Endofin acts as a Smad anchor for receptor activation in BMP signaling

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Summary
Signaling through receptors of the transforming growth factor β (TGFβ) superfamily is mediated by cytoplasmic Smad proteins. It has been demonstrated that Smad anchor for receptor activation (SARA) facilitates TGFβ and activin/nodal signaling by recruiting and presenting Smad2/3 to the receptor complex. SARA does not bind Smad1 and hence does not enhance bone morphogenetic protein (BMP) signaling. Here we report for the first time that the endosome-associated FYVE-domain protein endofin acts as a Smad anchor for receptor activation in BMP signaling. We demonstrate that endofin binds Smad1 preferentially and enhances Smad1 phosphorylation and nuclear localization upon BMP stimulation. Silencing of endofin by RNAi resulted in a reduction in BMP-dependent Smad1 phosphorylation. Moreover, disruption of the membrane-anchoring FYVE motif by point mutation led to a reduction of BMP-responsive gene expression in cell culture and Xenopus ectodermal explants. Furthermore, we demonstrate that endofin contains a protein-phosphatase-binding motif, which functions to negatively modulate BMP signals through receptor dephosphorylation. Taken together, our results suggest that endofin plays an important role in both positive and negative feedback regulation of the BMP signaling pathway.

Key words: BMP, Smad, SARA, Protein phosphatase, Osteoblast

Introduction
Growth factors of the TGFβ superfamily regulate cell proliferation, differentiation, migration and apoptosis and are crucial for the development and maintenance of many different tissues (Cheifetz, 1999; Francis-West et al., 1999). Bone morphogenetic proteins (BMPs) in this superfamily induce bone marrow mesenchymal stem cell differentiation into osteoblasts and enhance periodontal regeneration in surgically created defects. BMP ligands, as well as other ligands in the TGFβ superfamily, initiate cellular signaling by binding to type I and type II receptors, both of which are serine/threonine kinase receptors, thereby inducing the formation of a hetero-oligomeric receptor complex. The type II receptor then phosphorylates and activates the type I receptor, which subsequently transiently associates with and phosphorylates a subcell of a unique family of intracellular signaling molecules – the receptor-regulated Smads or R-Smads (Godin et al., 1999; Ghosh-Choudhury et al., 1994). The R-Smad Smad1 transduces signals from BMPs, whereas the R-Smads Smad2 and Smad3 mediate signals from the TGFβ/activin/nodal subfamily members. The recruitment of R-Smads to the receptor complex is the initial step of intracellular TGFβ signal transduction and plays a critical role in regulating signal transduction by the TGFβ receptor family.

TGFβ signaling can be enhanced by a protein, Smad anchor for receptor activation or SARA, which binds unphosphorylated Smad2 and Smad3 and recruits them to membranes (Tsukazaki et al., 1998). SARA contains a FYVE domain adjacent to its Smad-binding domain. The double zinc-finger motifs in FYVE domains have been shown to bind phosphatidylinositol 3-phosphate and anchor FYVE-containing proteins to cytoplasmic or endosomal membranes. This enables proteins containing the FYVE domain to recruit other molecules to membranes for endocytosis or relocation (Gaullier et al., 1998; Kutateladze and Overduin, 2001). The FYVE domain in SARA is required for recruitment of Smad2/3 to the appropriate subcellular locations that position it adjacent to the activated TβRI (Tsukazaki et al., 1998; Wu et al., 2000). Activation of TGFβ signaling causes phosphorylation of Smad2, subsequent dissociation from SARA, and nuclear translocation with the common Smad, Smad4 (Massague, 1998; Heldin et al., 1997).

