PP2A regulates BMP signalling by interacting with BMP receptor complexes and by dephosphorylating both the C-terminus and the linker region of Smad1

Luiza Bengtsson[1,*,‡], Raphaela Schwappacher[1,*], Martin Roth[2,§], Jan H. Boergermann[1], Sylke Hassel[2] and Petra Knaus[1,¶]

[1]Institute of Chemistry and Biochemistry, FU Berlin, Thielallee 63, 14195 Berlin, Germany
[2]Department of Physiological Chemistry II, University of Wuerzburg, Am Hubland, 97074 Wuerzburg, Germany

*These authors contributed equally to this work
‡Present address: MDC/FMP, Robert Roessle Strasse 10, 13125 Berlin, Germany
§Present address: Faculty of Life Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, UK
¶Author for correspondence (e-mail: knaus@chemie.fu-berlin.de)

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Summary

Phosphorylation of Smads is a crucial regulatory step in the signal transduction pathway initiated by bone morphogenetic proteins (BMPs). Although the dephosphorylation events terminating the pathway in the nucleus have been characterized, little is known about the dephosphorylation of Smads in the cytoplasm. In a proteomic screen for proteins interacting with the BMP type-II receptor, we found the regulatory Bβ subunit of PP2A. PP2A is one of the major serine/threonine phosphatases involved in cell-cycle regulation and signal transduction. Here, we present data showing that the Bβ subunit of PP2A interacts with both BMP type-I and type-II receptors. Furthermore, we demonstrate that several B subunits can associate with the BMP type-II receptor, independently of the kinase activity of the receptor and the catalytic subunit of PP2A. By contrast, the PP2A catalytic subunit is required for PP2A function at the receptor complex. This function of PP2A is the dephosphorylation of Smad1, mainly in the linker region. PP2A-mediated dephosphorylation of the BMP-Smad linker region leads to increased nuclear translocation of Smads and overall amplification of the BMP signal. Although other phosphatases identified within the BMP pathway are all shown to inhibit signalling, PP2A is the first example for a signalling stimulatory phosphatase within this pathway.

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Key words: BMP receptor, Phosphatase, PP2A, BMP2, Smad

Introduction

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β (TGFβ) superfamily of growth factors. BMPs regulate processes as diverse as dorsoventral patterning, cardiac development, ectopic cartilage and bone formation and are important for adult and embryonic stem cell fate and proliferation (reviewed by Bailey et al., 2007; Gazzerro and Canalis, 2006). The signal transduction cascade initiated by BMP ligand starts with activation of a heteromeric receptor complex consisting of two transmembrane serine/threonine kinases: the type-I (BMPRI) and type-II (BMPRII) receptors (Hartung et al., 2006). BMPRII, a constitutively active kinase, activates BMPRI by phosphorylation at the glycine and serine rich motif of BMPRI near the transmembrane domain . Activated BMPRI then phosphorylates the signal transducers Smad1, Smad5 and Smad8 (Smad1/5/8) at their C-terminal SSXS motif. Phosphorylated Smad1/5/8 form heteromeric complexes with co-Smad4 and translocate into the nucleus, where they are involved in transcriptional regulation of target genes (Cao and Chen, 2005; Derynck et al., 1998; Hoodless et al., 1996).

The TGFβ/BMP signal is highly dynamic: after ligand removal, phospho-Smad levels in the cell rapidly decline (Schmierer and Hill, 2005) and the receptors are degraded (Itoh and ten Dijke, 2007); both processes are necessary for spatial and temporal definition of the signal. Recent studies have identified several phosphatases that dephosphorylate Smads in the nucleus and thus terminate the TGFβ/BMP signal (Itoh and ten Dijke, 2007). For example, the pyruvate dehydrogenase phosphatase (PDP) and the small C-terminal domain phosphatases (SCPs) bind and dephosphorylate the SSXS motif of mammalian and *Drosophila* Smad1/5 (Chen et al., 2006; Knockaert et al., 2006). The phosphatase PPM1A antagonizes both TGFβ and BMP signalling by interacting with and dephosphorylating the very C-terminus of all R-Smads (Duan et al., 2006; Lin et al., 2006). Furthermore, the SCPs can also dephosphorylate the linker region of TGFβ/BMP R-Smads (Sapkota et al., 2007; Sapkota et al., 2006; Wrighton et al., 2006), which has distinct outcomes for the TGFβ versus BMP pathway (activation and inhibition, respective) (Sapkota et al., 2006). But how is the phosphorylation status of Smads regulated in the cytoplasm? Smads are not only phosphorylated by the activated type-I receptors, but also by a plethora of kinases from cross-talking pathways. Mitogen-activated protein kinases (MAPKs), cyclin-dependent kinases (CDKs), Jun N-terminal kinases (JNKs) and glycogen synthase kinase 3 (GSK3) all phosphorylate the linker of Smads in the cytoplasm, which results in inhibition of Smad translocation into the nucleus (Javelaud and Mauviel, 2005; Massague et al., 2005). Interestingly, both the C-terminal and the linker phosphorylation of Smads are induced upon stimulation with TGFβ and BMP (Sapkota et al., 2006). The MAPK and GSK3 linker-phosphorylated Smad1 is recognized by the ubiquitin ligase Smurf1, which not only targets the ubiquitinated Smad1 for degradation (Fuentealba et al.,

2007; Sapkota et al., 2007), but also inhibits its binding to the nucleoporin Nup214 (Sapkota et al., 2007). Thus, in order to efficiently translocate into the nucleus and transduce the TGFβ/BMP signal, Smads have to first overcome their inhibitory linker phosphorylation in the cytoplasm.

In our search for novel interactors of BMPRII (Hassel et al., 2004), we found the Bβ subunit of PP2A. The PP2A holoenzyme consists of three subunits: a catalytical subunit PP2A-C, a scaffolding subunit PP2A-A and a regulatory subunit PP2A-B (Lin et al., 1998; Ruediger et al., 1991). The PP2A-B subunit regulates the catalytical activity of the core complex and targets PP2A to different subcellular compartments and substrates (Kamibayashi et al., 1994; McCright et al., 1996; Schmidt et al., 2002; Strack et al., 1998; Tehrani et al., 1996). PP2A is involved in regulation of various cellular processes such as progression through the cell cycle, control of splicing and specification of tissue patterns (reviewed by Janssens and Goris, 2001; Moorhead et al., 2007). Several protein kinases such as protein kinase B [PKB/Akt (Andjelkovic et al., 1996)], p70-S6 kinase (Petritsch et al., 2000), protein kinase C [PKC (Srivastava et al., 2002)], calcium/calmodulin-dependent protein kinases [CaM kinases (Barnes et al., 1995)], MAPK/ERK kinase [MEK (Sontag et al., 1993)], p38-MAPK (Junttila et al., 2007) and extracellular signal-regulated kinases [ERKs (Alessi et al., 1995)] are regulated by PP2A through dephosphorylation.

PP2A belongs to the PPP family of phosphatases (Moorhead et al., 2007). Several members of the PPP family have been implicated in the regulation of TGFβ/BMP signalling. For example, the catalytic subunit of PP1 interacts with SARA [Smad anchor for receptor activation (Bennett and Alphey, 2002)] whereas the regulatory subunit of PP1, the growth arrest- and DNA damage-inducible protein GADD34, interacts with Smad7 and the TGFβ type-I receptor TGFβRI. GADD34-PP1c not only dephosphorylates TGFβRI (Shi et al., 2004) but is also recruited by the FYVE-domain protein endofin to BMPRIa to dephosphorylate this receptor (Shi et al., 2007). Furthermore, the Bα and Bβ subunits of PP2A bind and become phosphorylated by TGFβRI (Griswold-Prenner et al., 1998). Most recently, Bα and Bδ have been shown to have opposite roles in TGFβ signalling: Bα activates the pathway by stabilizing Activin type-I receptor (ALK4) protein levels whereas Bδ represses the pathway by downregulating ALK4 activity (Batut et al., 2008). Finally, inhibition of PP2A enhances the C-terminal phosphorylation of TGFβ-Smads (Van Berlo et al., 2005).

