Chapter 5

Soluble endoglin regulates signaling by bone morphogenetic protein 9

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In preparation

Abstract

Endoglin is a type III co-receptor of the transforming growth factor β (TGF-β) family. It plays a critical role in angiogenesis, and mutations in endoglin can lead to the vascular disease hereditary hemorrhagic telangiectasia. The soluble form of endoglin (sEng) has been found in human sera and enhanced levels correlate with the vascular disorder preeclampsia. In this study we have expressed and purified the extracellular domain (ECD) of human endoglin using human cells. This recombinant sEng was secreted in both monomeric and dimeric forms which could both bind to the TGF-β type receptor activin receptor-like kinase (ALK)1 ECD and bone morphogenetic protein (BMP)9. Our results indicate that monomeric sEng has multiple effects on BMP9 induced signaling, and does not only function as a ligand trap. Further research on the underlying molecular mechanisms by which sEng affects BMP signaling might be helpful for resolving the action of sEng in preeclampsia and other related vascular diseases.

Introduction

Human endoglin is present in cells as a 180 kDa homodimeric disulfide-linked transmembrane glycoprotein. Based on its cDNA sequence endoglin is predicted to have a large extracellular domain, a hydrophobic transmembrane domain, and a short cytoplasmic tail (1). It is mainly expressed in proliferating endothelial cells (ECs) and is a critical factor for vascular remodeling and angiogenesis (2).

Endoglin was initially characterized as a co-receptor for transforming growth factor β (TGF-β) (3). TGF-β is the prototype of a large structurally related cytokine family, including two other TGF-β isoforms, activins and bone morphogenetic proteins (BMPs), and signals via type I and type II transmembrane serine/threonine kinase receptors (4). Endoglin interacts with TGF-β1, TGF-β3, activin A, BMP2 and BMP7 in the presence of the respective receptors (5). Interestingly, it can interact with BMP9 and BMP10 in the absence of type I and type II receptors (6).

A soluble form of endoglin (sEng) has been found in particular in the serum of patients with cancers and preeclampsia (7, 8). In carcinoma tissues, a 80 kDa sEng was found to be shedded from the membrane bound endoglin through proteolytic cleavage by matrix metalloproteinase (MMP) 14 at position 586, indicating that nearly the whole extracellular domain of endoglin is released (9). Also in the case of preeclampsia, a systemic syndrome associated with pregnancy hypertension and proteinuria (8, 10, 11), the detected sEng was postulated to be derived from the membrane bound endoglin through proteolysis by MMP14, but the molecular weight of preeclampsia derived sEng is around 65 kDa (8, 12).

BMP9 is the only BMP family member circulating in a biological active concentration in the blood, and it plays an important role in human angiogenesis (13, 14). The vascular disorder phenotypes of patients with BMP9 mutations show overlap with the phenotype of HHT patients (15). BMP9 specifically activates the EC-specific type I receptor activin receptor-like kinase (ALK)1, but it can bind to three TGF-β family type II receptors: BMP type II receptor (BMPR2), activin A receptor type II A (ACVR2A) and ACVR2B (16-18). BMP9 signaling is measured by analyzing the activation/phosphorylation and expression of the downstream effectors of these TGF-β type I receptors, i.e. SMAD1/5 phosphorylation and ID1 gene expression.

Recently two groups reported mechanistic studies on the role of sEng. One group analyzed Fc chimeras containing amongst others the full-length endoglin extracellular domain (ECD) (sEng-Fc); the other examined recombinant sEng ECD without an Fc domain (6, 19). Both studies showed that endoglin ECD can directly bind to BMP9 and BMP10 with a high affinity. Moreover endoglin ECD and ALK1 were found to bind to different sites of BMP9 (6, 19). The recombinant sEng-Fc was found to inhibit vascular endothelial growth factor (VEGF) induced vessel formation in the chick chorioallantoic membrane assay and to reduce VEGF/ fibroblast growth factor (FGF)-induced blood vessel sprouting *in vivo* (6). It was proposed that sEng functions as a ligand trap to block the binding of BMP9 and BMP10 to TGF-β type II receptors to inhibit BMP9 and BMP10 signaling, thereby perturbing vascular homeostasis (6, 19).