Recent data suggest that SARA can also act as a negative regulator of TGFβ signaling. In Drosophila, the catalytic subunit of protein phosphatase 1 (PP1c) has been shown to bind SARA and negatively regulate signaling through Dpp, a BMP homologue (Bennett and Alphey, 2002). Expression of a mutant SARA with a mutation in the PP1c-binding domain (F678A) resulted in hyperphosphorylation of the type I receptor and elevated expression of dpp target genes (Bennett and Alphey, 2002). In our previous studies, we also found that SARA facilitates dephosphorylation of TβRI in cell culture through a Smad7-mediated negative feedback loop. The inhibitory Smad7 recruits a protein phosphatase holoenzyme, GADD34-PP1c, to inhibit TGFβ–induced cell cycle arrest and confer cell resistance to TGFβ in response to UV irradiation. SARA facilitates the formation of GADD34-PP1c by presenting PP1c to this holoenzyme, and thus acts as a component of Smad7-mediated negative feedback loop of this signaling pathway (Shi et al., 2004). SARA is therefore a...
central regulator of TGFβ signaling and can act to either promote or inhibit TGFβ signals.

The enhancement of TGFβ/activin signaling by another FYVE-domain protein, Hgs/Hrs, further indicates that other SARA-like molecules may participate in the regulation of TGFβ/activin signaling (Miura et al., 2000). By analogy, it would be anticipated that BMP signal transduction might also employ SARA-like regulators to help bring Smad1 to the receptor complexes; however, up to now, no SARA-based mechanism had been identified for the BMP pathway. In this study, we present evidence indicating an important role for the endosome-associated FYVE-domain protein endofin in BMP signaling (Seet and Hong, 2001; Seet et al., 2004; Seet and Hong, 2005). We show that endofin binds preferentially to Smad1 and regulates Smad1 signaling by modulation of its phosphorylation and nuclear translocation upon BMP activation. Endofin also has a functional PP1c-binding domain, which recruits PP1c and facilitates dephosphorylation of the type I BMP receptor. Our results identify for the first time a BMP-specific Smad anchor as a receptor activation molecule that plays a role in transduction and negative feedback regulation of BMP signals.

Results
Endofin interacts with Smad1 and PP1c

Endofin has been shown to localize to early endosomes, as does SARA; but it does not bind to Smad2 or influence TGFβ signals (Seet and Hong, 2001). The relatively high divergence of the Smad-binding domain suggested that endofin might bind R-Smads other than the TGFβ-specific Smad-2, such as the BMP-specific Smad1. To test this possibility directly, we performed a co-immunoprecipitation assay using the lysate of COS cells transfected with endofin and Smad1 or Smad2. We found that Smad1, but not Smad2, was co-precipitated with endofin (Fig. 1A), suggesting that endofin preferentially binds to Smad1. To further confirm the interaction in situ, we performed a protein-fragment complementation assay (PCA) using yellow fluorescent protein (YFP) as a marker. The principle of the PCA strategy is that complementary fragments (F1 and F2) of a reporter protein (such as YFP) will fold into an active form only when brought together in close proximity by fusion to two proteins that interact with each other (Remy et al., 2004; Li et al., 2006). In our experiments, we fused Smad1 and endofin to N-terminal (a.a. 1-158, YFP1) and C-terminal (a.a. 159-239, YFP2) fragments of YFP, respectively. When expressed alone, neither YFP1-Smad1 nor endofin-YFP2 produced fluorescent signals in the cells. Co-expression of the vectors encoding YFP1 and YFP2 did not generate fluorescent signals either (Fig. 1B). When the fusion proteins were co-transfected into the COS cells, however, we observed strong yellow fluorescence (Fig. 1B). The intensity of the fluorescence signal is similar to that observed when Smad1 and its interacting protein Hoxa1 were co-transfected in a similar way in parallel (Fig. 1B). These results indicated that Smad1 and endofin interact in transfected cells. To confirm this interaction, we also examined the localization of tagged Smad1 and endofin in transfected cells by fluorescence immunocytochemistry. Endofin and Smad1 co-localized in the cytosol in a punctate pattern (Fig. 1C), suggesting that the two proteins interact at intracellular vesicles in the cells. To eliminate the possibility of artifacts generated by the overexpression system, we also analyzed endogenous proteins by using antibodies against Endofin and Smad1. As shown in the merged panels in Fig. 1C, the endogenous proteins were also co-localized in the cytosol of the cells. All our data are consistent with the idea that endofin and Smad1 associate with each other in the cell.