In our studies, we focused on the role of PP2A and its regulatory B subunits in the modulation of BMP signalling. We found that PP2A-B subunits are associated with BMP receptors, regardless of the receptor complex activation status. PP2A can dephosphorylate BMPRII, and, most importantly, the B subunit seems to target PP2A to activated Smads. PP2A dephosphorylates BMP Smads mainly in the linker region, which results in increased translocation of Smads into the nucleus and enhanced BMP signalling. Thus, PP2A is a cytoplasmic Smad phosphatase and a positive regulator of BMP signalling.

Results

The B subunit of PP2A associates with the cytoplasmic domain of the BMP type-II receptor

In order to identify proteins associated with BMPRII, we performed GST pulldown from ^{35}S-labelled C2C12 cell lysates using either BMPRII short form [BMPRII-SF, a naturally occurring splice form of BMPRII lacking the tail region (Rosenzweig et al., 1995)] or BMPRII-tail domain (amino acid 501-1038) fused to GST as bait (Hassel et al., 2004). Co-precipitating proteins were identified by mass spectrometry. Among the GST-BMPRII-tail-associated proteins was the murine orthologue of the regulatory subunit B, β isoform, of PP2A (PP2A-Bβ; S1). Absence of a protein in a proteomic screen is often the result of technical detection problems, thus, to check whether the interaction BMPRII-Bβ really is restricted to the BMPRII tail region, we repeated the GST pulldown from C2C12 cell lysates and analyzed precipitated protein complexes by western blotting using antibodies recognizing all PP2A-B isoforms (Fig. 1A). We found that endogenous B subunits specifically interacted with both BMPRII-SF and the BMPRII-tail (Fig. 1A, lanes 3 and 4).

Next, we tested whether PP2A-B subunits interact with endogenous BMP receptors. As a positive control, we used the TGFβ receptors (TGFβRI and TGFβRII), as PP2A-Bα and PP2A-Bβ have been shown to associate with both TGFβRI and TGFβRII (Griswold-Prenner et al., 1998). We used antibodies covalently coupled to protein-A-Sepharose to precipitate endogenous TGFβ and BMP receptors from C2C12 cell lysates (Fig. 1B). As expected, we found PP2A-B subunit co-precipitating with TGFβRI and TGFβRII (Fig. 1B, lanes 4 and 5). Furthermore, we found B subunits associated with BMPRIa and BMPRII (Fig. 1B, lanes 6 and 7). Additionally, a larger or modified B subunit specifically associated only with TGFβRII. Thus, PP2A-B subunits appear to have a general affinity towards receptors of the TGFβ superfamily.

Different PP2A-B subunits can interact with BMPRII independent of both the kinase activity of BMPRII and the catalytic subunit of PP2A

Since PP2A-B subunits can bind all TGFβ family receptors that we tested, we wondered whether BMPRII can bind all B subunits.

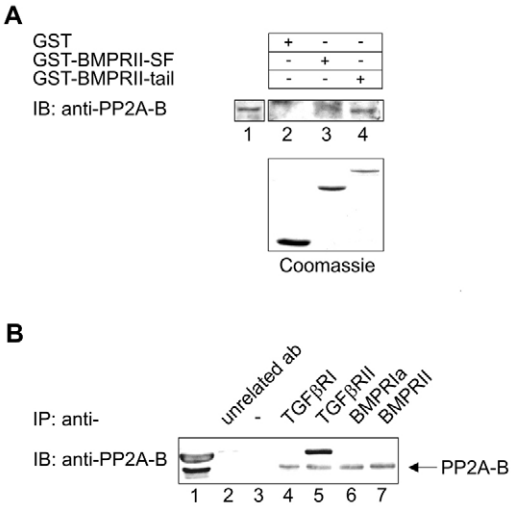

Fig. 1. PP2A-B subunit interacts with BMP/TGFβ receptors. (A) PP2A-B interacts with BMPRII-tail and BMPRII-SF. Recombinant GST-BMPRII-tail, GST-BMPRII-SF or GST alone was incubated with C2C12 cell lysates. Bound proteins were separated by SDS-PAGE and PP2A-B was detected by immunoblotting with anti-PP2A-B antibody (upper panel). Lane 1 shows the expression of PP2A-B subunit in C2C12 cells. Lower panel shows the input of total GST fusion protein. (B) Endogenous PP2A-B subunit interacts with endogenous BMP and TGFβ receptors. C2C12 cell lysate was incubated with the indicated antibodies which were covalently bound to protein-A-Sepharose. Lane 1, the PP2A-B subunit expression level; lane 2, unrelated antibody with subsequent protein-A-Sepharose incubation; lane 3, protein-A-Sepharose alone; lane 4, anti-TGFβRI; lane 5, anti-TGFβRII; lane 6, anti-BMPRIa; lane 7, anti-BMPRII). Precipitates were analyzed using SDS-PAGE and immunoblotting with anti-PP2A-B antibody.

Furthermore, we wanted to know whether the activity of the receptor or the phosphatase is a prerequisite for interaction. To check this, we overexpressed the wild-type (wt) BMPRII-LF or kinase-dead BMPRII-LF [BMPRII-LF-K230R (Hassel et al., 2003)] along with the B subunits α, β and γ in HEK293T cells and immunoprecipitated either the respective B subunits or the receptor (Fig. 2A-C). Additionally, we used a deletion mutant of PP2A-Bγ (PP2A-Bγ-Δ26-38) which cannot interact with PP2A catalytic and scaffolding subunits (Strack et al., 2002) (Fig. 2B).

Both wt and kinase-dead BMPRII-LF co-precipitated with the Bβ subunit indicating that the receptor activity is not required for the interaction (Fig. 2A, lanes 6 and 7). Accordingly, stimulation of cells with BMP2 has no impact on the strength of interaction (data not shown). The highly homologous B subunits α and γ (supplementary material Fig. S1) also interacted with BMPRII-LF, implying that the binding site on BMPRII is not tailored to a specific isoform of B subunit (Fig. 2B,C). Furthermore, the Bγ deletion mutant could still interact with BMPRII-LF (Fig. 2B, lane 9) indicating that the association between BMPRII and PP2A-B is mediated by neither A nor C subunits and is therefore independent of PP2A phosphatase activity. We confirmed these results in a GST-pulldown with GST-fused BMPRII-SF and -tail and ^{35}S-labelled wt and mutant B subunits (data not shown).

PP2A can attenuate BMPRII phosphorylation

While characterizing the interaction between TGFβRI and PP2A-Bα, Griswold-Prenner and co-workers did not detect any effect of Bα on the phosphorylation status of the receptor (Griswold-Prenner et al., 1998). Also, our attempts to assess the impact of PP2A-Bβ on phosphorylation status of BMP receptors using in vivo phosphorylation were unsuccessful (data not shown). In order to have some indication of whether dephosphorylation of BMPRII by PP2A is formally possible, we performed in vitro dephosphorylation assays. We immunoprecipitated BMPRII-LF from HEK293T cells overexpressing the HA-tagged receptor and incubated the receptor in the presence or absence of purified PP2A and [γ-^{32}P]ATP (Fig. 3A,B). The phospho-signal was quantified relative to the amount of precipitated receptor (Fig. 3C). Although we did not use any kinase inhibitors in this experiment, we found that phosphorylation of BMPRII-LF was attenuated in the presence of purified PP2A (Fig. 3B,C). By contrast, when we performed similar experiments on BMPRI (dephosphorylation of immunoprecipitated BMPRIa activated by BMP2; Fig. 3D), we could not detect any effect of PP2A on the phosphorylation status of the type-I receptor (Fig. 3E). Thus, binding of PP2A-B can dephosphorylate and/or inhibit phosphorylation of BMPRII but not BMPRI, at least in vitro.

PP2A is a Smad phosphatase

Activated BMPRI phosphorylates Smad1/5/8 on the C-terminal SSXS-motif (Kretzschmar et al., 1997b). Thus, to investigate the effects of Bβ on downstream effectors of the BMP pathway, we first assessed its impact on the C-terminal phosphorylation of endogenous Smad1/5/8. We overexpressed PP2A-Bβ in C2C12 cells and observed a decrease in BMP2-induced C-terminal Smad1/5/8 phosphorylation as compared to mock-transfected cells (Fig. 4A, compare lanes 2 and 4). The effect was readily seen, even without having to use BMPRI inhibitors. Accordingly, treatment of C2C12 cells with 1 nM okadaic acid, an inhibitor of PP2A catalytic subunit (Cohen et al., 1990), caused a modest, but reproducible increase in BMP2-induced C-terminal phosphorylation of Smad1/5/8 (Fig. 4B).

