Bone morphogenetic proteins (BMPs), originally identified by their ability to cause bone differentiation (1), are signaling molecules that belong to the transforming growth factor-β (TGF-β) superfamily. Presently, the biological functions of BMPs have been greatly expanded. BMPs regulate skeletal development as well as many non-osteogenic developmental processes, such as mesodermal patterning, left-right symmetry, neuronal patterning, and hematopoiesis (2–5). Accumulating evidence indicates that BMPs play an important role in the regulation of stem cell properties (6–8). Signals from BMP ligands are transduced through binding to type I and II receptors on the cell surface, where type II receptors activate type I receptors, which in turn phosphorylate the downstream Smad1, Smad5, and Smad8. The phospho-R-Smads (P-Smads) form a complex with Smad4 and translocate into the nucleus, where they bind to the Smad binding sites and cooperate with other transcription factors to regulate BMP-induced gene expression (5, 9, 10).

Despite substantial effort devoted to understanding the actions of BMP/TGF-β and Smads, the precise regulation of Smads remains enigmatic. Regulation of Smads can be accomplished via various post-translational mechanisms, including phosphorylation and ubiquitin-dependent modifications (11, 12). Among these, BMP-induced phosphorylation of Smad1/5/8, which is carried out by the BMP type I receptor (BMPRIA or BMPRIB) and occurs at the C-terminal SXS motif of Smad1/5/8, represents the most critical step in Smad signaling. The SXS phosphorylation triggers a cascade of intracellular events from Smad complex assembly in the cytoplasm to transcriptional control in the nucleus.

The reversible phosphorylation and dephosphorylation represents a fundamental strategy used by eukaryotic organisms to regulate a battery of biological functions. The cellular protein phosphorylation state is modulated by protein kinases and phosphatases. Until recently, how Smad functions are fine-tuned by Smad dephosphorylation remains poorly understood. R-Smads undergo continuous nucleocytoplasmic shuttling, and their export from the nucleus requires dephosphorylation (13, 14). A recent work identifies pyruvate dehydrogenase phosphatase (PDP) as a Smad phosphatase in the decapentaplegic pathway in Drosophila (15). On the other hand, we have found that PPM1A (but not PDPs) acts as a phosphatase for TGF-β-activated Smad2 and Smad3 (16).

In this study, we have explored whether Smad1 is dephosphorylated by PPM1A. We have demonstrated that PPM1A physically interacts with and dephosphorylates Smad1. Furthermore, overexpression of PPM1A attenuates or abolishes BMP-induced transcription, and conversely RNA interference-mediated knockdown of PPM1A enhances BMP signaling. Collectively, our study suggests that PPM1A plays an important role in controlling BMP signaling through catalyzing Smad dephosphorylation.

EXPERIMENTAL PROCEDURES

Plasmids—Expression plasmids for epitope-tagged Smads have been previously described (17, 18). PPM1A was obtained
by PCR and cloned into the EcoRI (5') and SalI (3') of pRK5His or pRK5F (derivative of pRK5 (Genentech). Point mutants (D239N or R174G substitution) and N-terminal deletions (deletions of the first 28, 35, or 111 amino acids) of human PPM1A were also obtained by PCR and similarly cloned into pRK5F. GCGCGlux (19), Id1-luc (20), and Xvent-luc (21) were kindly provided by Kohei Miyazono, Peter ten Dijke, and Christof Niehrs, respectively.

**Cell Culture, Cell Transfection, Immunoprecipitation, and Western Blotting**—HEK293T, HaCaT, Mv1Lu-tTA (22), HepG2, and C2C12 cells were cultured and transfected using Lipofectamine (Invitrogen) as previously described (16, 17). Stable HaCaT cells with PPM1A knockdown and stable Mv1Lu cells with tet-off expression of PPM1A have been described elsewhere (16).