To gain further insight in the molecular mechanisms by which sEng regulates BMP9/SMAD signaling, we expressed and purified recombinant sEng containing the full-length endoglin ECD using human embryonic kidney 293 cells stably expressing Epstein-Barr virus nuclear antigen (HEK EBNA), and investigated the biochemical interaction of this sEng with BMP9 and ALK1 ECD. In addition, we investigated the effect of recombinant sEng on BMP signaling and examined the role of ALK1, TGFβR2 and BMPR2 therein.

Results

Expression and purification of human sEng from human cells

The full-length extracellular region of human endoglin (Figure 1A) was cloned in a pCEP4 C-terminal histidine-tag (His-tag) vector and transfected into human HEK EBNA cells. The supernatant of the transfected cells was collected and the secreted endoglin was purified by column chromatography. Non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot analysis with an anti-endoglin antibody indicates that both dimeric (D) and monomeric (M) forms of sEng were present in the supernatant, with molecular weights of around 150 kDa and 80 kDa respectively (Figure 1B). In addition, a higher molecular weight protein complex was detected that likely represents a tetrameric form of sEng, albeit in a lower ratio compared to the D and M forms (Figure 1B). The different forms of sEng were further purified by nickel nitrilotriacetic acid column chromatography. The size of the D form of sEng was found to be comparable with that of recombinant

endoglin from R&D systems (Figure 1C). Moreover, both the R&D endoglin and the D form of purified sEng could be dissociated into the (monomeric) M form upon reduction with dithiothreitol (DTT) (Figure 1C). This is consistent with previous reports in which endoglin was found to be a covalently dimerized protein with disulfide cysteine linkages at position 350 and 582 (19, 20). In summary, our results showed that the full-length ECD of endoglin can be produced as a soluble form in human cells, and that it seems to exist in a dimeric and monomeric configuration.

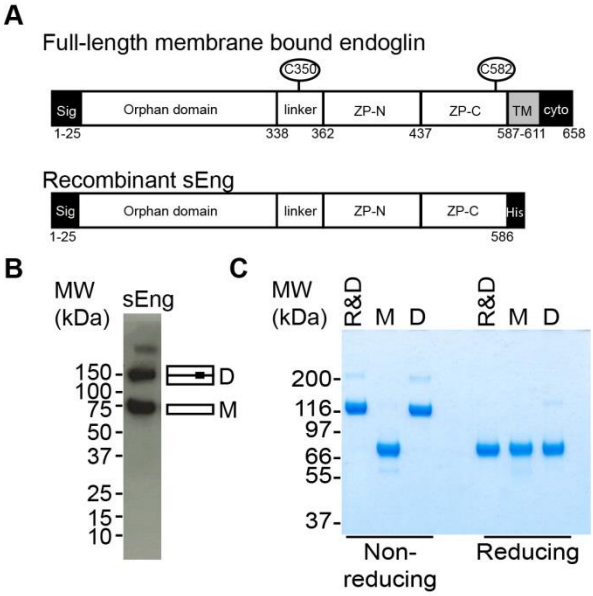

Figure 1. Western blot analysis of recombinant sEng. A, Schematic diagrams of full-length membrane bound endoglin and recombinant sEng with His-tag used in this study with numbers indicating the amino acid residues. Full-length endoglin is cleaved by MMP14 at position 586 to release 80 kDa sEng with the complete extracellular domain. Cys350 and Cys582 may mediate the dimerization of endoglin via disulfide linkage. Sig, signal peptide; TM, transmembrane region; ZP, zona pellucida; C350, cysteine 350; C582, cysteine 582; cyto, cytoplasmic; His, histidine tag. **B,** sEng expression in dimeric and monomeric forms. Supernatant of sEng expressing cells was separated on non-reducing SDS-PAGE and detected with an endoglin antibody; the diagram on the right shows a schematic drawing of sEng. Molecular weight markers are indicated on the left side. **C,** SDS-PAGE analysis of recombinant human endoglin (R&D Systems) and 1.5 µg purified sEng dimers and monomers, under non-reducing

