2002, Takahashi *et al.,* 2000, Tanegashima *et al.,* 2000). To investigate the contribution of each conserved domain of SIP1 on repression of *Xbra* expression, we carried out whole-mount *in situ* hybridization analysis on embryos overexpressing the 4 deletion mutants (Fig. 3). All embryos that were injected with *MT-XSIP1* mRNA showed suppression of *Xbra* expression on the side of injection (n = 66) (Fig. 3B). Expression of *Xbra* was also inhibited in every embryo injected with one of the 4 domain-deletion mutants (MT-XSIP1-ΔNZf, n = 62; MT-XSIP1-ΔSBD, n = 66; MT-XSIP1-ΔHD-CBS, n = 61; MT-XSIP1-ΔCZf, n = 68; Fig. 3C-F). These results indicate that no single functional domain of SIP1 is responsible for suppression of *Xbra* expression and that multiple domains may be independently involved in *Xbra* suppression. Our findings also suggests that SIP1 suppresses *Xbra* expression by a mechanism that is different from suppression of BMP signaling.

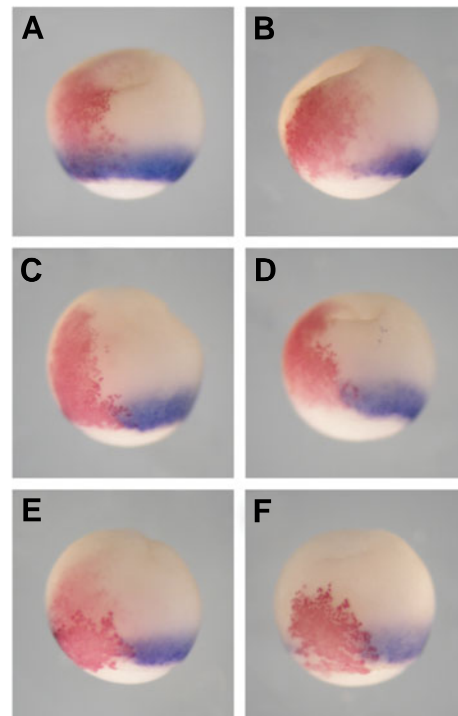


Fig. 3. *Xbra* expression was downregulated by overexpression of XSIP1 deletion mutants. *Each mRNA (500 pg) was co-injected with* lacZ *mRNA into the marginal region of one blastomere of 4-cell-stage embryos. The injected embryos were cultured until the sibling embryos reached stage 11.* Xbra *expression was downregulated in the region where mRNA was injected (marked by Red-Gal staining). The embryos shown were injected with* lacZ *alone* **(A)**, MT-SIP1 **(B)**, MT-ΔNZf **(C)**, MT-ΔSBD **(D)**, MT-ΔHD-CBS **(E)**, *or* MT-ΔCZf **(F)**.

Experimental Procedures

Embryos

Xenopus laevis embryos were obtained by artificial fertilization and were cultured in 10% Steinberg's solution (SS) at 20°C. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1956).

Plasmid constructs

The plasmids pCS2-MT-XSIP1, pCS2-MT-XSIP1-ΔNZf, pCS2-MT-XSIP1-ΔSBD, pCS2-MT-XSIP1-ΔHD-CBS and pCS2-MT-XSIP1-ΔCZf were created by PCR and subcloned into pCS2-MT vector. The deleted

regions were amino acids 1 to 406 for MT-XSIP1-ΔNZf, amino acids 408 to 505 for MT-XSIP1-ΔSBD, amino acids 507 to 880 for MT-XSIP1-ΔHD-CBS and amino acids 882-1214 for MT-XSIP1-ΔCZf (see Fig. 1).

To construct pGEM-Sox2, *Sox2* was amplified by PCR using the forward primer 5'- TCTGCCAGCCTTTGCTCC-3' and the reverse primer 5'- CACATGTGCGACAGAGGC-3' and cloned into pGEM®-T Easy vector (Promega, Madison, Wis). All constructs were verified by sequencing.

Microinjection and animal cap dissection

Microinjection was performed in 100% SS containing 5% Ficoll. mRNA was synthesized using SP6 mMESSAGE mMACHINE (Ambion, Austin, Tex) with linearized pCS2-XSIP1 (Eisaki *et al.,* 2000), pCS2-MT-XSIP1, pCS2-MT-XSIP1-ΔNZf, pCS2-MT-XSIP1-ΔSBD, pCS2-MT-XSIP1-ΔHD-CBS, pCS2-MT-XSIP1-ΔCZf and pCS2-NLS-lacZ (Takahashi *et al.,* 2000). Animal caps were dissected at stage 9 and were cultured in 100% SS containing 0.1% bovine serum albumin for RT-PCR analysis and whole-mount *in situ* hybridization.

RT-PCR analysis and real-time RT-PCR

Total RNA was extracted from *Xenopus* embryos using Isogen (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized using 1 μg of total RNA with oligo-(dT) primer and SuperScript™ II RT (Invitrogen, Carlsbad, Calif). One-twentieth of the cDNA was used as a template for RT-PCR. Real-time RT-PCR was performed on an ABI PRISM 7700 (Applied Biosystems, Foster City, Calif) using SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) according to the QuantiTect SYBR Green Kit instructions. *Elongation factor 1α (EF-1α)* was used as an internal control and the relative expression amount of each gene was normalized to the expression amount of *EF-1α*. The results are averages of 3 independent experiments and error bars indicate SEM. Primer sequences were as follows:
N-CAM
forward 5'-CACAAGGGGAACCTAGTG-3' and
reverse 5'-CTATTAGAAGGTACCCGC-3';
NRP1
forward 5'-CTGTGAGAGGCCGATCTC-3' and
reverse 5'-GTTCTCTCTACACGAAAC-3';
Vent2
forward 5'-GTTCTTTGGTGTGTACGG-3' and
reverse 5'-GCAGGTAGAGCATCTGAA-3';
EF-1α
forward 5'-TTGCCACACTGCTCACATTGCTTGC-3' and
reverse 5'-ATCCTGCTGCCTTCTTTTCCACTGC-3'.

Whole-mount in situ hybridization

Whole-mount *in situ* hybridization analysis was performed according to Harland (Harland, 1991). Antisense RNA probes were synthesized with the templates pGEM-Sox2 and pSP73-Xbra (Smith *et al.,* 1991). For linage tracing, NLS-lacZ mRNA was co-injected and the embryos were stained with Red-Gal (Research Organics, Cleveland, Ohio) before *in situ* hybridization.

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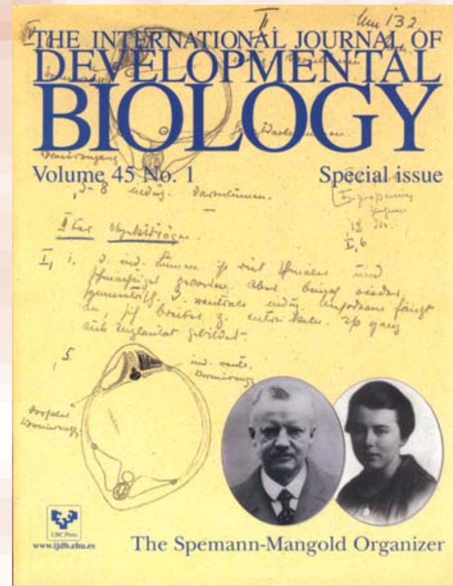

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