Phosphorylation of the linker region of Smads is an important determinant of their cellular localization and activity (Kretzschmar et al., 1997a; Pera et al., 2003; Sapkota et al., 2007). Several cellular kinases are known to phosphorylate the Smad linker region. Sites

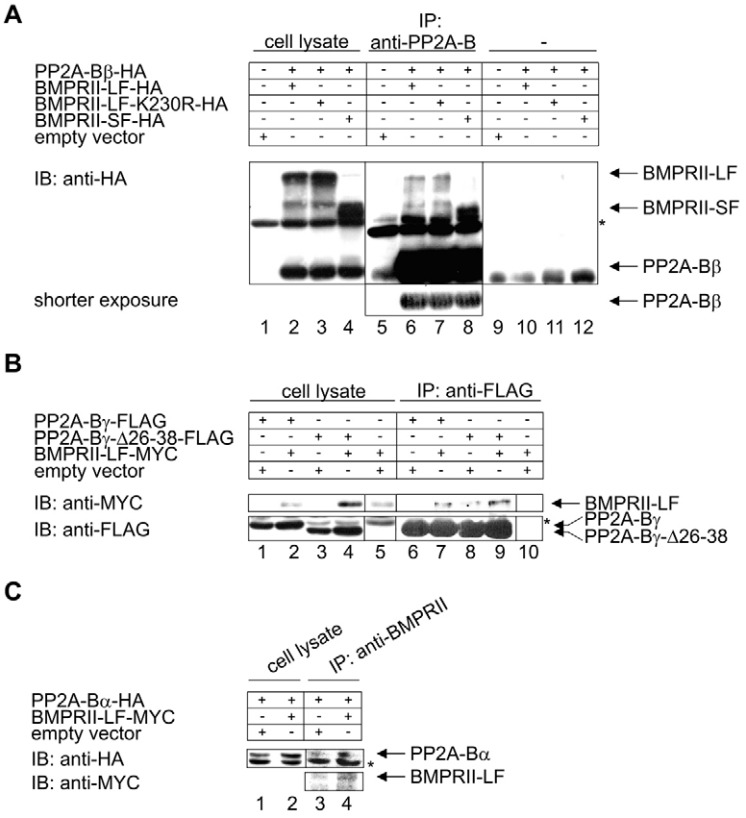

Fig. 2. Different PP2A-B subunits interact with BMPRII independently of receptor kinase activity and of the PP2A catalytic activity. (A) The association of PP2A-Bβ subunit with BMPRII is not inhibited by a kinase-dead mutation in BMPRII. HEK293T cells were transfected with the indicated constructs, lysed and incubated with anti-PP2A-B antibody. Co-precipitated BMPRII-LF (lane 6), BMPRII-LF-K230R (lane 7) and BMPRII-SF (lane 8) were analyzed by SDS-PAGE followed by immunoblotting with anti-HA antibody. Mock-transfected cells (lane 5) and incubation with protein-A-Sepharose beads alone (lanes 9-12) served as controls. An aliquot of each lysate was used to control for protein expression with anti-HA antibody (lanes 1-4). The lower panel shows a shorter exposure of immunoprecipitated PP2A-Bβ. The asterisk indicates an nonspecific band. (B,C) PP2A-B-BMPRII interaction is not PP2A-B isoform-specific and does not depend on phosphatase activity of PP2A. HEK293T cells were transfected with the indicated constructs. Immunoprecipitation was performed by incubation with (B) anti-FLAG antibody to precipitate PP2A-Bγ (lanes 6 and 7) and PP2A-Bγ-Δ26-38 (lanes 8 and 9) or (C) anti-BMPRII antibody covalently linked to protein-A-Sepharose to precipitate BMPRII coexpressed with PP2A-Bα. After separation by SDS-PAGE, protein complexes were examined by immunoblotting with (B) anti-MYC antibody (lower panel) or (C) anti-HA antibody (upper panel). Expression controls in B used anti-MYC and anti-FLAG antibodies (lanes 1-5), and in C anti-HA antibody. The asterisks indicate nonspecific bands.

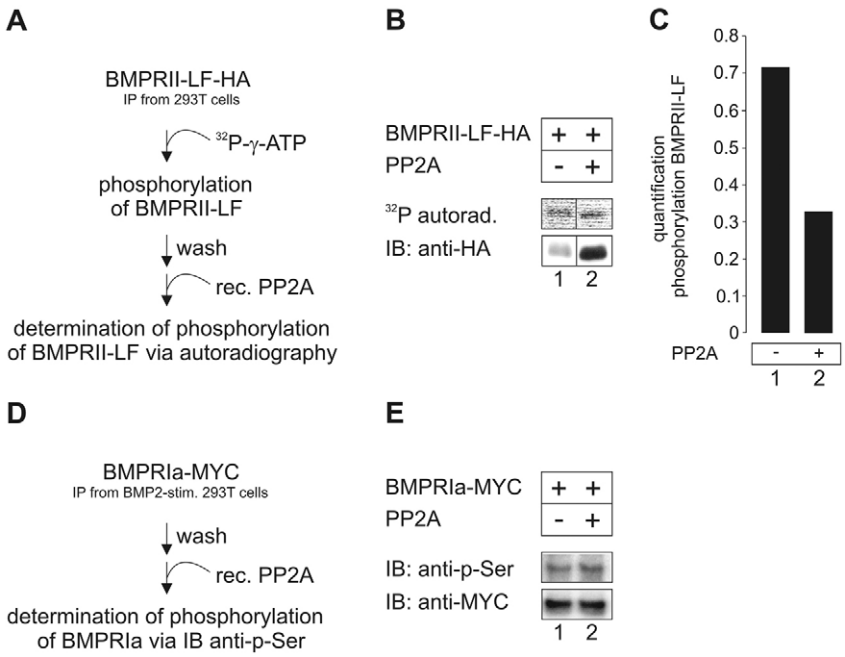

Fig. 3. PP2A dephosphorylates BMPRII. (A) Scheme of the in vitro phosphorylation-dephosphorylation assay of BMPRII. (B) Immunoprecipitated BMPRII-LF was subjected to in vitro phosphorylation using [γ-^{32}P]ATP. The dephosphorylation assay was started by the addition of recombinant PP2A to the phosphorylated receptor. Incorporation and removal of [γ-^{32}P]ATP was monitored by autoradiography (upper panel), and the amount of the bead-coupled BMPRII-LF by immunoblotting with anti-HA antibody (lower panel). (C) Quantification of the results in B. Phosphorylation of BMPRII-LF was quantified relative to the amount of BMPRII-LF protein using ImageJ. (D) Scheme of the in vivo and in vitro phosphorylation-dephosphorylation assay of BMPRIa. (E) After BMP2 stimulation, phospho-BMPRIa was immunoprecipitated and subjected to in vitro dephosphorylation by recombinant PP2A. Serine phosphorylation of the receptor was measured by immunoblotting (upper panel). Lower panel shows the amount of bead-coupled BMPRIa.