and reducing conditions followed by Coomassie staining. D, dimer form; M, monomer form.

Both D and M sEng can form complexes with BMP9 and BMP9/ALK1

To investigate whether the purified forms of sEng can interact with BMP9 and ALK1, we incubated it with purified recombinant BMP9 and ALK1 and analyzed complex formation on native PAGE. His tagged BMP9 (HBMP9) and ALK1 ECD were produced as described before and they can form a complex on native gel (21). We observed that both D and M forms of sEng can form complexes with HBMP9, as upon co-incubation with BMP9 the two bands of sEng shifted to higher molecular weight bands (Figure 2A and 2B, band 2, 3). sEng can form complexes in the presence of both ALK1 ECD and BMP9 (Fig. 2A and 2B, band 4, 5), but not with ALK1 ECD alone (unpublished data). SDS-PAGE analysis of bands 2-5 confirmed that both the D and M forms of sEng formed complexes with BMP9 and BMP9/ALK1 (Figure 2B and 2C).

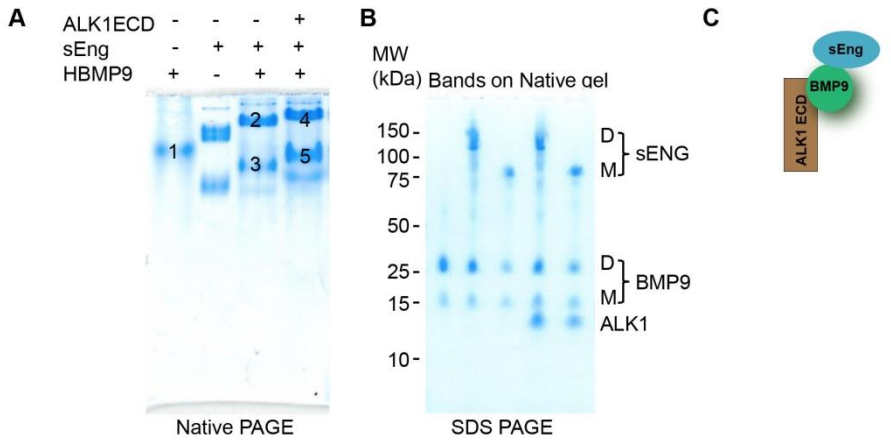

Figure 2. sEng forms complexes with BMP9 and BMP9/ALK1. A, Purified proteins were mixed and run on 10% native PAGE at 100V for 3 hours and visualized by Coomassie staining. Both sEng dimer and monomer can form complexes with His tagged (H) BMP9 and BMP9/ALK1 in the native PAGE. **B,** Analysis of the complexes shown in panel A: bands 1-5 were cut out and boiled in loading buffer with SDS for 20 min before loading in the non-reducing SDS-PAGE. D, dimer, M, monomer. **C,** Schematic diagram of the monomeric sEng complexed to BMP9-ALK1 ECD in the plasma membrane.

