SANE, a Novel LEM Domain Protein, Regulates Bone Morphogenetic Protein Signaling through Interaction with Smad1*

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G. Praveen Raju, Neviana Dimova, Peter S. Klein†§¶, and Hui-Chuan Huang*‡

From the Cell & Molecular Biology Graduate Group, the Department of Medicine, and the Howard Hughes Medical Institute, the University of Pennsylvania Medical Center, Philadelphia, Pennsylvania 19104

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily that play important roles in bone formation, embryonic patterning, and epidermal-neural cell fate decisions. BMPs signal through pathway specific mediators such as Smads1 and 5, but the upstream regulation of BMP-specific Smads has not been fully characterized. Here we report the identification of SANE (Smad1 Antagonistic Effector), a novel protein with significant sequence similarity to nuclear envelop proteins such as MAN1. SANE binds to Smad1/5 and to BMP type I receptors and regulates BMP signaling. SANE specifically blocks BMP-dependent signaling in Xenopus embryos and in a mammalian model of bone formation but does not inhibit the TGF-β Smad2 pathway. Inhibition of BMP signaling by SANE requires interaction between SANE and Smad1, because a SANE mutant that does not bind Smad1 does not inhibit BMP signaling. Furthermore, inhibition appears to be mediated by inhibition of BMP-induced Smad1 phosphorylation, blocking ligand-dependent nuclear translocation of Smad1. These studies define a new mode of regulation for intracellular BMP/Smad1 signaling.

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily that were first identified by their ability to induce ectopic bone formation in the soft tissue of rats (1). Because then BMP family members have been identified in diverse organisms from Drosophila and Caenorhabditis elegans to mammals and, in addition to their roles in bone formation, have been shown to play critical roles in embryonic patterning and neural induction (2–7).

The importance of BMP-related factors in embryonic development has been elucidated by extensive analysis in model organisms. For example, in Drosophila, the BMP orthologue decapentaplegic (dpp) plays a critical role in dorsal-ventral patterning of the embryo as well as patterning of the imaginal discs (8). In C. elegans, BMPs regulate the size of the organism as well as patterning of the male tail (9). A role for BMPs in vertebrate development was first suggested by observations that ectopic expression of BMPs causes expansion of ventral tissues and reduction of dorsal and anterior structures in Xenopus embryos (10, 11) and ectodermal explants (12). In support of this, BMP-4 is expressed in a ventral-lateral crescent in the marginal zone of gastrula stage embryos (12). BMPs have also been implicated in neural cell fate determination in Xenopus (13, 14).

The importance of BMP signaling in bone formation and in vertebrate development has been confirmed by genetic analyses in mouse and zebrafish (2, 3, 5, 15). Thus, mutation of the BMP-5 (short ear mutant) and BMP-7 genes in mice disrupt multiple aspects of skeletal development (16), whereas mice lacking BMP-2, BMP-4, or the BMP type I receptor, ALK-3, die in utero prior to the onset of bone formation, supporting an additional role for BMPs in earlier developmental processes, including mesoderm induction and neural-epidermal cell fate decisions (2). In zebrafish, a forward genetic screen for defects in early embryonic development yielded a series of dorsaled mutants, including mutations in genes encoding BMP 2b and 7 (swirl and snailhouse), the BMP receptor ALK5 (lost-a-fin), and other extracellular and intracellular components of this tightly regulated signaling pathway (e.g. Smad5/somitaban and tolloid/mini-fin) (15, 17).

Similar to other TGF-β superfamily members (4, 5), BMPs bind to a heteromeric receptor complex composed of type II and type I receptors; the type II receptor phosphorylates the type I receptor, thus activating the type I receptor kinase, which in turn phosphorylates cytoplasmic Smad proteins (4, 18, 19). Phosphorylated Smad proteins form a multimeric complex that translocates to the nucleus and activates transcription through interaction with DNA binding proteins or through direct DNA binding.

Smad proteins can be divided into three broad groups, the pathway-restricted Smads, the common-mediator Smads, and the antagonistic Smads (4, 18). The pathway restricted Smads are regulated by specific TGF-β superfamily members (20). For example, Smad1 and Smad5 are specifically activated by BMPs, which lead to phosphorylation of these Smads on carboxyl-terminal serines (21). Similarly, activin/nodal/TGF-β ligands cause phosphorylation of analogous serines in Smad2 and Smad3 (22, 23). Overexpression of Smad1 in Xenopus mimics ectopic BMP signaling, inducing ventral mesoderm from ectodermal explants (24–26), whereas Smad2 can induce dorsal mesoderm, similar to the effect of activin- or nodal-related proteins (reviewed in Ref. 5).