In addition to the Smad-binding motif, all known SARA homologs also contain a canonical PP1c-binding motif (K/R)xVxF or (K/R)VxF (Bennett and Alphey, 2002) and this motif is involved in negative feedback regulation of Dpp signaling in Drosophila. We therefore examined whether endofin can also associate with PP1c. Co-immunoprecipitation analyses indicated that endofin indeed interacts with PP1c. Interestingly, our results further showed that overexpression of PP1c enhanced the interaction of endofin with Smad1 (Fig. 1D). As PP1c can down-regulate BMP signaling resulting in hypophosphorylation of BMP signal components, our observation implies that endofin may preferentially bind to unphosphorylated Smad1. To verify this hypothesis, we assayed for endogenous interaction of endofin with Smad1, phosphorylated Smad1 (pSmad1) and PP1c in the presence or absence of BMP2. We observed that BMP2 stimulated binding of endofin to PP1c, and the phosphorylated form of Smad1 has very low affinity for endofin (Fig. 1E). To examine the specificity of the interaction between endofin and Smad1, the interaction of SARA and Smad1 was also detected. BMP2 does not stimulate the binding of SARA with either Smad1 and phosphorylated Smad1. Our results confirm that endofin, but not SARA, binds preferentially to the inactive form of Smad1.

To further determine whether the putative Smad- and PP1-binding domains (SBD and PBD respectively) mediate the interaction of endofin with Smad1 and PP1c, we performed co-immunoprecipitation experiments with mutant endofin. Three mutants were generated that contain point mutations in FYVE and PBD domains or deletion of the SBD motif (Fig. 2A). We found that disruption of the SBD or mutation of FYVE domain resulted in a diminishment of the Smad1-endofin interaction (Fig. 2B), while mutation of the PBD almost abolished the interaction between PP1c and endofin (Fig. 2C). The results demonstrate that SBD and PBD mediate the binding of endofin to Smad1 and PP1c respectively.

Endofin facilitates dephosphorylation of the BMP type I receptor by PP1

Expression of a PP1c-binding mutant of SARA has been shown to result in hyperphosphorylation of the type I receptor and elevation of expression of a target of TGF-β signaling (Bennett and Alphey, 2002). To assess whether endofin has a similar activity, we performed an in vivo phosphorylation assay. As shown in Fig. 3A, overexpression of endofin reduced phosphorylation of the type I BMP receptor ALK3 in response to BMP2, whereas the mutant endofin with impaired PBD enhanced ALK3 phosphorylation. Phosphatase inhibitor 1, an inhibitor for PP1c, also increased ALK3 phosphorylation. The results suggest that endofin can regulate ALK3 activation through its interacting phosphatase PP1c.

In our previous study of SARA, we found that dephosphorylation of the type I TGFβ receptor ALK5 is mediated by a protein phosphatase regulatory subunit GADD34, which binds to ALK5 through a bridging mechanism involving an inhibitory Smad, Smad7 (Shi et al.,...
To see whether a similar mechanism applies to endofin, we tested the interaction of the BMP type I receptors ALK3 and ALK6 with GADD34, as well as the potential requirement for inhibitory Smads Smad6 and Smad7. We found that both ALK3 and ALK6 readily interact with GADD34 without any auxiliary factors (Fig. 3B), and sequential co-immunoprecipitation further clarified that although ALK3 and ALK6 bind inhibitory Smads, they do not form a ternary complex with GADD34 and Smad6 or Smad7 (Fig. 3C). Our data indicate that unlike ALK5, ALK3 and ALK6 directly associate with the regulatory subunit of protein phosphatase 1 in the absence of inhibitory Smads.
Endofin modulates Smad1 phosphorylation and nuclear translocation

To examine the functional significance of the endofin-Smad1 interaction, we next performed a loss-of-function study, using small interfering RNA. Knockdown of endofin using specific siRNA resulted in a reduction in the level of phosphorylation of Smad1 (Fig. 4A), which corroborated our hypothesis that endofin acts as a SARA-like protein in BMP signaling and is required for Smad1 phosphorylation. Notably, endofin appears to utilize a mechanism similar to that employed by SARA in which the PP1c-binding motif acts to negatively regulate phosphorylation of R-Smad.