Immunoprecipitations were carried out as described previously (16, 17). 293T cells were transiently transfected with cDNAs for HA-Smad1 and FLAG-PPM1A (for Smad1-PPM1A interaction) or FLAG-Smad1 and Myc-Smad4 (for Smad1-Smad4 interaction). Anti-HA or anti-FLAG antibodies were used to immunoprecipitate Smad1 from transfected cell lysates. To detect Smad1-bound PPM1A, the immunoprecipitated proteins were separated by SDS-PAGE, transferred onto nitrocellulose membrane, and immunostained with primary antibodies and finally detected by horseradish peroxidase-conjugated secondary antibodies and visualized by chemiluminescence (Pierce).

Antibodies against Smad1 (Cell Signaling) and PPM1A (Abcam) were used at 1:1000 dilutions in Western blotting assays to detect levels of endogenous proteins. P-Smad1 (Cell Signaling) was used to measure the level of P-Smad1.

**Glutathione S-Transferase (GST) in Vitro Binding Assay**—Production and purification of the GST-PPM1A fusion protein was done following the manufacturer's instructions (Amersham Biosciences). In vitro translation of PPM1A and in vitro binding assays were essentially carried out using the TNT kit (Promega) as previously described (16, 17).

Real-time Quantitative RT (qRT)-PCR—Total RNAs were prepared using TRIzol reagent (Invitrogen) from HaCaT
Dephosphorylation of Activated Smad1 by PP2C

A

HA-Smad1
Flag-PPM1A
IP: HA
IB: Flag
PPM1A
Smad1
WCL
IB: Flag
PPM1A

B

Input (1%) WT D239N R174G
His-PPM1A
D239N R174G GST GST-Smad1 GST-Smad1N GST-Smad1L

C

His-PPM1A
(1%)
WCL
IB: Flag
GST-GST-Smad1 GST-Smad1N

FIGURE 2. PPM1A physically interacts with Smad1. A, HA-Smad1 and Flag-PPM1A were transfected in 293T cells. Smad1 was immunoprecipitated (IP) with anti-HA antibodies and then subjected to SDS-PAGE and Western blots (IB) with anti-FLAG antibodies to detect the Smad1-bound PPM1A. WCL, whole cell lysate. B, direct interaction between PPM1A and Smad1. In vitro translated, 35S-labeled His-PPM1A or D239N and R174G mutants were incubated with purified glutathione bead-bound GST protein or GST-Smad1 fusion protein. The precipitated complex was subjected to SDS-PAGE followed by autoradiography. The quality of GST fusions is shown in supplemental Fig. S1. C, PPM1A interacts with both the N and C termini of Smad1. In vitro translated, 35S-labeled His-PPM1A was incubated with an equal amount of GST proteins as follows: GST alone, GST-Smad1 full-length, N-terminal region (amino acids 1–144), linker region (amino acids 144–268), or C-terminal region (amino acids 268–465) as described in B. The quality of the GST fusions is shown in supplemental Fig. S1.

RESULTS

PPM1A Dephosphorylates Smad1—In a search to study Smad2/3 dephosphorylation in the phospho-SXS motif, we recently identified PPM1A as the phosphatase for Smad2 and Smad3 that is responsible for termination of TGF-β signals (Lin et al., (16)). Considering the highly conserved nature of the SXS motif in all R-Smads, we reasoned that PPM1A might also recognize the SXS motif in the BMP-activated Smad1. To test this, BMP-induced Smad1 phosphorylation was analyzed in HeLa cells that were transfected with HA-Smad1 and His-PPM1A. The phosphorylation level of Smad1 (P-Smad1) was determined by a phospho-SXS motif-specific antibody. In the absence of PPM1A, the level of P-Smad1 increased upon 1 h of BMP2 stimulation (Fig. 1A, lane 2). In contrast, co-transfection of His-PPM1A abolished BMP-induced Smad1 phosphorylation (Fig. 1A, lane 3), suggesting that PPM1A either prevents Smad1 phosphorylation or directly dephosphorylates Smad1. To rule out the possibility that PPM1A dephosphorylates BMP receptors to prevent Smad1 phosphorylation and activation, we used a mutant of BMP receptor ALK3(Q233D) that constitutively activates Smad1. Clearly, P-Smad1 level was increased by ALK3(Q233D) (Fig. 1A, lane 5), and this increase was abolished by the co-expression of PPM1A (Fig. 1A, lane 6).