Effects of sEng on BMP9 induced signaling

To gain more insight into how monomeric sEng regulates BMP9 signaling, we assessed SMAD phosphorylation in human ECs treated with BMP9 or BMP9/sEng complexes. Human microvascular endothelial cells (HMEC-1) were serum starved overnight, and (without washing) subsequently stimulated with BMP9 (0.1 ng/ml), or with BMP9 that was first pre-incubated with increasing concentrations of the purified sEng monomer (BMP9/sEng) for 15 minutes at room temperature. Cells were stimulated for 15 minutes to detect the immediate signaling responses without involving new protein synthesis. The phosphorylation status of SMAD1/5 (pSMAD1/5) was measured by western blot analysis. We observed that our monomeric sEng partially inhibits BMP9 induced pSMAD1/5 activity at the high concentration (200 ng/ml), as observed by others who studied the effect of recombinant sEng on BMP9 signaling (6, 19), and unexpectedly, but reproducibly, also at low doses of sEng (4 ng/ml). However, at concentrations ranging from 8 to 16 ng/ml we did not find that monomeric sEng inhibits but weakly activates BMP9/SMAD1 signaling (Figure 3A). The weak stimulatory effect was reproducible, but the exact dose of BMP9 by which this was observed differed between experiments. Next, we analyzed BMP9 and BMP9/monomeric sEng for their ability to induce an Id1-derived BMP responsive element (BRE) driven luciferase reporter in C2C12 mouse myoblast cells. As these cells do not express ALK1, we first transfected the cells with a human ALK1 expression vector or control vector, and subsequently with the BRE reporter. After overnight serum starvation, the transfected C2C12 cells were stimulated with increasing concentrations of BMP9 or BMP9 pre-incubated with monomeric sEng (16 ng/ml) for 8 hours. Both BMP9 and BMP9/sEng induced luciferase responses in the presence of ALK1, and there was a trend that the preformed BMP9/sEng complex is more potent than BMP9 to induce luciferase activity at multiple BMP concentrations (Figure 3B). Thus, these experiments indicate that that monomeric sEng has multiple effects on BMP9 induced signaling, and does not only inhibit signaling by acting as a ligand trap that blocks the binding of BMP9 to its receptors.

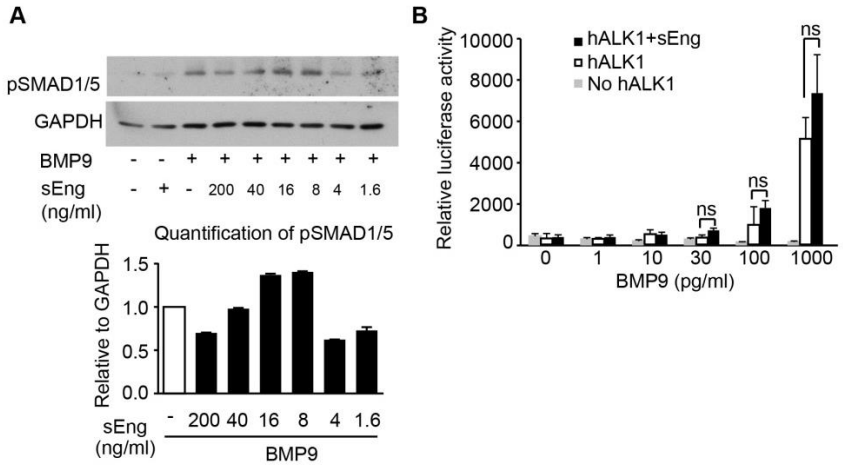

Figure 3. Effects of sEng on BMP9 induced signaling. A, The effect of sEng monomer on BMP9 induced pSMAD1/5 in human microvascular endothelial cells. **B,** sEng promotes BMP9/ALK1 mediated signaling in a BRE-luciferase assay in C2C12 mouse myoblast cells. All the experiments were repeated 3 times, and one of the representative results is shown. Values are presented as mean and SD from 3 technique replicates. Data are representative of three independent experiments.