As phosphorylation of Smad1 enables its translocation into the nucleus, we next tested whether endofin also regulates the nuclear translocation of Smad1. Staining of phosphorylated Smad1 in C2C12 cells expressing endofin confirmed that endofin enhanced Smad1 phosphorylation and nuclear translocation upon simulation of BMP2. Overexpression of SARA, however, did not have a significant effect on Smad1 activation and localization (Fig. 4B).

Endofin regulates expression of BMP downstream genes in C2C12 cells and mineralization in human MSCs

To further examine the effect of endofin on BMP signal transduction, we generated stable cell lines derived from C2C12 and human MSCs infected with retroviruses that express endofin, endofin mutants or the control green fluorescent protein (GFP). The influence of stable endofin expression on BMP-induced downstream gene transcription was monitored in these cell lines using a BMP-responsive reporter construct (9xSBE-luc). As shown in Fig. 5A, wild-type endofin slightly enhanced the expression of the BMP-responsive luciferase gene. The FYVE(C753S) mutant, which leads to cytosolic mislocalization of endofin and possibly its associated proteins including Smad1, decreased the reporter gene transcription. As expected, deletion of the Smad-binding domain (EndofinΔSBD) also inhibited BMP-induced gene expression. By contrast, mutation of the PP1c-binding domain PBD(F872A) enhanced transcription of the reporter transcription; a finding that is consistent with our observation that this mutant increased ALK3 phosphorylation (Fig. 3A).

BMPs were originally purified from bone matrix and have been shown to regulate bone development and induce osteoblast differentiation. We therefore examined the effects of expression of mutant endofin on BMP-stimulated expression of the osteoblast differentiation marker, alkaline phosphatase (ALP), as well as osteoblast-mediated mineralization. C2C12 cells with stable overexpression of the PBD(F872A) mutant showed higher ALP activity than cells infected with wild-type endofin (Fig. 5B,C). Similarly, human MSCs with stable overexpression of the PBD(F872A) mutant showed enhanced mineralization (Fig. 5D).

Regulation of BMP-dependent mesodermal induction by endofin in Xenopus embryos

BMPs play diverse roles during early Xenopus development, especially in embryonic dorsoventral patterning. When overexpressed, BMPs can induce ventral types of mesoderm in Xenopus ectodermal explants (animal caps). To determine

![Fig. 2. Identification of the endofin interaction domain in Smad1 and PP1c. (A) Schematic representation of wild-type and mutant endofin. Three mutants were generated to disrupt these conserved domains: FYVE domain (C753S), Smad-binding domain (deletion of amino acids 814-860) and PP1c-binding domain (F872A). (B) Deletion of Smad-binding domain in endofin diminishes its interaction with Smad1. COS1 cells were transfected with Flag-Smad1, HA-tagged endofin and its mutants. Immunoblots in the two lower panels show the protein expression levels of the COS1 cell lysates. The upper panel shows anti-HA immunoprecipitates probed with anti-Flag antibody. (C) Co-IP of PP1c with endofin and the PBD(F872A) mutant. HA-tagged endofin and its mutant with disrupted PP1c-binding domain were co-transfected with PP1c into COS1 cells. The lysate was subject to immunoprecipitation with anti-HA. Immunoblots indicating the protein expression levels of the lysates of COS1 cells are shown in the two lower panels. The upper panel shows anti-HA immunoprecipitates probed with anti-Flag antibody.
whether endofin can modulate BMP signaling in vivo, we examined the effect of endofin overexpression on BMP-dependent endogenous mesodermal marker expression. In this experiment, we injected BMP4 RNA with or without RNAs encoding endofin or its mutants in animal poles of two-cell stage embryos and dissected animal caps from injected embryos at blastula stages 8.5-9. The caps were incubated to gastrula stages before total RNA was extracted for use in a reverse transcription (RT)-PCR assay of marker gene expression. As shown in Fig. 6, BMP2 induced the expression of the early mesodermal markers Xbra, Xhox3 and Xwnt8. Expression of wild-type endofin did not affect the level of induction of these genes, possibly because of the presence of a high level of endogenous \textit{Xenopus} endofin-like molecule. Expression of the FYVE(C735S) mutant led to a dramatic inhibition of Xbra, Xhox3 and Xwnt8, whereas expression of the endofin H9004 SBD mutant resulted in a modest reduction in the marker gene expression (Fig. 6). By contrast, expression of the PBD(F872A) mutant enhanced the expression of Xbra, Xhox3 and Xwnt8: a result further supporting the notion that recruitment of PP1c by endofin plays an important role in negative feedback regulation of BMP signaling in vivo.