Smad1, Smad5, and Smad8 are three R-Smads transducing BMP signals in vertebrates. After having established that PPM1A dephosphorylates Smad1, we then carried out an experiment to test whether PPM1A also dephosphorylates Smad5 and Smad8. The results of analysis showed that PPM1A dephosphorylated P-Smad5 (Fig. 1B) and P-Smad8 (Fig. 1C), which were recognized by the same P-Smad1 antibody (αPS1).

We next determined whether the phosphatase activity of PPM1A is essential in reducing Smad1 phosphorylation. PPM1A point mutants at its catalytic domain (D239N and R174G) were generated (23). As shown in Fig. 1D, both D239N and R174G mutants were unable to eliminate ALK3(Q233D)-induced P-Smad1. In addition, three N-terminal deletion mutants (Met-29, Met-36, and Met-112) of PPM1A were tested for their Smad1-dephosphorylating activity in 293T cells. We found that all three deletion mutants lost their activity toward Smad1 dephosphorylation (Fig. 1E). These results suggest that PPM1A reduces the P-Smad1 level through its phosphatase activity.

To further determine whether the effect of PPM1A on P-Smad1 accumulation is due to direct dephosphorylation of P-Smad1, an in vitro phosphatase assay was performed using purified PPM1A and P-Smad1 (Fig. 1F). P-Smad1 and PPM1A (wild type or mutants) were separately purified by immunoprecipitation from 293T cells, which were transfected with either F-Smad1 or His-PPM1A. Results in Fig. 1F showed clearly that the P-Smad1 level was reduced by wild-type PPM1A but not by either the D239N or R174G mutant, suggesting PPM1A directly dephosphorylates Smad1.

To exclude the possibility that PPM1A causes reduction in Smad1 phosphorylation dependent on the 26 S proteasome, the proteasome inhibitor MG-132 was included. Inhibition of the
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26 S proteasome by MG-132 did not reverse the effect of PPM1A on the P-Smad1 level (Fig. 1G). This suggests that proteasome-dependent degradation does not contribute to PPM1A-induced loss of Smad1 phosphorylation.

Finally, we examined the effect of PPM1A on the dephosphorylation of endogenous Smad1. Mv1Lu cell lines that stably express PPM1A under the control of the tetr-off promoter were established. Withdrawal of doxycycline (−Dox), a tetracycline derivative, induced PPM1A expression (Fig. 1H, lanes 3 and 4). As a control, BMP2 treatment (1 h) resulted in a strong increase in the endogenous P-Smad1 level (lane 2). However, BMP2-induced Smad1 phosphorylation was eliminated when PPM1A expression was induced (−Dox) (Fig. 1H, lane 4).

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We next determined the effect of PPM1A on BMP-regulated Smad-dependent gene transcription. To this end, we used a synthetic reporter construct GCCG-lux, which contains multiple repeats of the GCCG motif and specifically responds to BMP stimulation but not to TGF-β or activin (19). As shown in Fig. 3C, BMP2 treatment caused an increased GCCG-lux reporter activity in HepG2 cells. In contrast, the presence of PPM1A completely blocked BMP2-dependent expression of GCCG-lux.

FIGURE 4. Knockdown of PPM1A enhances BMP signaling. A, lysates from HaCaT cells stably expressing control short hairpin RNA or shPPM1A were used for Western blot analysis with the indicated antibodies. BMP2 treatment was done for 1 h. B, shPPM1A enhances BMP2-induced Id1-luc reporter activity. Expression of zPPM1A (zebrafish PPM1A) is resistant to the effect induced by shPPM1A. HepG2 cells were transfected with a plasmid for control short hairpin RNA or shPPM1A, together with Id1-luc plasmid. BMP2 treatment and luciferase assay were as described under "Experimental Procedures." C, shPPM1A enhances BMP2-induced p21-luc reporter activity. D, knockdown of PPM1A increases the level of P-Smad1 in response to BMP2. E, enhanced expression of p21 in shPPM1A cells as assessed by qRT-PCR. BMP2 treatment, RNA extraction, and qRT-PCR data analysis were as described under "Experimental Procedures." F, enhanced expression of Id1 in shPPM1A cells as assessed by qRT-PCR.