Both TGFβR2 and BMPR2 may mediate BMP9 regulated signaling

BMP9 strongly binds to ALK1 and BMPR2 and weakly binds to the type II receptors ACVR2A and ACVR2B, but not TGFβR2 (17, 18). The role of full-length (membrane bound) endoglin in regulating BMP9 signaling is largely dependent on the type II receptors involved (22), which raises the question whether sEng might utilize different type II receptors than full-length endoglin to alter downstream SMAD signaling. To answer this question, we first knocked down ALK1, BMPR2, TGFβR2 and ACVR2B by siRNA in HMEC-1 cells. Receptor knockdown efficiency was verified by the quantitative PCR (qPCR) analysis (Figure 4A). HMEC-1 cells were subsequently stimulated with BMP9 for 15 minutes. Compared to the cells treated with control siRNA the induction of pSMAD1/5 was impaired in the ALK1, BMPR2 and TGFβR2 knockdown cells but not in the ACVR2B depleted cells (Figure 4B). This suggests that ALK1, BMPR2 and TGFβR2 might be required for BMP9 signaling in HMEC-1 cells. To further study this, we performed crosslinking experiments with ^{125}I-BMP9. COS-1 cells were transfected with expression plasmids encoding a mutant TGFβR2 containing a truncated intracellular domain with a

Myc tag (TGFβR2 Δ-Myc), HA-tagged wild-type ALK1 (ALK1-HA) and full-length membrane bound endoglin (Figure 4C). After incubation with ^{125}I-BMP9 for 3 hours and crosslinking, the receptor and ligand complexes were immunoprecipitated with a Myc antibody (Figure 4C). TGFβR2 was found to bind to BMP9 in the presence of ALK1 or endoglin in this overexpression system (Figure 4C). This indicates that TGFβR2 ECD might transduce BMP9 and endoglin controlled signaling. Additional receptor interaction experiments with labeled sEng need to be performed for further investigations.

Discussion

In this study we expressed and purified recombinant sEng containing a full-length ECD in human cells, and showed that both monomeric and dimeric sEng forms complexes with BMP9 and ALK1 ECD *in vitro*. We also showed that TGFβR2 ECD can bind to BMP9 in the presence of overexpressed ALK1 or endoglin. In our experimental conditions monomeric sEng did not inhibit BMP9 induced signaling at a physiological concentration (around 16 ng/ml). This might be due to the fact that sEng together with BMP9 can bind to ALK1 (and possibly TGFβR2) and participate in signaling itself at the cell membrane. Another explanation might be that dimeric sEng is a more potent scaffold than monomeric sEng, but this remains to be established.

The soluble form of endoglin has been hypothesized to scavenge BMP9 and thereby affect the delicate balance of angiogenesis (6, 19). However, our study indicates that it may also function in a different way, which appears to be concentration dependent. The studies demonstrating an inhibitory effect of sEng on BMP signaling were performed with relatively high concentration of sEng. For instance, Alt *et al.* showed that 10,000 ng/ml sEng blocks BMP9 induced *ID1* gene expression in HMEC-1 (19). Castonguay *et al.* showed an inhibitory effect of human sEng-Fc on BMP9 induced luciferase activity in a human rhabdomyosarcoma cell line with IC50 of 83.6 ng/ml based on the molecular weight of sEng-Fc (6). However, it is difficult to compare their results with ours, since they used a different cell type and the Fc fusion protein used might bring about unspecific protein interactions. It is possible that excessive sEng scavenges BMP9 to inhibit the downstream signaling, but that at lower concentrations sEng might also promote downstream signaling. Importantly, the median value of sEng in the plasma of normal pregnant women is 5.6 ng/ml; for

patients with preeclampsia the median value is around 73.7 ng/ml for early-onset patients and 31.1 ng/ml for late-onset patients (23).