for MAPKs (Kretzschmar et al., 1997a; Pera et al., 2003) and GSK3 (Fuentealba et al., 2007; Sapkota et al., 2007) were found in the BMP Smad linker, whereas the linker of TGFβ Smads has also been shown to be phosphorylated by JNK, CDKs, PKC and CamKs (reviewed by Feng and Derynck, 2005). Furthermore, PP2A is a known 'counter player' of the MAPKs (Junttila et al., 2007), therefore, we investigated whether PP2A acts as a Smad linker phosphatase. We overexpressed either empty vector or the PP2A-Bβ subunit in C2C12 cells and treated the cells with BMP2 and/or okadaic acid. We then assessed phosphorylation status of the endogenous BMP Smad linker region by using an antibody that specifically recognizes MAPK/CDK-phosphorylated sites (Holmes and Solomon, 1996; Sapkota et al., 2006; Songyang et al., 1996). As previously shown (Sapkota et al., 2007; Sapkota et al., 2006), activation of the BMP signal transduction pathway resulted in an accumulation of MAPK-phosphorylated BMP Smads (Fig. 4C, compare lanes 1 and 2, panels a and b). Interestingly, treatment of the cells with okadaic acid alone resulted in a similar increase of phosphorylated MAPK sites on Smads (Fig. 4C, lane 4, panels a and b). Furthermore, the effects of BMP2 and okadaic acid were additive (Fig. 4C, lane 3, panels a and b). By contrast, overexpression of the PP2A-Bβ subunit (Fig. 4C, lanes 5-8) led to an inhibition of the BMP2-induced MAPK phosphorylation of Smads (Fig. 4C, lane 6, panels a and b). This effect could be partially reversed by a simultaneous treatment with okadaic acid (Fig. 4C, lane 7, panels a and b). Neither overexpression of Bβ subunit nor inhibition of PP2A by okadaic acid had any effect on the Smad1 protein levels in cells (Fig. 4C, panel e). To further confirm the involvement of PP2A in the dephosphorylation of the Smad linker region, we partially inactivated PP2A by knocking down levels of the scaffolding subunit Aα in C2C12 cells. We chose to target Aα for several reasons: (1) all tested B subunits tested were able to interact with BMPRII, thus we would presumably have to downregulate all of them to see an effect; (2) our attempts to downregulate C subunits led to cell lethality; and (3) knocking-down the A subunit would at least limit the functionality of the PP2A holoenzyme. As expected, lowering the levels of Aα in C2C12 cells (Fig. 4D), led to an increased Smad linker phosphorylation before and after BMP2 treatment (Fig. 4E, lanes 2 and 4).

SCPs have recently been shown to dephosphorylate BMP Smads in the linker region, on serine residues detectable by the phospho-MAPK antibody (anti-p-linker antibody) that we are using in the current study. Thus, it is formally possible, that the dephosphorylation effects we are observing are due to indirect activation of SCPs by PP2A. To exclude this possibility, we performed an in vitro phosphorylation and dephosphorylation assay. We incubated purified, recombinant Smad1 N-terminally fused to MBP with purified Smad linker kinase ERK2 (Kretzschmar et al., 1997a; Pera et al., 2003), as well as with CDK1 and CaMKII as negative controls, in the presence of [γ-^{32}P]ATP (Fig. 4F) and unlabelled ATP (Fig. 4G). As expected, Smad1 was in vitro phosphorylated mainly by ERK2 (Fig. 4F,G). After completed phosphorylation, the kinase was washed off and half of the sample was further incubated with purified PP2A (Fig. 4G, lanes 4-6). After SDS-PAGE and western blotting, phosphorylation status of the Smad1 linker was detected with the anti-phospho-linker antibody (Fig. 4G, panels a and b). As expected, ERK2 phosphorylated Smad1 at the linker. Furthermore, in accordance with our in vivo experiments, PP2A completely dephosphorylated the ERK2-phosphorylated linker region (Fig. 4G, compare lanes 3 and 6, panel a). Thus, we conclude that PP2A directly dephosphorylates BMP Smads in their linker region without the involvement of SCPs. But what about the C-terminus? Unfortunately we were unsuccessful at expressing recombinant BMPRIa, the Smad C-terminus kinase, and could thus not perform the same direct dephosphorylation assay as for the linker. Instead, we immunprecipitated endogenous Smad1 from C2C12 cells stimulated with BMP2 and, after extensive washing, we incubated the precipitates either with or without recombinant PP2A. The reactions were then divided, separated on SDS-PAGE, western blotted and the phosphorylation status of either the C-terminus or the linker of Smad1 was detected using specific antibodies (Fig. 4H). According to our results, PP2A is almost twice as effective