BMP signaling is highly regulated in Drosophila and in vertebrates (20), as evidenced by the marked defects in embryonic development observed when these regulatory components are mutated in flies, zebrafish, or mice (2–5,15). Thus extracel-
Co-immunoprecipitation Analysis—Synthetic mRNA was generated by in vitro transcription (Ambion) and injected into Xenopus oocytes (5 ng) or embryos (1 ng). Xenopus oocyte or embryo (blastula stage) extracts were prepared as previously described (31) and immunoprecipitated with either anti-myc 9E10 monoclonal antibody or anti-Smad1 polyclonal antibody (T-20 or H-465) (Santa Cruz Biotechnology). Immunoprecipitates were analyzed by immunoblotting using antibodies to the myc epitope (9E10), Smad1 (H-465 or T-20), Smad2 (N-19), Smad5 (D-20) (Santa Cruz Biotechnology), and Smad4 and pSmad1 (Upstate Biotechnology). The SANE monoclonal antibody was generated by immunizing with the SANE-C fragment fused in-frame to the carboxyl terminus of glutathione S-transferase (GST) according to standard procedures. Four hybridoma clones were selected from initial screening and tested for specificity to SANE. The monoclonal antibodies react with the endogenous SANE protein of 98.5 kDa (as a single band) in Xenopus in Western blots, with myc-tagged full-length SANE (lower electrophoretic mobility), and with the SANE-C fragment but not with SANE-3C, indicating that the antibodies specifically react with an epitope in the SANE carboxyl terminus. Antibody binding to endogenous SANE in Western analysis is blocked by co-incubation with GST-SANE-C, protein (data not shown).

Xenopus Embryo Manipulations, RT-PCR Analysis, and in Situ Hybridization—mRNA for SANE constructs (1 ng) was injected into a single ventral blastomere of Xenopus four-cell embryos. Embryos were allowed to develop until tadpole stage for analysis of dorsal-ventral patterning phenotypes. For ectodermal explant assays, mRNA for SANE constructs (0.5 and 1 ng) was injected into the animal pole of Xenopus one-cell embryos, and ectoderm was explanted at stage 8. Explants were harvested at stage 23 for analysis of neural markers (NCAM, otx2, and XAG) or for the dorsal mesodermal marker (muscle actin) as previously described (14). For rescue of SANE, Smad1 mRNA (4 ng) was co-expressed with SANE mRNA (1 ng) in explants, and RT-PCR was performed as described above.

For mesoderm induction assays, BMP-4 (0.5 ng) or activin βB (10 ng) mRNA was co-injected with SANE mRNA (1 ng) into Xenopus one-cell embryos, ectoderm was explanted at stage 8, and explants were harvested at stage 10.5 explants for analysis of mesodermal markers (brachyury, un9, and goosecoid) as previously described (32).
expression was assessed by RT-PCR, as described, using primers for neural markers, NCAM, otx2, and XAG, or for the mesodermal markers, muscle actin, goosecoid (gsc), brachyury (Xbra), or Xunt8. In situ hybridization was performed as described previously (33, 34).

Stable C2C12 Cell Lines—C2C12 myoblasts were transfected with pCS2MT-SANE constructs or pCS2LacZ as control with pCR3.1 (Invitrogen), which contains the neomycin-selectable marker, in a ratio of 10:1 μg of DNA using LipofectAMINE (Invitrogen). Myoblasts were mock treated or treated with BMP-4 protein (50 μg/ml) or TGF-β1 (1 ng/ml) and stained for alkaline phosphatase as previously described (35). Alkaline phosphatase-positive cells were scored as a percentage of total cell number as determined by counting cell nuclei. At least 300 cells in multiple fields were counted.

Luciferase Reporter Assays—XVent2 luciferase reporter or XMix2 luciferase reporter plasmids (50 pg) were co-injected with either BMP-4 or activin βb mRNA alone or with SANE or SANE-ΔC mRNA (500–1500 pg) in the animal pole of one-cell Xenopus embryos. Extracts were made at late blastula stage (stage 9), and luciferase activity was measured according to standard protocols using the Monolight 3010 luminometer (BD Pharmingen).

Smad1 Nuclear Translocation Assay—NIH 3T3 cells were transiently transfected with expression vectors containing myc-tagged SANE. After 24 h to allow for gene expression, cells were starved and treated with BMP-4 protein (20 ng/ml, R&D Systems). Cells were fixed and stained by immunofluorescent methods as described previously (25) using an anti-Smad1-specific antibody (Upstate Biotechnology). Nuclear versus cytoplasmic localization of endogenous Smad1 was assessed by counting 200–400 cells per group. This experiment was repeated three times with similar results.