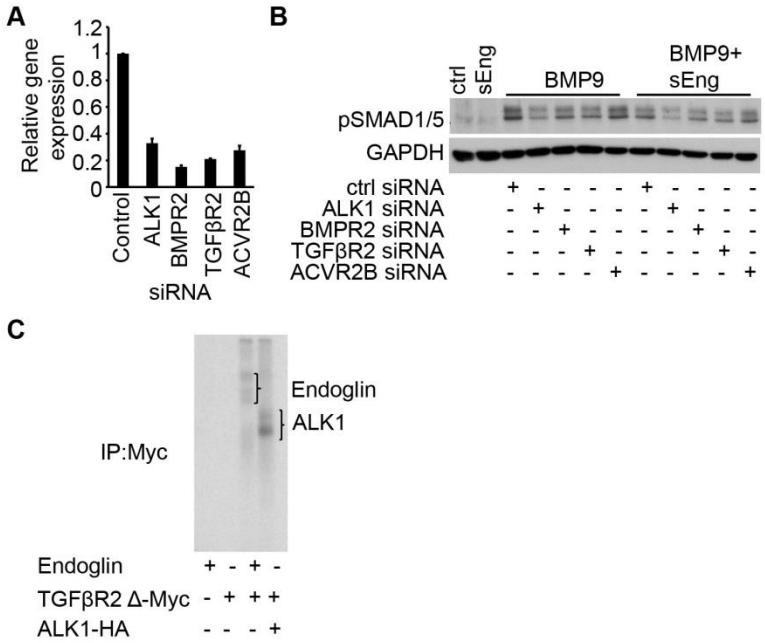

Figure 4. TGFβR2 is possibly involved in BMP9 signaling. A, Verification of the knockdown efficiency of the indicated constructs by qPCR in HMEC-1 cells two days after the transfection. Scramble siRNA (Control) was tested in all qPCR primers set, which is defined as 1, and all values were accordingly adjusted to the control. **B,** Knockdown of ALK1, BMPR2, TGFβR2 in HMEC-1 impairs BMP9 induced BMP/SMAD signaling. **C,** BMP9/endoglin/TGFβR2 and BMP9/ALK1/TGFβR2 complexes were detected on the cell surface of transfected COS-1 cells after affinity labeling with ^{125}I-BMP9. Data are representative of three independent experiments in Figure 4A and 4B.

The involvement of TGFβR2 might be another reason why we did not observe a strong inhibitory effect of sEng in our study. Upton *et al.* showed that the type II receptors are critical for balancing BMP9 signaling via ALK1, as BMP9 signals through BMPR2 to induce Smad1/5 phosphorylation while ACVR2A mediates BMP9/Smad2 activation in ECs (22). As mentioned above, we found that endoglin ECD forms complexes with BMP9 and ALK1 ECD, and that BMP9 can bind to TGFβR2 in the presence of excessive ALK1 or

full-length (membrane bound) endoglin. Moreover, knockdown of TGFβR2 by siRNA inhibited BMP9/SMAD signaling. Previous research also showed that endoglin, ALK1 and TGFβR2 form functional complexes to interact with several proteins associated with endothelial barrier maintenance and cell migration (24). We therefore hypothesize that BMP9 and (monomeric) sEng can bind TGFβR2 at physiologically relevant concentrations of sEng; these BMP9/sEng complexes might signal through ALK1/TGFβR2 and result in non-inhibitory effects on BMP signaling. However, receptor binding experiments with monomeric sEng need to be performed to validate binding to the various type II receptors involved in BMP9/sEng signaling. This might help to explain how sEng contributes to the pathogenesis of various vascular diseases like preeclampsia.

Materials and Methods

Cell culture and reagents

Human HMEC-1 cells were maintained in MCDB 131 (Invitrogen) supplied with 10 ng/ml epidermal growth factor (Millipore), 1 ug/ml hydrocortisone (Sigma) and 15% fetal bovine serum (FBS). C2C12 mouse myoblast cells and COS-1 cells were grown in DMEM with 10% FBS. All cells were cultured at 5% CO_2. The antibodies used for immunoblotting were phosphorylated SMAD1/5 antibody (1:1000, Cell signaling Technology, Danvers, MA, USA), Endoglin antibody (R&D system), Myc antibody (Santa Cruz) and GAPDH antibody (1:40,000, Sigma).