**Discussion**

The FYVE domain protein, SARA, was identified as a key molecule that anchors Smad2/3 and promotes receptor activation of the TGFβ signaling pathway (Tsukazaki et al., 1998; Bennett and Alphey, 2002; Wu et al., 2000). The similarities among members of the TGFβ superfamily prompted us to search for evidence of a SARA-like molecule associated with BMP signaling. GenBank search for sequences with homology to functional regions of SARA identified a FYVE domain protein, KIAA0305, which had a C-terminal region that was closely related to the C-terminus of SARA. KIAA0305, also known as endofin (Seet and Hong, 2001), was first sequenced by investigators at the Kazusa Institute in Japan, but its function remained unknown. The conserved domain structures between SARA and endofin suggest that endofin may function like SARA as a membrane anchor protein for R-Smads; however, the Smad-binding domain is highly divergent between the two proteins. Accordingly, it has been shown that although endofin localizes to early endosomes, it does not bind to Smad2 nor influence TGFβ signals (Seet and Hong, 2001). We therefore investigate the possibility that endofin acts like SARA but binds different R-Smads. Here, we report that using co-immunoprecipitation, protein-fragment complementation assay and immunolocalization approaches, we observed consistently that endofin preferentially binds to Smad1, not Smad2, suggesting that endofin may act as a Smad anchor for receptor activation in BMP signaling. This idea is consistent with our finding that endofin, but not SARA, facilitated the BMP signalling, and
Endofin knockdown with siRNA reduced the level of Smad1 phosphorylation.

The specificity of the Smad anchor for receptor activation proteins may be determined by critical residues in the Smad-binding domains, which define preferential interactions with TGFβ- or BMP-specific R-Smads (Wu et al., 2000). The crystal structure of the Smad2 MH2 domain bound by the Smad-binding domain of SARA has revealed that the MH2 domain interacts with an extended proline-rich motif consisting of a proline-rich coil, an α helix and a β strand in the Smad-binding domain of SARA (Wu et al., 2000). The sequence of this region differs in endofin and SARA, which may explain why the two proteins bind distinct R-Smads. Furthermore, it has been shown that an asparagine residue (N381) in Smad2 makes extensive contacts with the Smad-binding domain of SARA (Wu et al., 2000). Notably, in Smad1 this residue is a serine. Substitution of N381 in Smad2 with serine interferes with the binding of SARA to Smad2 and leads to a failure of SARA to properly localize the mutant Smad2 for activation. We also found that substitution of the serine at position 379 in Smad1 with asparagine interfered with the binding of endofin to Smad1 (our unpublished data). This would suggest that a serine at this position is critical for contact of Smad1 with endofin.