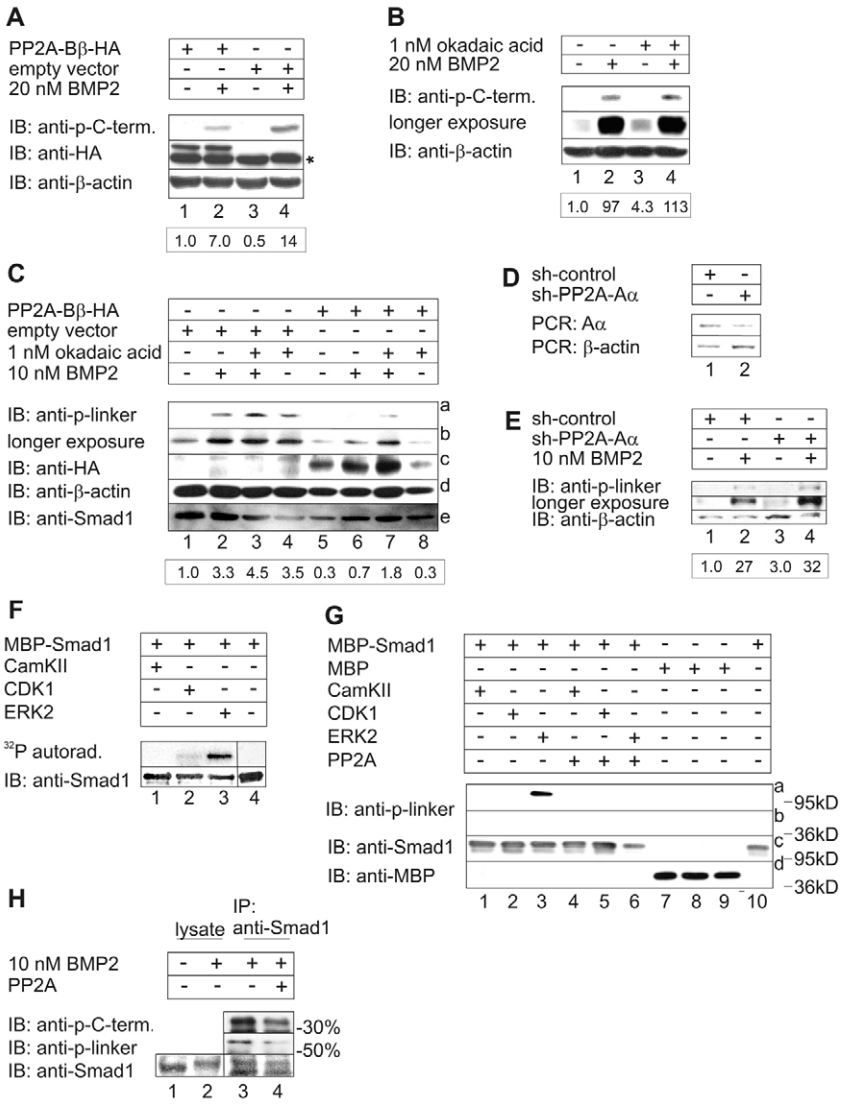

Fig. 4. PP2A dephosphorylates BMP-Smads. (A) BMP2-induced C-terminal Smad1/5/8 phosphorylation is attenuated by PP2A-Bβ. C2C12 cells were transfected with HA-tagged PP2A-Bβ (lanes 1 and 2) or empty vector (lanes 3 and 4) and stimulated with BMP2 (lanes 2 and 4) or left untreated (lanes 1 and 3). Cell lysates were analyzed by immunoblotting for phospho-Smad1/5/8 levels. β-actin was used as a loading control (lower panel) and anti-HA antibodies as a control for PP2A-Bβ expression (middle panel). The asterisk indicates a nonspecific band. Numbers below the lanes are the ratio of phospho-Smad to actin signal quantified by ImageJ (also in B,C and E). (B) BMP2-induced Smad1/5/8 phosphorylation is enhanced by inhibition of PP2A. C2C12 cells were stimulated with BMP2 (lane 2) or BMP2 and okadaic acid (lane 4) or okadaic acid (lane 3) or left untreated (lane 1). After SDS-PAGE, cell lysates were immunoblotted with anti-p-Smad1/5/8 (C-term.) antibody (upper panels). Equal loading was monitored by anti-β-actin antibody (lower panel). (C) PP2A-Bβ overexpression induces dephosphorylation of the linker region of Smad1. C2C12 cells overexpressing HA-tagged PP2A-Bβ (lanes 5-8) or empty vector (lanes 1-4) were starved and treated without ligand (lanes 1 and 5), with BMP2 (lanes 2 and 6), with BMP2 and okadaic acid (lanes 3 and 7) or okadaic acid alone (lanes 4 and 8). The lysates were analyzed by SDS-PAGE and immunoblotting for the phosphorylated linker region of BMP-Smads using an anti-MAPK substrate antibody (anti-p linker; panel a). Panel b shows a longer exposure of the anti-p-linker blot. Expression and loading was controlled for using anti-HA antibody (panel c), anti-β-actin antibody (panel d), and anti-Smad1 antibody (panel e). (D) Control of downregulation of endogenous PP2A-Aα using RNAi. The mRNA levels of PP2Aα in C2C12 cells transfected with either control non-silencing or PP2A-Aα-specific shRNAs were examined by RT-PCR using PP2A-Aα subunit-specific oligonucleotides. β-actin served as control. (E) Downregulation of Aα subunit enhances Smad linker phosphorylation. C2C12 cells were transfected with a plasmid encoding shRNA against PP2A-Aα. Level of Smad linker phosphorylation was monitored as in C. (F) CDK1 and ERK2 phosphorylate Smad1 in vitro. The upper panel shows the autoradiography of phosphorylated Smad1 after treatment with CamKII (lane 1), CDK1 (lane 2) and ERK2 (lane 3). The lower panel shows the amount of Smad1 in the sample. (G) PP2A dephosphorylates Erk2-mediated linker phosphorylation of Smad1 in vitro. MBP-Smad1 immobilized to amylose resin was in vitro phosphorylated. MBP alone served as control for nonspecific phosphorylation (lanes 7-9). PP2A-mediated dephosporylation was started by the addition of PP2A to the samples (lanes 4-6). Following SDS-PAGE, Smad linker phosphorylation was analyzed using immunoblot anti-p linker (panels a and b). Anti-Smad1 (panel c) and anti-MBP antibody (panel d) show the amount of MBP-Smad1 and MBP in each sample. (H) PP2A dephosphorylates BMP2-mediated C-terminally and linker-phosphorylated Smad1 in vitro. After BMP2 treatment, endogenous Smad1 was immunoprecipitated and subjected to in vitro dephosphorylation using recombinant PP2A. The precipitates were analyzed by SDS-PAGE and immunoblotting with anti-P-C-terminal and anti-p-linker antibodies. Anti-Smad1 immunoblotting monitors the amount of Smad1 in each sample. The percentages of the PP2A-mediated reduction in phosphorylation are given on the right. The intensities of the phospho signals and the Smad1 signals of a representative experiment were measured with ImageJ.

at dephosphorylating the linker as the C-terminus of Smad1 (30% versus 50% reduction of the phospho-signal).

To investigate whether dephosphorylation of the Smad linker is dependent on phosphorylation of its C-terminus or vice versa, we overexpressed either wt or mutant Smad1 in BMP2-stimulated HEK293T cells and immunoprecipitated these with Smad1 antibody. We then dephosphorylated the precipitates with recombinant PP2A and checked the level of remaining C-terminus or linker phosphorylation by specific antibodies. First of all, this experiment underlined the specificity of our phospho-linker antibody – the phospho-linker signal is absent in a Smad1 linker mutant (Smad1-LM) with its MAPK sites mutated to alanine (4SP/AP) (Kretzschmar et al., 1997a) (supplementary material Fig. S2). Interestingly, C-terminal phosphorylation is increased in the Smad1 linker mutant (supplementary material Fig. S2, compare grey columns 2 and 5) and the linker phosphorylation in the C-terminal Smad1 mutant (Smad1-CM; AAVA) (Kretzschmar et al., 1997) (supplementary material Fig. S2, compare black columns 8 and 11). Dephosphorylation of the C-terminus of overexpressed Smad1 protein seems independent of linker mutations (supplementary material Fig. S2, compare grey columns 2,3 and 5,6), however, the linker region is more readily dephosphorylated when the C-terminus is mutated (supplementary material Fig. S2, compare black columns 8, 9 and 11,12). These results, together with the results in Fig. 4H, indicate that the linker region is the preferred site of dephosphorylation by PP2A.

PP2A promotes Smad translocation into the nucleus

Phosphorylation in the Smad linker region and/or inhibition of the C-terminal phosphorylation has been shown to counteract the

activation of Smads and their subsequent translocation into the nucleus (Feng and Derynck, 2005). To investigate the effect of PP2A on Smad localization, we treated C2C12 cells with BMP2 with or without 1 nM okadaic acid and used immunofluorescence staining to visualize endogenous Smad1 and co-Smad4 (Fig. 5). As published (Lagna et al., 1996; Liu et al., 1996), activation of the BMP pathway lead to accumulation of both Smad1 and -4 in the nucleus (Fig. 5, second panels). Stimulation with BMP2 plus inhibition of PP2A by okadaic acid and thus simultaneous increase of C-terminal and linker phosphorylation of Smads lead to cytoplasmic accumulation of both Smad1 and Smad4 (Fig. 5, third panel). The accumulation of Smad4 in the cytoplasm is probably the result of its presence in the heterotrimeric, phosphorylated, but not translocated Smad complexes. Since increased C-terminal phosphorylation would normally lead to enhanced nuclear translocation of Smads, the linker phosphatase function of PP2A seems to dominate over the C-terminal phosphatase function.

PP2A is mainly a Smad linker phosphatase

Smads translocate into the nucleus in order to regulate gene expression (Shi and Massague, 2003). To check whether dephosphorylation of Smads by PP2A has functional consequences on the level of gene expression, we analyzed the effect of PP2A-B subunits on the activity of a BMP-regulated promoter. We measured the activity of luciferase transcriptionally controlled by the *BMP responsive element* (*BRE*) (Korchynskyi and ten Dijke, 2002) in C2C12 cells overexpressing PP2A-Bβ and -Bγ (Fig. 6). Expression of wt PP2A-Bβ and wt PP2A-Bγ resulted in an enhancement of Smad-dependent BMP2 signalling (Fig. 6A, compare columns 2, 4 and 6). By contrast, overexpressing the PP2A-Bγ-Δ26-38 mutant had no effect on BMP2-dependent transcription compared to mock-transfected cells (Fig. 6A, compare columns 2 and 8). The enhanced activity of the *BRE* promoter caused by overexpression of PP2A-Bβ could be partially reversed by treatment with okadaic acid (Fig. 6B, compare columns 6 and 7), which correlates with our western blotting data (Fig. 4E,F). Additionally, downregulation of Aα-subunit lead to ~50% decrease of promoter activity (Fig. 6C). Thus, the decreased level of BMP Smad linker phosphorylation caused by targeting of PP2A to Smads via the B subunits and via the BMP receptor complexes enhances Smad signalling. This strongly suggests, that PP2A preferentially dephosphorylates the Smad linker, in accordance with our previous data (Figs 4 and 5). Dephosphorylation of the Smad C-terminus would result in a decrease in luciferase activity.

In order to study the contribution of the linker dephoshorylation by PP2A to the overall signal output, we 'uncoupled' linker and C-terminal dephosphorylation by using Smad1 mutants (as in supplementary material Fig. S2). As expected, expression of wt Smad1 together with Bβ resulted in higher *BRE* promoter activity than overexpressing wt Smad1 alone (Fig. 6D, compare black columns 2 and 4). As expected, linker-mutated Smad1 is transcriptionally more active than the wt Smad1 (Fig. 6D, compare columns 6 and 2). Furthermore, expression of a Smad1 linker mutant together with Bβ leads to a reduction in promoter activity (Fig. 6D, compare columns 8 and 6). This result demonstrates that dephosphorylation of Smad1 C-terminus by PP2A. The C-terminal mutant of Smad has much lower transcriptional activity than the wt (Fig. 6D, compare columns 10 and 2). Consequently, expression of Smad1 C-terminal mutant together with Bβ leads to a slight increase of the promoter activity (Fig. 6D, compare columns 12 and 10). This result shows the dephosphorylation of Smad1 linker by PP2A. Since overexpression of Bβ results in an enhanced transcriptional activity of endogenous and overexpressed Smad1, we conclude that the function of PP2A as a Smad linker phosphatase overrides its role in attenuation of C-terminal Smad phosphorylation.