RESULTS

Cloning of Xenopus SANE—Full-length Xenopus Smad1 was used as bait in a two-hybrid screen of a Xenopus oocyte cDNA library (36). Partial cDNAs from twelve positive clones were isolated and sequenced. One of the partial clones, which was isolated three times, was sufficient to interact with Smad1 by co-immunoprecipitation analysis in Xenopus embryos. This partial clone was used to isolate a cDNA that includes myc-tagged SANE. After 24 h to allow for gene expression, cells were starved and treated with BMP-4 protein (20 ng/ml, R&D Systems). Cells were fixed and stained by immunofluorescent methods as described previously (25) using an anti-Smad1-specific antibody (Upstate Biotechnology). Nuclear versus cytoplasmic localization of endogenous Smad1 was assessed by counting 200–400 cells per group. This experiment was repeated three times with similar results.

SANE Interacts with Smad1—Endogenous SANE protein, with Smad1 in vivo in Xenopus embryos. Lysates from stage 9 embryos were immunoprecipitated with antibodies to SANE (lanes 1), a Smad1-specific antibody (lane 2), an antibody that recognizes Smad1, 2, 3, 5, and 8 (lane 3), or a control antibody (anti-myc, lane 4); immunoprecipitates were Western blotted with the SANE (upper panel) or Smad1 (lower panel) antibodies. The presence of mouse IgG heavy chain in SANE immunoprecipitates, which runs in a position close to Smad1, partially obscured the Smad1 band in lane 1, lower panel. B, SANE constructs used in co-immunoprecipitation analysis. All SANE constructs are tagged at the amino terminus with the myc epitope. The carboxyl-terminal 123 amino acids are deleted in SANE-ΔC constructs. C, SANE interacts with the carboxyl-terminal region of SANE. myc-tagged SANE and Smad1 were co-expressed in Xenopus oocytes. Extracts were immunoprecipitated with an anti-Smad1 antibody and Western blotted with anti-myc antibody (upper panel). Extracts were blotted with anti-myc antibody to confirm expression of all three SANE constructs (lower panel).

Specific antibody, with an antibody that recognizes multiple Smad family members (including Smad1, 2, 3, 5, and 8) or with a control antibody. Western blotting with monoclonal antibodies to SANE (Fig. 2A, upper panel, lanes 2 and 3) shows that SANE co-immunoprecipitates with Smad1, confirming that endogenous SANE and Smad1 interact in vivo. (Note that Smad1 protein migrates close to and is partially obscured by the mouse IgG heavy chain; Fig. 2A, lower panel, lane 1.) Furthermore, the region of SANE that interacts with Smad1 lies within the carboxyl-terminal 130 amino acids of SANE. This was shown by co-expressing a myc-tagged SANE carboxyl-terminal frag-
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Fig. 3. a, Smad1 constructs used in co-immunoprecipitation analysis. Smad1 N contains only the MH1 domain (amino acids 1–200), Smad1 C encodes the MH2 domain (amino acids 310–464), and Smad1 LC encodes the linker and the MH2 domain of Smad1 (amino acids 201–464). In Smad1-asp and Smad1-ala, the three carboxyl-terminal serines (amino acids 461, 462, and 464) are changed to aspartic acid or alanine residues, respectively. b, SANE interacts with the carboxyl-terminal domain of Smad1. Smad1-N (lane 1), Smad1-C (lane 2), or Smad1-LC (lane 3) was expressed with SANE in Xenopus oocytes. Extracts were Western blotted with an anti-Smad1 antibody (middle panel) or with anti-myc antibody for SANE expression (lower panel). c, Extracts were immunoprecipitated with an anti-Smad1 antibody, and analyzed by Western blot using an anti-myc antibody (upper panel). SANE was expressed alone (lane 1) or with either Smad1-ala (lane 2) or Smad1-asp (lane 3) in Xenopus oocytes. Extracts were immunoprecipitated with an anti-Smad1 antibody and Western blotted either with an anti-Smad1 antibody (middle panel) or with anti-myc antibody (upper panel). d, Specificity of SANE interaction with Smad proteins. Myc-SANE was expressed in Xenopus oocytes or embryos either alone or with hemagglutinin (HA) epitope-tagged Smads 1, 2, 4, and 5, or FLAG-tagged Smad3 (lanes 11 and 12). Extracts were immunoprecipitated with anti-HA or anti-FLAG antibodies and Western blotted with an anti-myc antibody to detect co-immunoprecipitation of myc-SANE (upper panel) or with anti-HA or FLAG antibodies to confirm immunoprecipitation of the respective Smad proteins (middle panel; note that Smad 3 migrated close to the IgG band). Cell lysates removed prior to immunoprecipitation were Western blotted with an anti-myc antibody to confirm equal SANE expression (lower panel).