The SARA-like proteins appear to be capable of both enhancing and dampening the level of phosphorylation of certain phospho-proteins. Based on our results, it seems that when ligand binding initiates the signaling events, the endofin recruits the Smad effectors thereby enabling their phosphorylation, subsequent nuclear translocation and further regulation of downstream gene expression (Tsukazaki et al., 1998; Bennett and Alphey, 2002; Shi et al., 2004). To balance the stimulated signaling, PP1c is recruited by endofin for dephosphorylation of Smad1 and this enables the signaling.

**Fig. 4.** Endofin modulates Smad1 phosphorylation and nuclear translocation. (A) Silencing of endofin expression by RNA interference inhibited Smad1 phosphorylation. C2C12 cells were transfected with Flag-tagged Smad1 and either vector, construct for GFP or endofin siRNA. On the third day following 3-hour BMP2 treatment, Flag-tagged Smad1 was precipitated with anti-flag and its phosphorylation level was detected with anti-phospho-Smad1. (B) Endofin, but not SARA, regulates BMP-specific phosphorylation and intracellular translocation of Smad1. C2C12 cells were transfected with either HA-SARA or HA-Endofin, then stimulated with BMP (100 ng/ml) for 3 hours. After fixation with paraformaldehyde (PFA), cells were immunostained with either anti-HA (green) or anti-phospho-Smad1 (red). Nuclei were visualized using Hoechst 33342. Representative images are shown. Bar, 20 μm.
Fig. 5. Endofin regulates expression of BMP downstream genes and mineralization in human MSCs. (A) Transcriptional response assay. C2C12 cells stably expressing GFP (control), or endofin or its mutants were transfected with BMP signaling reporter construct 9XSBE-luc. Transfected cells were incubated in the presence or absence of BMP2 (200 ng/ml). Luciferase activity was normalized and plotted as the mean ± s.d. of triplicates from a representative experiment. *$P<0.05$ compared with the second group. (B) Alkaline phosphatase activity assay. C2C12 cells stably expressing GFP (control) or endofin or its mutant PBD(F872A) were cultured in 24-well plates treated with or without BMP2 and harvested at day 5 for alkaline phosphatase activity assay. Relative alkaline phosphatase activity was normalized and plotted as the mean ± s.d. of triplicates from a representative experiment. *$P<0.05$ compared with second group. (C) Alkaline phosphatase staining. C2C12 were cultured and treated as in alkaline phosphatase activity assay and harvested at day 3. After fixation with 4% paraformaldehyde, cells were subjected to alkaline phosphatase staining. Bar, 20 μm. (D) Overexpression of mutated endofin with disrupted PP1c-binding domain enhanced mineralization activity of human MSCs (von Kossa Assay). Human MSCs were cultured in Dulbecco’s Modified Eagle’s Medium: low glucose, 1X penicillin-streptomycin, 10% fetal bovine serum (BioWhittaker, MSC serum), 10 mM β-glycerol phosphate, 50 μM Ascorbic acid 2-phosphate (AsAP) and 200 ng/ml BMP2. Mineralization assay was performed at day 24. After silver nitrate was added, a calcium deposit was visible as a black structure or focal dot. i, GFP; ii, GFP+BMP2; iii, Endofin+BMP2; iv, Endofin-PBD(F872A)+BMP2. Bar, 30 μm.