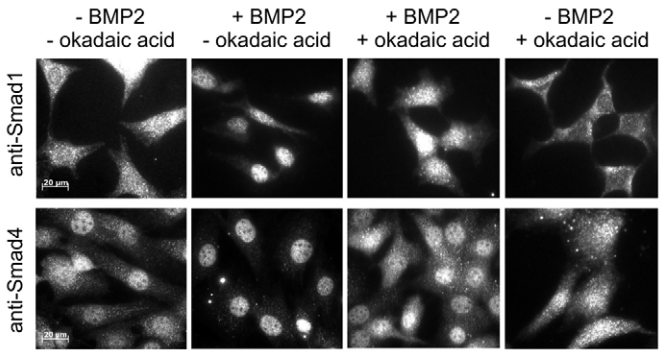

Fig. 5. Inhibition of PP2A represses Smad nuclear translocation. C2C12 cells were either left untreated, stimulated with BMP2, BMP2 and okadaic acid or okadaic acid alone, as indicated. After cell fixation and permeabilization, localization of endogenous Smad1 and co-Smad4 was detected using anti-Smad1 (upper panels) and anti-Smad4 antibodies (lower panels).

Discussion

Several Smad phosphatases have recently been identified: the PDPs, SCP1-3 and PPM1A (Chen et al., 2006; Duan et al., 2006; Knockaert et al., 2006; Lin et al., 2006). PDP and PPM1A belong to the family of metal-ion-dependent phosphatases (Lu and Wang, 2008), whereas SCPs are homologous to the RNA polymerase II phosphatase (Kamenski et al., 2004; Yeo et al., 2005). All of these phosphatases terminate the TGFβ/BMP signal in the nucleus. However, somewhat paradoxically, stimulation with BMP induces not only the activating C-terminal Smad phosphorylation (Kretzschmar et al., 1997b), but also an inhibitory linker phosphorylation via MAPK (Sapkota et al., 2006). Linker phosphorylation is inhibitory in two ways: (1) translocation of Smads into the nucleus is prevented, and (2) linker-phosphorylated Smads are degraded. Phosphorylated MAPK sites in the Smad1 linker serve as recognition motifs for GSK3. GSK3-phosphorylated Smad1 in turn is recognized by Smurf1, which ubiquitinates Smad1 and targets it for proteosomal degradation (Fuentealba et al., 2007; Sapkota et al., 2007). Furthermore, binding of Smurf1 to MAPK-phosphorylated linker inhibits Smad1 interaction with Nup214 (Sapkota et al., 2007), which normally promotes nuclear translocation of Smads (Xu et al., 2003). The crucial step in this cascade is the initial MAPK-dependent phosphorylation, which thus has to be removed to enable the translocation of Smads into the nucleus and their execution of BMP responses. We found that PP2A is the cytoplasmic BMP Smad linker phosphatase, which removes this crucial MAPK phosphorylation site in Smad1/5/8.

There have been several reports of involvement of an okadaic-acid-sensitive phosphatase in the regulation of TGFβ signalling. For example, okadaic acid treatment of various cell types results in increased Smad2 and Smad3 C-terminal phosphorylation levels (Cao et al., 2003; Lin et al., 2006; Van Berlo et al., 2005). Interestingly, overexpression of a constitutive active and thus ligand- and TGFβRII-independent TGFβRI, abolishes the okadaic acid effect (Bennett and Alphey, 2002; Shi et al., 2004). Thus, the receptor complex appears important for the okadaic-acid-sensitive phosphatase to be able to dephosphorylate the C-terminus of

Smads. Indeed, both PP2A and PP1 subunits have been found to associate with either TGFβRI or TGFβRII, which in each reported case had consequences either for the phosphorylation status of the receptor or the phosphatase. For example, the regulatory subunit of PP1, GADD34, was found to interact with Smad7. Smad7 in turn, binds to the TGFβ receptor complex, which results in recruitment of the catalytic C-subunit of PP1 to TGFβRI and dephosphorylation thereof. Furthermore, even without the assistance of Smad7 or Smad6, the interaction of GADD34/PP1c with TGFβRI leads to dephosphorylation of TGFβRI (Shi et al., 2004). By contrast, the Bα subunit of PP2A itself becomes phosphorylated upon binding to TRI (Griswold-Prenner et al., 1998). The function of B subunits in TGFβ signalling seems to be the stabilization of the receptor complex, as both the knockdown of Bα and overexpression of Bδ lead to degradation of the type-I receptor (Batut et al., 2008). Confirming the previous studies, we found an interaction between the PP2A-B subunits and TGFβ type-I and type-II receptors in vivo. Additionally, we established that the B subunits interact with both BMP type- I and type- II receptors. Furthermore, we showed that PP2A can dephosphorylate BMPRII. In vivo, this might translate into less activated BMPRI by BMPRII and therefore reduced C-terminal phosphorylation of Smads, which we also observed. This effect was not dramatic; however, we never had to block BMPRI activity in order to detect it. Thus, regulation of receptor complex activity by PP2A explains the previously reported (Lin et al., 2006) requirement of the receptor complex for the activity of okadaic-acid-sensitive phosphatase on the C-terminus of Smads. Similar mechanism has been reported for the PP2A-unrelated phosphatase Dullard (Satow et al., 2006). Dullard binds BMPRII and dephosphorylates BMPRI, which results in attenuation of C-terminal BMP-Smad phosphorylation (Satow et al., 2006).

In contrast to TGFβ signalling, the positive role of PP2A in the regulation of BMP signalling can be attributed to its function as a Smad linker phosphatase. We show that PP2A, containing its regulatory subunit B, dephosphorylates the ERK-phosphorylated linker of Smad1 in vivo and in vitro. In fact, this effect was so robust, that it was readily seen on endogenous BMP Smads and without the use of ERK inhibitors. Interestingly, PP2A dephosphorylates the same sites in Smad1 as the previously reported Smad linker phosphatase SCP (Sapkota et al., 2006). However, there are some important differences between these two phosphatases. First, SCP is a nuclear phosphatase (Sapkota et al., 2006), whereas Bβ, the subunit required for directing PP2A to BMP receptors and Smads, has a pancellular distribution (data not shown). Second, dephosphorylation of the linker region by SCP resets Smad1 to its basic level leading to attenuation of signalling (Sapkota et al., 2006), whereas dephosphorylation by PP2A enhances the BMP signal. Thus, although both phosphatases dephosphorylate the same phospho sites, they do so with different purposes: PP2A to start and SCP to terminate the signal.

In summary, we propose that the PP2A regulatory subunit Bβ is a constitutive interactor of BMP receptors. Bβ recruits the catalytic subunit to the receptors complex where it dephosphorylates BMPRII. Dephosphorylated BRII may be less active in binding and