Smad1, like Smads 2, 3, and 5, is regulated by receptor-mediated phosphorylation of carboxyl-terminal serine residues (4, 18, 19). To examine whether this region of Smad1 is involved in SANE binding, Smad1 deletion constructs (Fig. 2C) were expressed with SANE in Xenopus oocytes and Smad1-SANE complexes were immunoprecipitated with anti-Smad1 antibodies followed by immunoblotting for SANE. This analysis demonstrates that SANE interacts with the carboxyl-terminal third of Smad1, which includes the MH2 domain and the conserved serines that are phosphorylated by the BMP type I receptor (Fig. 3B). Furthermore, SANE fails to interact with a Smad mutant in which the carboxyl-terminal serines were mutated to alanines (Smad1-ala) but does interact if these serines are changed to aspartic acid (Smad1-asp) (Fig. 3C), suggesting that the carboxyl-terminal sequence of Smad1 is important for interaction with SANE. However, neither BMP-4 nor chordin overexpression affected the level of SANE-Smad1 interaction (data not shown).

To address the specificity of SANE interaction with Smad1, we also performed co-immunoprecipitation analysis with the activin/Vg1 and TGF-β-specific Smads 2 and 3, the TGFβ superfamily-shared Smad4, and the BMP-specific Smad5 (Fig. 3D). Smads 1, 2, 3, 4, or 5 were co-expressed with myc-epitope tagged SANE (mycSANE) in Xenopus embryos. Smads were immunoprecipitated followed by immunoblotting for mycSANE. This analysis shows that SANE strongly interacts with the BMP-specific Smads, Smad1 and Smad5, and binds weakly, if at all, with TGF-β specific Smad2 or Smad3 (Fig. 3D). These findings are supported by functional studies in Xenopus and mammalian cells (see below).

SANE Specifically Inhibits BMP Signaling—To test the effect of SANE on BMP- and Smad1-regulated processes, we used several well-established assays of BMP/Smad1 signaling in Xenopus. Activators of the BMP pathway such as BMPs and Smad1 cause expansion of ventral mesoderm, whereas inhibitors, such as chordin, noggin, follistatin, gremlin, dominant-negative BMP receptor, and Smad6, cause expansion of dorsal mesoderm (4, 5, 39). Expression of SANE mRNA in ventral blastomeres (Fig. 4A), over a narrow dose range, results in partial duplication of the dorsal axis, like other BMP pathway
inhibitors. Similarly, ventral expression of SANE-C (Fig. 4C), which alone is sufficient to bind Smad1, results in partial axis duplication. Injection of SANE-C mRNA into ventral blastomeres does not alter dorsal-ventral patterning (Fig. 4B), though this was limited by toxicity at higher doses. Secondary axis induction by SANE is reversed by co-injection of Smad1 (Fig. 4D), suggesting that SANE specifically interferes with the BMP/Smad1 pathway and inhibits the pathway at or upstream of the level of Smad1. Expression of SANE-FL, SANE-ΔC, and SANE-C in dorsal blastomeres did not affect dorsal-ventral patterning (Table I) consistent with the low affinity of SANE for activin/TGFβ specific Smad2.

To test whether SANE can block ectopic BMP signaling and to assess further the functional specificity of inhibition by SANE, we examined whether SANE could inhibit mesoderm induction by either BMP-4 or activin in ectodermal explants. Fertilized eggs were injected with mRNA encoding BMP or activin with or without SANE mRNA. Animal pole explants were dissected at the blastula stage, cultured until stage 10, and expression of mesodermal markers was assessed by RT-PCR. SANE blocks BMP-dependent induction of ventral mesoderm (Fig. 4E), as assessed by expression of the ventral mesodermal markers Xbra and Xwnt8. BMP induction of Xbra (Fig. 4E, lane 4) was inhibited by co-expression of SANE (lane 5), but activin-mediated induction of gsc and Xbra (Fig. 4F, lane 9) was not affected by SANE (lane 11). SANE mRNA alone (lanes 3 and 10) did not induce mesodermal markers. Lanes 1 and 6 show expression of Xbra, Xwnt8, and gsc in whole embryos (stage 10.5). EF-1α is used as a loading control. G and H, direct inhibition of BMP signaling by SANE. G, an XVent.2 promoter luciferase construct was co-injected with BMP-4 mRNA (lane 1) and together with increasing doses of SANE mRNA (lanes 2–4). H, similarly, an activin-responsive Xmix.2 promoter luciferase construct was co-injected with activin-βB mRNA (lane 6) and together with increasing doses of SANE mRNA (lanes 7–9). β-Galactosidase mRNA (lanes 5 and 10) was co-injected as control. Embryos were harvested at stage 10.5 for analysis of luciferase activity.