Fig. 6. Regulation of BMP-dependent mesodermal induction by endofin in *Xenopus* embryos. Capped RNAs encoding endofin (wild type and mutants) and BMP4 were synthesized in vitro and injected alone or in combination into both animal poles of two-cell-stage embryos. The ectodermal explants (animal caps) from injected embryos were dissected at blastula stages (stage 8.5-9) and cultured until gastrula stages (stage 11) before total RNA was extracted. RT-PCR was performed using the primers for different mesodermal marker genes. EF1-α serves as a loading control. Although mutations in the FYVE and SBD domains reduced expression of BMP-responsive genes, the mutation in the PBD domain enhanced BMP-induced marker gene expression.
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Back to a basal unstimulated state. Endofin preferentially binds unphosphorylated R-Smads. Therefore, once most R-Smads are phosphorylated and dislocated from endofin, PP1c will readily bind to endofin (Shi et al., 2004). The availability of R-Smads and phosphorylated R-Smads or the ratio of the two determines whether endofin facilitates BMP signaling positively or negatively. Thus, the immediate question is the identity of the substrate of the PP1c once it is recruited to endofin. Smad1 is not likely to be the substrate because it dissociates from endofin once phosphorylated (Bennett and Alphay, 2002; Shi et al., 2004). However, as shown previously, GADD34, a PP1 regulatory subunit (He et al., 1996; Novoa et al., 2001), interacts with Smad7 to direct the PP1 holoenzyme to the type I receptor of TGFβ, ALK5 for its dephosphorylation (Shi et al., 2004). Here we demonstrated a Smad7-independent interaction between GADD34 and the type I receptor of BMP. Endofin, acting as a Smad anchor for receptor activation, delivers PP1c to GADD34 to form a holoenzyme that dephosphorylates BMP type I receptor. This dual function also explains why overexpression of wild-type endofin and SARA has only a modest affect on phosphorylation of Smads and osteoblast differentiation (Fig. 4B, Figs 5 and 6) (Bennett and Alphay, 2002; Shi et al., 2004). These dual regulatory mechanisms ensure the proper level of signaling activity. However, once the dephosphorylation activity is blunted by mutation, the positive regulatory effect of the Smad anchor for receptor activation becomes evident immediately (Figs 5 and 6). Endofin and its mutants affected the expression of the osteoblast differentiation marker alkaline phosphatase, osteoblast-mediated mineralization as well as transcription of endogenous BMP-responsive genes in vivo in Xenopus upon BMP stimulation (Figs 5 and 6), which suggests that endofin utilizes membrane anchoring (FYVE domain) and PP1c dephosphorylation (PBD domain) to modulate the levels of BMP signaling during in vitro osteoblast differentiation and in vivo Xenopus development.

TGFβ signaling is modulated mainly through regulation of the phosphorylation status of key components of the signaling pathway (Massague, 1998; Derynck, 1994). Continuous receptor activity is required to maintain localization of active Smads in the nucleus and for TGFβ-induced transcription (Inman et al., 2002). This implies that depletion of active Smads in the nucleus is a prerequisite for dampening of the signaling. It has been reported that both Smads and receptors can be degraded in the cytoplasm through proteosome degradation (Datto and Wang, 2005; Kuratomi et al., 2005; Di Guglielmo et al., 2003). However, accumulating evidence suggests that R-Smads are recycled and that R-Smads are shuttled continuously between the nucleus and cytoplasm (Xu et al., 2002; Inman et al., 2002). After phosphorylation by a type I receptor and translocation into the nucleus, the R-Smads reappear in the cytoplasm unphosphorylated by a dephosphorylation mechanism. Recently, such a phosphatase was finally identified (Knockaert et al., 2006; Lin et al., 2006). In addition to the direct dephosphorylation mechanism of R-Smads, we identified a BMP-specific inhibitory mechanism by which endofin recruits PP1 complex to dephosphorylate type I receptors. As a result, like SARA, endofin indirectly regulates the R-Smads phosphorylation level positively and negatively in controlling R-Smads recycling, and maintains balanced BMP signaling. Thus, upon BMP stimulation and Smad1 phosphorylation, endofin releases the phosphorylated Smad1 for its translocation to the nucleus. At the same time, there is a signal-dependent increase in binding of PP1c to endofin for negative feedback inhibition of the BMP signals. Endofin-regulated dephosphorylation occurs in a similar fashion to that of SARA, but with a distinct mechanism: inhibitory Smad7 is not involved, since type I receptors of BMPs directly interact with GADD34 in the absence of inhibitory Smads (Fig. 3). Furthermore, unlike direct dephosphorylation of R-Smads by their phosphatase (Knockaert et al., 2006; Lin et al., 2006), SARA- and/or endofin-mediated dephosphorylation is at the receptor level, upstream of R-Smads. It is unclear whether endofin interaction with Smad1 and/or PP1c is also modulated by other proteins, and whether this constitutes another level of control for BMP signals in a cell-type-specific manner.