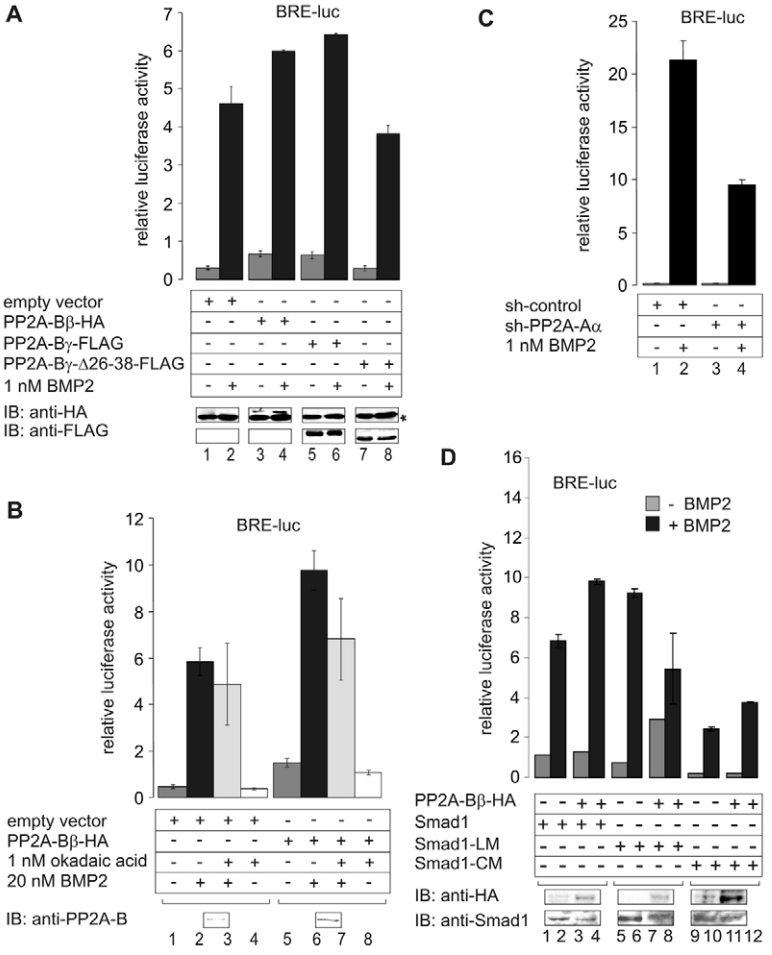

Fig. 6. PP2A is a BMP-Smad linker phosphatase. (A) Different PP2A-B subunits together with PP2A catalytic subunit enhance the activity of a BMP2-regulated promoter. C2C12 cells were transfected with the indicated constructs (empty vector, lanes 1 and 2; PP2A-Bβ-HA, lanes 3 and 4; PP2A-Bγ-FLAG, lanes 5 and 6; PP2A-Bγ-Δ26-38-FLAG, lanes 7 and 8) and co-transfected with *BRE*-luc and a reference reporter. Cells were starved and stimulated with BMP2 (black columns) or left untreated (grey columns) and the luciferase activity was measured. An aliquot of each lysate was taken to monitor expression of PP2A-B subunits using anti-HA and anti-FLAG antibodies. Standard deviations result from the mean of two measurements, the result was reproduced in three independent experiments. (B) Inhibition of PP2A activity partially reverses the positive regulative effect of the B-subunits. The experiment was carried out as in A. Cells were starved and stimulated with BMP2 (black columns), BMP2 and okadaic acid (light grey columns), okadaic acid alone (white columns) or left untreated (dark grey columns). Luciferase activities were measured as described above and samples overexpressing empty vector or PP2A-Bβ-HA were pooled to determine PP2A-Bβ expression by anti-PP2A-B antibody. Standard deviations result from the mean of six measurements from two experiments. (C) Downregulation of Aα subunit enhances the activity of a BMP2-regulated promoter. Cells expressing shRNAs against PP2A-Aα were assayed as in A. (D) PP2A has different signalling outcomes on Smad1 linker and C-terminal mutants. Cells expressing the indicated constructs were analyzed as in A.

A

		–	BMP2	BMP2 oa	oa
Smad1 C-term. phosphorylation		-	+	++	-+
Smad1 linker phosphorylation		-	+	+++	+
localization Smad1	c	+	-	+	+
	n	-+	++	+	-+
Smad1 transcriptional activity		-	+	-+	-

B

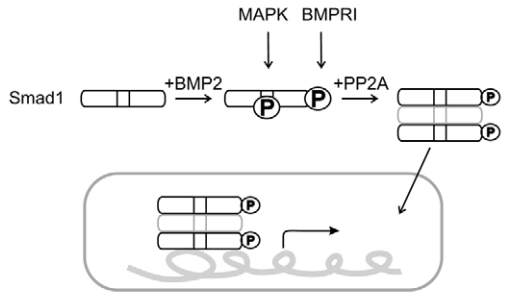

Fig. 7. Model for mechanism of action of PP2A in the regulation of BMP signalling. (A) Schematic summary of our results. (B) Upon BMP2 stimulation, both MAPK and BMPRI become activated and phosphorylate R-Smads in the linker region and at the C-terminus, respectively. PP2A targeted to receptor complexes and Smads via its regulatory subunit causes slight dephosphorylation of the C-terminus and a more thorough one of the linker region. Only the linker-dephosphorylated Smad complexes can translocate into the nucleus and activate BMP target genes.

activating BMPRI. This function of PP2A appears to be independent of the BMP ligand and may have the purpose of keeping receptors in a basal, inactive state. After stimulation with BMP, BMPRI is activated by BMPRII and phosphorylates BMP Smads at the C-terminus. Less activated BMPRI leads to attenuation of the C-terminal phosphorylation of Smads. C-terminally phosphorylated Smads oligomerize with co-Smad4. At the same time, the MAPK cascade is activated, which is responsible for the phosphorylation of the linker region of BMP Smads. Bβ targets PP2A to the linker region of Smads. After removal of the linker phosphorylation, the Smad complexes can translocate into the nucleus and activate BMP-induced gene expression (Fig. 7A,B).

Materials and Methods

Plasmids

GST-BMPRII-SF and GST-BMPRII-tail were described previously (Hassel et al., 2004). HA-BMPRII-LF, MYC-BMPRII-LF, HA-BMPRII-SF and HA-BMPRII-LF-K230R were described previously (Gilboa et al., 2000; Hartung et al., 2006; Nohe et al., 2002). PP2A-Bα and PP2A-Bβ were kindly provided by K. Schmidt (University of Basel, Switzerland) (Schmidt et al., 2002) and subcloned into pcDNA3.1 providing an N-terminal HA-tag (Invitrogen, Carlsbad, CA). PP2A-Bγ-FLAG and PP2A-Bγ-Δ26-38-FLAG were kindly provided by S. Strack (University of Iowa, IA) (Strack et al., 2002). MBP-Smad1 in pMAL p2C (New England Biolabs, Ipswich, MA) was a generous gift from O. Huber (Charité, Berlin, Germany). Smad1, FLAG-Smad1-4SP/AP and FLAG-Smad1-AAVA were described previously (Akiyoshi et al., 1999; Kretzschmar et al., 1997a; Kretzschmar et al., 1997b). Vector encoding short hairpin RNAs (shRNAs) for the Aα subunit were constructed using the BLOCK-iT Pol II miR RNAi Kit and the oligonucleotides Mmi548752 and Mmi548753 (Invitrogen). A non-coding shRNA vector provided in the kit was used as a negative control.

Antibodies

The following antibodies were used for immunoprecipitation: anti-PP2A-B (0.2 μg; Millipore, MA), anti-FLAG (2.5 μl; Sigma-Aldrich, Hannover, Germany), anti-BMPRII [rabbit polyclonal serum FB-60 raised against a peptide within the kinase domain of BMPRII (Gilboa et al., 2000; Nohe et al., 2002)], anti-BMPRIa (rabbit polyclonal serum FB-14 raised against a juxta-membrane peptide of BMPRIa), anti-TGFβRI antibody [rabbit polyclonal serum VPN44A raised against a juxta-membrane peptide of TGFβRI (Rotzer et al., 2001)], and anti-TGFβRII [rabbit polyclonal serum FB-260, raised against a juxta-membrane peptide of TGFβRII (Rotzer et al., 2001)]. The anti-receptor antibodies were cross-linked to protein-A-Sepharose (Sigma-Aldrich) with DMS (dimethyl suberimidate dihydrochloride; Pierce, Rockford, IL) before use. For immunoblotting the following antibodies, diluted as indicated in TBST (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.1% v/v Tween) were used: anti-PP2A-B (1:1000; Millipore), anti-HA (1:1000; Roche Diagnostics, Mannheim, Germany), anti-FLAG (1:2000; Sigma-Aldrich), anti-β-actin (1:5000-1:10,000; Sigma-Aldrich), anti-MYC [1:1000 (Bengtsson and Otto, 2008)], anti-Smad1 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA or 1:1000; Cell Signalling Technology), anti-MBP (1:1000; Cell Signalling Technology, Danvers, MA), anti-p-Smad1/5/8 (1:1000; Cell Signalling Technology), and anti-MAPK/CDK substrate (1:1000; Cell Signalling Technology). The blots were developed with appropriate HRP-conjugated secondary antibodies followed by ECL reaction.