**Table I**

| Samples             | Injection site | Axis duplication | No. of embryos |
|---------------------|----------------|------------------|-----------------|
| Uninjected          |                |                  | 96              |
| SANE ΔC             | Dorsal         | 0                | 70              |
| SANE C              | Ventral        | 6                | 89              |
| SANE C-term         | Dorsal         | 8                | 78              |
| SANE C-term         | Ventral        | 83               | 71              |
| SANE FL             | Dorsal         | 2                | 84              |
| SANE FL             | Ventral        | 49               | 91              |
| SANE FL plus Smad1  | Ventral        | 23               | 43              |
| Smad1               | Ventral        | 0                | 35              |

**Fig. 4.** SANE specifically inhibits BMP signaling (A–D). Induction of partial secondary axes by ventral expression of SANE in Xenopus embryos. A, SANE-FL (full-length SANE); B, SANE-ΔC; or C, SANE-C mRNA (1 ng) was injected into a single ventral blastomere of four-cell embryos. D, SANE-FL mRNA was co-injected with Xenopus Smad1 mRNA (4 ng). SANE had no effect when injected into dorsal blastomeres (see Table I). E and F, SANE inhibits BMP but not activin-mediated mesoderm induction in Xenopus ectodermal explants. BMP-4 (E) or activin (F) mRNA were injected with or without SANE into the animal pole of fertilized eggs, animal cap explants were removed at stage 9, and expression of mesodermal markers was assessed by RT-PCR at stage 10+. Induction of Xbra and Xwnt8 by BMP-4 (E, lane 4) was inhibited by co-expression of SANE (lane 5), but activin-mediated induction of gsc and Xbra (F, lane 9) was not affected by SANE (lane 11). SANE mRNA alone (lanes 3 and 10) did not induce mesodermal markers. Lanes 1 and 6 show expression of Xbra, Xwnt8, and gsc in whole embryos (stage 10.5). EF-1α is used as a loading control.
somal marker Xwnt8 and the pan-mesodermal marker brachyury (40, 41) but has no effect on mesoderm induction by activin (Fig. 4F), assessed by expression of brachyury and the dorsal mesodermal marker goosecoid (42). Thus SANE specifically interferes with the BMP/Smad1 pathway and does not disrupt activin signaling.

To test whether SANE inhibits activation of direct targets of BMP, we utilized luciferase reporters containing response elements from the Xenopus Vent.2 gene, a direct transcriptional target of BMP signaling (43), and the Mix.2 gene, a direct target of activin signaling (44, 45). SANE inhibits BMP-4-dependent activation of the Vent.2-luciferase reporter in a dose-dependent manner (Fig. 4G) but does not inhibit activin-dependent activation of Mix.2 (Fig. 4H). SANE also does not inhibit TGF-β-dependent activation of the 3TP-Lux promoter reporter in Mv1Lu cells (data not shown), an assay that has been used extensively to measure direct transcriptional response to TGF-β (23). These data support a specific inhibitory role for SANE in the regulation of direct transcriptional targets of BMP signaling.

Additionally, BMP signaling has been shown to induce epidermal cell fate at the expense of neural cell fate in Xenopus ectodermal explants (13, 14). Thus, expression of inhibitors of the BMP signaling pathway causes neural induction in ectodermal explants, and this has been widely used to characterize putative BMP inhibitors. Expression of SANE in ectodermal explants mimics other BMP antagonists, with formation of a cement gland (Fig. 5A) and induction of multiple neural markers (Fig. 5B), including NCAM, a pan-neural marker, otx2, an anterior neural marker, and XAG, a marker of anterior neural and cement gland cell types. Expression of SANE–C also causes neural induction, whereas the SANE–AC construct, which does not bind Smad1, does not induce neural markers, suggesting that this effect is mediated through interaction with Smad1. SANE did not induce dorsal mesoderm, as seen by the absence of muscle actin expression, suggesting that the induction of neural fates by SANE is direct, rather than a secondary consequence of dorsal mesoderm induction. These results in the Xenopus ectodermal explant assay are consistent with the inhibition of BMP signaling by SANE.

**SANE Inhibits BMP Signaling in Mammalian Cells—**SANE also inhibits BMP signaling in a mammalian cell culture model of osteoblast differentiation. The mouse C2C12 myoblast cell line responds to BMP by differentiating into an osteoblast lineage (35). Thus, stable C2C12 cell lines expressing either full-length myc-tagged SANE, SANE–C, or β-galactosidase as control were established, and populations of transfected C2C12 cells were screened for transgene expression by Western blot analysis. These cell lines were then treated with BMP-4 protein and stained for alkaline phosphatase activity, indicative of osteoblast differentiation (Fig. 6). As reported previously, BMP-4 induces bone-specific alkaline phosphatase in control cells expressing β-galactosidase (Fig. 6, A and D). However, expression of SANE–C inhibited BMP-4 mediated induction of alkaline phosphatase by more than 10-fold (Fig. 6, B and D). Expression of full-length SANE also inhibited alkaline phosphatase induction (~5- to 6-fold; Fig. 6, C and D). SANE did not block formation of myotubes upon serum withdrawal and did not block TGF-β1-mediated inhibition of myogenic differentiation (data not shown). Inhibition of BMP-4 signaling in C2C12 myoblasts by SANE and SANE–C is consistent with their inhibitory activity in Xenopus and again supports the specificity of SANE for Smad1 and the BMP pathway.