Materials and Methods

Antibodies and reagents

For endogenous co-immunoprecipitation, with the assistance of Cytomol Corp. (Mountain View, CA) we developed a rabbit anti-endofin polyclonal antibody raised against a peptide (amino acids 41-59, CSVSVSELASSQRTSLLPKD) in the N-terminus of human endofin. All other antibodies were obtained from commercial sources: monoclonal anti-Flag M2 and anti-β-actin (Sigma-Aldrich), anti-HA (Babco), anti-phospho-Smad1 (Ser463/465) rabbit polyclonal IgG (Upstate), anti-phospho-Smad2 (Ser465/467) rabbit polyclonal IgG (Biosource), mouse monoclonal anti-Smad1 and monoclonal anti-PP1 (Santa Cruz). Texas-Red-conjugated and FITC-conjugated (donkey anti-mouse, donkey anti-rabbit) secondary antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA).

cDNA constructs and retroviral vectors

The human cDNA of endofin (KIAA0305) was kindly provided by the Kazusa DNA Research Institute and cloned into the pcDNA3 vector. For FYP fragment fusion expression vectors, Smad1 and endofin were subcloned respectively at the 3' and 5' end of the N-terminal fragment FYP1 (a.a. 1-158) and C-terminal fragment FYP2 (a.a. 159-239) of FYP (Clontech, Palo Alto, CA) into the pcDNA3.1 vector. The mutations in the FYP2 domain and PP1c-binding domain were generated by site-directed mutagenesis using the Quik-Change® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The deletion mutant endofinASBD was generated by PCR. We used the murine stem cell virus (MSCV) vector, pMSCVneo (Clontech), to generate cell lines expressing endofin or its mutants. Endofin and its mutants were subcloned into pMSCVneo, and the viruses were prepared by transfection of these retroviral constructs into a packaging cell line, 293GPF, using Lipofectamine. Human mesenchymal stromal cells (MSCs) were infected with these viruses, and stable expression of endofin or its mutants was achieved through neomycin resistance gene selection. pMSCVneo-EFGP was used as a transfection and infection control.

Immunoprecipitation and immunoblotting

Cells transfected by Lipofectamine (Gibco-BRL) were lysed with radioimmunoprecipitation buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 1% Nonidet-P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μM PMSF) and phosphatase inhibitors (10 mM sodium orthovanadate, 50 mM inhibitor-1 and 50 mM sodium β-glycerophosphate). Lysates were immunoprecipitated with incubation with the appropriate antibodies, followed by adsorption to protein-G-Sepharose. Immunoprecipitates were separated by SDS-PAGE, blotted onto a PVDF membrane (Bio-Rad Laboratories), and visualized by enhanced chemiluminescence (ECL Kit; Amersham Biosciences).

Small interfering RNA (siRNA) constructs and transfection

To generate the construct for silencing endogenous endofin expression, a 21-nucleotide oligo (oligo 1) corresponding to nucleotides 390-410 of the mouse endofin coding region was first inserted into ApaI–HindIII-digested pBSU6/S (Sui et al., 2002). The inverted motif that contains the six-nucleotide spacer and five Ts (oligo 2) was then subcloned into the EcoRI–HindIII sites of the intermediate plasmid to generate BS–U6–si-Endofin for endofin silencing. The sequence of oligo 1 is 5'-GGTAA CTTAG TGCAT GCCAC A-3', (forward) and 5'-AGCTT GTGCC ATGCC ATGA CTAAG TTACC-3' (reverse). The sequence of Oligo 2 is 5'-AGCTT GTGCC ATGCC ATGA CTAAG TTACC-3' (reverse) and 5'-AAATC AAAA GGTTA ACTTCA GTGCC TGCCA CA -3' (reverse). For the control construct for silencing of green fluorescence protein (GFP) expression, pBS-U6-