sEng expression and purification

The human endoglin extracellular domain was cloned into pCEP4 with a C-terminal His-tag and verified by DNA sequencing. The endoglin ECD construct was transfected into HEK EBNA cells by polyethylenimine as described before (21). Cells were changed into CDCHO expression medium without serum the following day, and conditioned media was harvested after 3–4 days. To purify sEng, the conditioned medium was run over a nickel nitrilotriacetic acid column (GE Healthcare), followed by a Q sepharose and S200 gel filtration column.

Transfections and quantitative real-time PCR analysis

HMEC-1 cells were transfected using DharmaFECT 1 (Thermo Scientific, Pittsburgh, PA, USA) according to the manufacturer's instructions. Total RNA was isolated using the RNAII isolation kit (Machery Nagel, Düren, Germany) according to the manufacturer's instructions. 1 µg RNA was reverse transcribed by using the RevertAid H Minus First strand cDNA synthesis kit (Fermentas, St.Leon-Rot, Germany) according to manufactures instructions. Quantitative real-time PCR (qPCR) analysis was performed using the Roche LightCycler 480 and the relative expression levels of the genes of interest were determined in triplicate for each sample using the $2^{-\Delta\Delta CT}$ method. Values were normalized to *ACTIN* expression.

qPCR primers

Markers	Forward primer (5'-3')	Reverse primer (5'-3')
ACTIN	AATGTCGCGGAGGACTTTGATTGC	AGGATGGCAAGGGACTTCCTGTAA
ALK1	ATGACCTCCCGCAACTCGA	TAGAGGGAGCCGTGCTCGT
ACVR2B	ATGTGGACATCCATGAGGAC	TGAAGATCTCCCGTTCACTC
BMPR2	AACTGTTGGAGCTGATTGGC	GGTTTGCAAAGGAAAACAC
TGFβR2	GTCCTTCAAGCAGACCGATGTC	CGTCTCACACACCATCTGGATG

^{125}I-BMP9 binding assay

Iodination of BMP9 was performed according to the chloramine T method and cells were subsequently affinity-labeled with the radioactive ligand as described before (25). In brief, cells were pre-incubated with the radioactive BMP9 on ice for 3 hours. After incubation, crosslinking was performed using 0.27 uM disuccinimidyl suberate (DSS) and 0.07 uM bis(sulfosuccinimidyl)suberate

(BS3, Pierce) for 15 minutes. Cells were washed and lysed. Cell lysates were incubated with Myc antibody overnight and immune complexes were precipitated by adding protein G sepharose (GE healthcare). Samples were boiled and subjected to SDS-PAGE.

Transcriptional reporter assay

C2C12 cells were seeded in a 96-well plate and transiently transfected with a human ALK1 expression construct or empty vector using Lipofectamine reagent (Invitrogen) according to the manufacturer's protocol. 24 hours later, the cells were transfected with BRE-luciferase reporter construct. An expression plasmid for β-galactosidase was co-transfected and used to correct for transfection efficiency. Cells were serum-starved overnight and stimulated with the respective ligands the next day for eight hours. Cells were washed, lysed and luciferase and β-galactosidase activity were determined. Each transfection was carried out in triplicate and representative experiments are shown.

Standard SDS–PAGE, non-reducing SDS–PAGE, and native PAGE

Standard SDS-PAGE was prepared with reducing reagent DTT or β-mercaptoethanol and denatured by SDS. Non-reducing SDS-PAGE was prepared without DTT, but with SDS. For native PAGE, protein samples were prepared without SDS denaturation and DTT reduction, and there was no SDS or DTT included in the gel casting buffers or in the running buffer for electrophoresis. The gels were either stained by Coomassie blue or used for Western blotting. Immunoblotting was performed as previously described, using standard techniques (26).

Acknowledgements

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