**SANE Inhibits Phosphorylation and Nuclear Translocation of Smad1**—To begin to understand the mechanism by which SANE inhibits BMP signaling, we examined the effect of SANE on Smad1 phosphorylation. BMP-4 mRNA was injected alone or with SANE mRNA into fertilized eggs, embryos were harvested at stage 10.5, and Smad1 phosphorylation was assessed by Western blotting using antibodies specific to Smad1 phosphoepitope at the carboxyl terminus (46). BMP-4 increased phosphorylation of endogenous Smad1 as described previously (Fig. 7, lane 2), and SANE reduced BMP-dependent Smad1 phosphorylation in a dose-dependent manner (Fig. 7, lanes 3 and 4, upper panel) without changing the level of Smad1 protein (Fig. 7, middle panel).

Phosphorylation of Smad1 leads to its nuclear translocation and activation of BMP/Smad1-dependent gene expression; thus, to address the effect of SANE on Smad1 nuclear translocation, we utilized the Smad nuclear translocation assay that has been established in COS-1 and NIH3T3 cells (25, 47). NIH3T3 cells were transiently transfected with the full-length SANE expression plasmid. After allowing time for expression, cells were starved and then treated with BMP-4 protein to promote Smad1 nuclear translocation. Endogenous Smad1 is readily detected in the cytoplasm of ~95% of untreated cells by immunofluorescence (Fig. 8A, upper left, Fig. 8B) and over 95% of Smad1 translocates to the nucleus in control cells after treatment with BMP (Fig. 8, A and B). SANE inhibits BMP-
dependent nuclear translocation of endogenous Smad1 in 80% of the SANE-transfected cells. SANE also blocks nuclear translocation of overexpressed Smad1 in COS-1 cells but does not block TGF-β1-dependent Smad2 nuclear translocation under similar conditions (data not shown). These data are also consistent with the specificity of SANE for antagonizing the BMP pathway and offer a mechanism to explain SANE inhibition of BMP/Smad1 signaling.

**SANE Interacts with BMP Receptors**—The predicted amino acid sequence of SANE reveals a hydrophobic region between residues 438 and 458, indicating a potential transmembrane domain, and biochemical fractionation in *Xenopus* embryos (Fig. 9A) and COS-1 cells (not shown) shows that SANE protein localizes primarily to a detergent-soluble membrane fraction and not cytosolic or nuclear fractions. We therefore tested whether SANE can also interact with BMP receptors. SANE was expressed in *Xenopus* embryos with HA-epitope-tagged type I receptors specific for BMPs (ALK-3 and ALK-6), an activin/BMP type I receptor (ALK-2), or with an activin/TGF-β-specific type I receptor (ALK-4); SANE was immunoprecipitated and the type I receptors were detected by immunoblotting with HA antibodies. The BMP-specific type I receptors (ALK-3 and ALK-6) strongly associate with SANE (Fig. 9B, lanes 3 and 5), but interaction of SANE with ALK-2 and ALK-4 is barely detectable (Fig. 9B, lanes 7 and 9). This interaction is independent of the Smad1 binding region of SANE, because SANE-ΔC also associates with ALK-3 or ALK-6 whereas SANE-C, the Smad1 binding domain of SANE, does not (Fig. 9C, lanes 4–6). Thus, SANE interacts specifically with two components of the BMP signaling pathway.

**DISCUSSION**

Our data show that SANE specifically interacts with and regulates the BMP pathway. In support of this, SANE binds directly to BMP pathway-specific Smads1 and 5, but weakly, if at all, with activin/TGF-β-specific Smad2 and 3; binds strongly to type I BMP-specific receptors (ALK-3 and ALK-6); inhibits BMP signaling (but not activin or TGF-β) in multiple established assays of BMP signaling; and is expressed in a pattern consistent with a role in BMP/Smad1 signaling. Furthermore, the dorsalizing effects of SANE are reversed by co-expression of Smad1, indicating that the effect of SANE is specific to the BMP/Smad1 pathway and acts at or upstream of Smad1. Although ALK-2 is also a BMP receptor, the sequence of ALK-2 is sufficiently distinct from ALK-3 and -6 that it was not recognized as a BMP receptor until recently, suggesting that its structure is significantly different than ALK-3 and -6 (23, 48). ALK-3 and ALK-6 are type I receptors for BMP4 and BMP7, and ALK-2 is a type I receptor for BMP6 and 7 but not BMP4, which may suggest that ALK-3 and -6 have biological roles that are similar to each other but distinct from ALK-2 (48–50). This is consistent with our observation that SANE interacts with ALK-3 and -6 and not with ALK-2.