GST pulldown

Expression and purification of recombinant proteins, GST pulldown and identification of proteins interacting with glutathione-Sepharose-bound GST constructs (GST-BMPRII-SF, GST-BMPRII-tail and GST) were performed as described previously (Hassel et al., 2004). For confirmation of MALDI results, precipitated proteins were separated on SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-PP2A-B antibody. To determine the amount of GST fusion proteins a parallel gel was stained with Coomassie blue.

Co-immunoprecipitation

1×10^6 HEK293T cells grown in a 6 cm dish in DMEM plus 10% (v/v) FCS were transfected with 2-4 μg of each construct using polyethylenimine (PEI) (Boussif et al., 1995) as described previously (Hartung et al., 2006). After 24-48 hours incubation, the cells were either first starved for 3 hours [DMEM, 0.5% (v/v) FCS] and stimulated with 10 nM BMP2 (a generous gift from W. Sebald, University of Wuerzburg, Wuerzburg, Germany) for 30 minutes or lysed directly with Triton lysis buffer [1% (v/v) Triton X-100, 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM PMSF, protease inhibitors (Complete® EDTA free; Roche Diagnostics)]. Endogenous PP2A-B subunit was precipitated from 4×10^7 C2C12 cells grown in a 15 cm dish (4 ml lysate divided among six samples). Lysates were cleared by centrifugation for 10 minutes at 4°C and 16,000 ***g*** and incubated overnight at 4°C on an overhead incubator with the respective antibodies. Antibody-bound complexes were precipitated with protein-A-Sepharose and subjected to SDS-PAGE followed by immunoblotting with the respective antibodies.

Phospho-Smad assay

2×10^4 C2C12 cells per well of a 24-well or 1×10^5 C2C12 cells per well of a six-well plate were transfected with 0.2-1 μg DNA using Lipofectamine2000 (Invitrogen) or jetPEI (Biomol, Hamburg, Germany) according to manufacturer's instructions. After 24 hours, cells were starved [DMEM, 0.5% (v/v) FBS] for 2-24 hours and stimulated with 10-20 nM BMP2 and/or 1 nM okadaic acid (Sigma-Aldrich) for 30 minutes. Cells were lysed in 100 μl TNE-lysis buffer [150 mM NaCl, 20 mM Tris pH 7.5, 1 mM EDTA, 1 mM PMSF, protease inhibitors (Complete® EDTA free)], 10 mM sodium fluoride, 20 mM sodium pyrophosphate) containing 1% (v/v) Triton X-100. After centrifugation (16,000 ***g***, for 10 minutes at 4°C), the supernatants were subjected to SDS-PAGE and immunoblotting using the indicated antibodies. Phosphorylation levels were quantified relative to protein amounts using ImageJ [Wayne Rasband (National Institutes of Health, NIH); http://rsb.info.nih.gov/ij].

In vitro phosphatase assay

Receptors

To assay BMPRII-LF, HEK293T cells were transfected with BMPRII-LF and the receptor was immunoprecipitated using its HA-tag as described above. 20 μl of BMPRII-LF beads were supplemented with phosphorylation buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 100 μM $CaCl_2$, 1 mM DTT) and 5 ng recombinant PP2A (EMD Biosciences and Calbiochem, San Diego, CA). Phosphorylation-dephosphorylation was started by the addition of 5 μCi of [γ-^{32}P]ATP (Hartmann Analytic, Braunschweig, Germany) and 25 μM unlabelled ATP (Sigma-Aldrich) and allowed to proceed for 30 minutes at 30°C. Phosphorylated proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were exposed to phospho-imager screens (Fuji, Stamford, CT, USA) and subsequently immunoblotted with anti-HA antibody. To examine BMPRI, HEK293T cells were transfected with BMPRIa and stimulated with 10 nM BMP2 for 30 minutes. After immunoprecipitation with anti-MYC, 20 μl of BMPRIa beads were supplemented with dephosphorylation buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 100 μM $CaCl_2$, 1 mM DTT) and 5 ng recombinant PP2A. Following incubation for 30 minutes at 30°C the proteins were separated by SDS-PAGE and subjected to immunoblotting with anti-phospho-Ser and anti-MYC antibody.

Smads

To analyze linker phosphorylation of Smad1, either 2 μg recombinant MBP-Smad1, immobilized to amylose resin, or MBP alone were in vitro phosphorylated using

CamKII, CDK1 and Erk2 (New England Biolabs) for 30 minutes at 30°C, according to the manufacturer's instructions. Reaction was started by addition of either 25 μM ATP or 5 μCi [γ-^{32}P]ATP and 25 μM non-radioactive ATP. The non-radioactive samples were first extensively washed with phosphatase buffer [50 mM Tris, pH 7.5, 0.2% (v/v) β-mercaptoethanol, 0.1 mM EDTA] and then one half of each sample was supplemented with 1 ng PP2A in phosphatase buffer. Dephosphorylation proceeded at 30°C for 30 minutes. After separation by SDS-PAGE, the proteins were transferred to nitrocellulose membrane, which was then either probed with anti-MAPK substrate, anti-Smad1 and anti-MBP antibodies, or the radioactive signals were detected by autoradiography. Phosphorylation levels were quantified relative to protein amounts using ImageJ (Wayne Rasband, NIH). To compare C-terminal and linker phosphorylation, either endogenous Smad1 (from BMP2-stimulated C2C12 cells) or overexpressed Smad1 or Smad1 mutants (from BMP2-stimulated HEK293T cells) were immunoprecipitated using an anti-Smad1 antibody. 20 μl of bead-coupled Smad1 variants were supplemented with dephosphorylation buffer [50 mM Tris, pH 7.5, 0.2% (v/v) β-mercaptoethanol, 0.1 mM EDTA] and 5 ng recombinant PP2A. After incubation for 30 minutes at 30°C the proteins were separated on SDS-PAGE and subjected to immunoblotting with anti-c-C-terminal, anti-p-linker and anti-Smad1 antibodies.

Immunofluorescence

C2C12 cells were seeded in 24-well plates on coverslips in DMEM plus 10% (v/v) FBS. On the next day, cells were starved for 2 hours and then stimulated with 10 nM BMP2 and/or 1 nM okadaic acid for 24 hours or left untreated. Immunofluorescence staining was performed as described previously (Bengtsson and Wilson, 2006). Fluorescence images were obtained using an Axiovert 200M microscope (Zeiss, Jena, Germany) with a 63-fold magnification.

Reporter gene assay

2×10^4 C2C12 cells per well were seeded on a 24-well plate and transfected with 60 ng control reporter (RL-TK; Promega), 0.2 μg *BRE*-luc reporter (Korchynskyi and ten Dijke, 2002) and 0.2 μg of the indicated constructs using Lipofectamine® (Invitrogen) according to manufacturer's instructions. After 24 hours, cells were starved for 5 hours in DMEM plus 0.2-0.5% (v/v) FBS followed by stimulation with 1 nM BMP2 and/or 1 nM okadaic acid for 24 hours. Luciferase activity was measured according to manufacturer's instructions (Dual Luciferase Assay System; Promega) using a FB12 luminometer (Berthold Detection Systems, Pforzheim, Germany). Remaining lysates were pooled, concentrated if necessary, and separated using SDS-PAGE and immunoblotted. B subunits and Smad1 variants were visualized using anti-HA, anti-FLAG, anti-PP2A-B or anti-Smad1 antibodies.

Downregulation of PP2A-Aα

1×10^5 C2C12 cells per well were plated on a six-well plate. The cells were transfected with sh-PP2A-Aα or sh-control plasmids using Lipofectamine2000 according to the manufacturer's instructions. 72 hours post transfection, total RNA was isolated using Tri-fast (Peqlab, Erlangen, Germany) according to the manufacturer's protocol. Reverse transcription was done using MMLV (Promega) and oligo(dT) primer using a standard procedure. The presence of PP2A-Aα transcripts relative to actin was investigated by PCR using PP2A-Aα-specific (forward 5′-ATGCCACTATCATCCCCAAG-3′, reverse 5′-TGCCATACGAAGAACTGTGG-3′) and β-actin-specific (forward 5′-CGGAACGCGTCATTGCC-3′, reverse 5′-ACCCACACTGTGCCCATCTA-3′) oligonucleotides. The amplified DNA fragments were analyzed by agarose gel electrophoresis.

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