BMP signaling is highly regulated by a number of extracellular inhibitors, including chordin, noggin, follistatin, and gremlin, which were first identified in *Xenopus* as dorsalizing factors that specifically inhibit BMP function (2–5). Similar to these proteins, SANE blocks BMP-mediated ventral mesoderm...
induction and leads to formation of a partial secondary dorsal axis when expressed in ventral cells. In addition, SANE, like these other inhibitors (51–53), induces neural tissue in ectodermal explants and blocks BMP function in mammalian cells as well.

SANE bears superficial similarity to other proteins that interact with the TGF-β and BMP pathways. The BMP and activin membrane bound inhibitor (BAMBI) is a transmembrane pseudoreceptor that antagonizes both activin and BMP receptors and, like SANE, mimics other BMP inhibitors when overexpressed in Xenopus embryos (54). However, unlike SANE, BAMBI is not specific for the BMP pathway and is not known to interact with Smads. SARA, like SANE, interacts with both receptors and Smads but is specific for the activin/TGF-β pathway and does not interact with BMP pathway-specific Smads or BMP receptors (55). Furthermore, SARA shows no sequence similarity to SANE, and ectopic SARA expression does not inhibit TGF-β signaling, whereas SANE inhibits BMP signaling. Smurf1 and Tob, like SANE, specifically bind BMP pathway Smads and antagonize BMP signaling (29, 56). However, unlike SANE, Smurf1 and Tob do not interact with BMP receptors and do not inhibit Smad1 nuclear translocation; Smurf1 acts by accelerating Smad1 protein turnover and Tob sequesters Smads in nuclear bodies.

Intracellular Smad antagonists, such as Smads 6 and 7, are induced in response to BMP or TGF-β signaling and are proposed to act as feedback inhibitors of the respective pathways (57–60). These proteins appear to mediate their effects by binding to type I receptors and preventing pathway-specific Smad activation, although Smad6 has also been reported to sequester Smad1 in a nonfunctional complex (60–63). Thus, while SANE and Smad6 do not share obvious sequence similarity, they appear to have similar activities in the regulation of BMP signaling.

SANE shows no apparent sequence similarity to other proteins that interact with BMP-pathway specific Smads or BMP receptors, including BAMBI, Tob, Smurf1, OAZ, and BRAM1 (29, 54–56, 58, 60, 64). However, SANE does show sequence

**Fig. 8.** SANE prevents BMP mediated Smad1 nuclear translocation. A, SANE was expressed in NIH3T3 cells, and after 24 h, cells were treated with BMP-4 protein and analyzed by immunofluorescence for nuclear translocation of endogenous Smad1. 4′,6-Diamidino-2-phenylindole staining was used to identify nuclei (blue). Transfected SANE was visualized with rhodamine-labeled secondary antibody (red), and Smad1 was visualized with an fluorescein isothiocyanate-labeled secondary antibody (green). Endogenous Smad1 in nontransfected cells served as a positive control for BMP-dependent nuclear translocation. Arrows indicate cells transfected with SANE. B, quantitation of the effect of SANE on Smad1 nuclear translocation in the presence of BMP in NIH3T3 cells as shown in Fig. 9A. The number of cells counted is indicated at the top of each column. For the SANE-transfected group, only SANE-positive cells were counted.
Regulation of BMP Signaling by SANE

Fig. 9. SANE interacts with the BMP type I receptor. A, SANE is a membrane protein. Stage 10 embryos were lysed in the presence (+) or absence (−) of nonionic detergent (1% Triton X-100) and centrifuged, and supernatants were analyzed by Western blotting for endogenous SANE. In the presence of detergent, SANE is in the soluble fraction. B, SANE interacts with BMP-specific type I receptors: mRNA for HA-tagged type I BMP-specific receptors ALK-6 and ALK-3, the activin and BMP receptor ALK-5, and the activin/TGF-β receptor ALK-4 was injected with (+) or without (−) myc-tagged SANE-FL mRNA. Receptor complexes were immunoprecipitated with anti-HA antibodies and then Western-blotted for myc-SANE (upper panel). Expression of myc-SANE (middle panel) and HA-tagged receptors (lower panel) is shown by Western blotting of lysates. C, interaction between SANE and BMP receptor does not require the Smad1 interaction domain of SANE. Myc-SANE-FL (S), SANE-ΔC (ΔC), or SANE-C (C) was co-expressed with ALK-6-HA or with β-galactosidase (β-gal). Receptor complexes were immunoprecipitated with anti-HA antibodies as above and then blotted for myc-SANE (left panel). ALK-6 associates with SANE-FL (lane 4) and SANE-ΔC (lane 5) but not with SANE-C (lane 6), which contains the Smad1 interaction domain of SANE. Expression of myc-SANE in lysates is shown by Western blot (right upper panel). The lower panel shows expression of ALK-6-HA.

SANE interacts with the BMP type I receptor. The data suggest that SANE is a membrane protein that interacts with two components of the BMP signaling pathway: the BMP type I receptor ALK-2, and the activin/TGF-β receptor ALK-6. This interaction does not require the Smad1 interaction domain of SANE. Expression of myc-SANE in lysates shows expression of ALK-6-HA.

Similarity with the nuclear envelope protein MAN1 (37). Like MAN1, SANE has an LEM domain near the amino terminus. In other LEM domains, this region has been shown to interact with the DNA binding protein BAF (barrier to autointegration factor) and with DNA (65). Except for the LEM domain, however, much of the N-terminal domain of MAN1, which is required for localization of the protein to the inner nuclear membrane, diverges significantly from the corresponding region of SANE (66). In addition, unlike MAN1, the hydrophyphy analysis of SANE is more consistent with a single transmembrane domain, placing the SANE amino terminus topologically outside the cell. The greatest sequence similarity between MAN1 and SANE is found in the carboxyl-terminal region, which includes the Smad1 binding domain of SANE, suggesting that this may represent a functionally conserved domain, and it is interesting to consider that the MAN1 carboxyl-terminal region could also interact with Smad1 to regulate BMP signaling, perhaps in the nucleus. Indeed, regulation of transcription has been suggested as a possible function for other LEM domain proteins (67). The presence in mouse and Xenopus of several expressed sequence tags encoding related, but distinct genes raises the intriguing possibilities that SANE is one of a family of TGF-β/Smad interacting genes and that other SANE-like molecules may regulate the TGF-β/Smad2 pathway.

SANE is expressed in cells that are likely to respond to BMP signaling, because the expression of SANE parallels Smad1 expression in most tissues. However, at the gastrula stage SANE is enriched in the dorsal marginal zone, similar to other BMP antagonists, such as chordin, noggin, and follistatin, whereas Smad1 mRNA is ubiquitous at this stage, consistent with a role for SANE as an inhibitor of BMP signaling in the dorsal marginal zone.

Our model for the mechanism of SANE action suggests that SANE interacts with the BMP type I receptor through its amino-terminal domain and with Smad1 through its carboxyl-terminal domain to inhibit BMP signaling. Inhibition of BMP signaling by SANE is supported by biochemical data, analysis in cultured cells and embryonic explants, and phenotypic analysis in intact embryos. Thus, biochemically, we have shown that SANE blocks phosphorylation of endogenous Smad1 by the BMP type I receptor. As a consequence, SANE prevents translocation of Smad1 to the nucleus and blocks BMP-dependent gene expression. This inhibition is clearly specific to the BMP/Smad1 pathway, because Smad2 translocation and Smad2-dependent transcriptional activation are not affected by SANE. We have also demonstrated that SANE antagonizes BMP-induced bone formation in C2C12 cells, BMP-induced ventral mesodermal gene expression and neural to epidermal cell fate specification in embryonic explants, and endogenous ventral mesodermal formation in intact embryos, but that it does not inhibit biological responses to activin or TGF-β. This evidence for SANE as an inhibitor of BMP signaling comes from gain-of-function studies in Xenopus and mammalian cells, similar to analysis of other BMP antagonists. However, these experiments do not rule out the possibility that SANE could act as an adaptor that mediates interaction between BMP type I receptor and Smad1 and that could either positively or negatively regulate signaling. In this scenario, SANE could recruit Smad1 to the type I receptor for its subsequent activation, thus acting in a positive role in BMP signaling. This adaptor model would predict that expression of SANE at lower doses may potentiate BMP signaling if endogenous SANE is limiting. However, we have not detected any enhancement of BMP-4 activity over a wide dose range of SANE RNA in the induction of mesodermal markers such as Xbra and XWnt8 or in the induction of Xvent-2-luciferase.2 Similarly, expression of SANE in dorsal blastomeres does not ventralize Xenopus embryos at any dose tested (Table I and data not shown). These data do not support a role for SANE as a positive regulator of BMP signaling; however, further studies, including loss-of-function experiments, will be needed to validate this hypothesis.

In summary, SANE interacts with two components of the BMP pathway, is expressed in the embryo in a pattern that closely parallels Smad1 expression, and specifically inhibits Smad1-dependent signaling in Xenopus embryos and mammalian cells. Inhibition by SANE in Xenopus embryos is functionally similar to the effects of other BMP inhibitors, such as chordin, noggin, follistatin, gremlin, and tob, yet SANE defines a new class of genes to regulate this pathway that uniquely

2G. P. Raju, P. S. Klein, and H.-C. Huang, unpublished data.
interacts with both BMP receptors and BMP pathway specific Smads.

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