Several BMP early target genes have been reported. Among them, Id-1 encodes a protein that acts as a positive regulator of cell proliferation and a negative regulator of cell differentiation (24). Xvent1 is another BMP direct target gene, which is a member of the vox/vent homeobox gene family and is involved in dorsoventral axis determination (25). We studied the effect of PPM1A on the transcriptional activity of these target genes. As shown in Fig. 3D, BMP2 treatment caused an increased promoter activity of Id-1 in HepG2 cells. Co-expression of PPM1A completely eliminated this induction. Moreover, PPM1A D239N and R174G mutants did not affect Id1 promoter activity. Similarly, expression of PPM1A profoundly inhibited the transcription of Xvent-luc reporter gene (Fig. 3E).

Knockdown of PPM1A Enhances BMP Signaling—Having established the inhibitory effect of PPM1A overexpression on BMP-induced Smad1 phosphorylation and Smad1-dependent transcriptional responses, we took a loss-of-function approach to knock down PPM1A expression by RNA interference. Expression of small interfering RNA against human PPM1A (shPPM1A494) could efficiently knock down the exogenous expression of human PPM1A but not zebrafish PPM1A (Fig. 4A). As a result, shPPM1A blocked Smad1 dephosphorylation induced by hPPM1A (lane 3). The effect of PPM1A knockdown could be rescued by the expression of zPPM1A (lane 5). zPPM1A, which was resistant to shPPM1A-mediated knockdown, led to Smad1 dephosphorylation in the presence of shPPM1A494 (lane 6).

We also assessed the effect of PPM1A knockdown on BMP2-induced promoter activity. When the shPPM1A plasmid was transfected in HepG2 cells, Id1 promoter activity was markedly increased compared with control cells (Fig. 4B). Furthermore, expression of zPPM1A abolished the increased induction of Id promoter activity by shPPM1A (Fig. 4B). We also noticed that HepG2 cells exhibited a weak p21 response to BMP2. Notably, shPPM1A could sensitize the cells to respond to BMP2, and conversely, zPPM1A completely shuts off BMP2-induced p21 response (Fig. 4C).
We have stably knocked down PPM1A expression in HaCaT cells (Fig. 4D). In PPM1A-depleted HaCaT-stable cells, called shPPM1A-stable cells, the level of P-Smad1 induced by BMP2 was profoundly higher than that in control cells (Fig. 4D, lanes 2 and 4). To characterize the effect of PPM1A knockdown on BMP transcriptional responses, we examined the levels of p21 and Id1 mRNAs in HaCaT stable cells. p21 and Id1 are two representative Smad-dependent BMP target genes (20, 26). In control cells, BMP2 induced a gradual increase in the p21 mRNA level over a period of 24 h, at which time it reached 2.5-fold higher than the basal level. Notably, knockdown of PPM1A in shPPM1A cells rendered cells more sensitive to BMP2, which induced a higher level of p21 mRNA (Fig. 4E). Similar results were obtained on BMP2-induced Id1 mRNA as higher and better induction of Id1 mRNA was observed in shPPM1A cells (Fig. 4F). Taken together, these results suggest that knockdown of PPM1A enhances Smad-dependent transcription responses.

**DISCUSSION**

It is generally accepted that phosphorylation in the SXS motif of R-Smads is the most critical intracellular event in the BMP/TGF-β signal transduction pathways. Conversely, dephosphorylation of the SXS motif should provide a counter-mechanism terminating or antagonizing the functions of activated Smads. This study describes the identification and characterization of PPM1A as a phosphatase for BMP-activated Smad1. Our initial study on PPM1A-mediated dephosphorylation of Smad2/3 (16) led us to investigate whether PPM1A also acts as a Smad1 phosphatase. Considering the highly conserved sequences upstream of the distal SXS motif among all R-Smads, it is not surprising that all R-Smads are dephosphorylated by the same phosphatase. PPM1A physically interacts with Smad1 in both co-immunoprecipitation and GST pulldown assays. PPM1A dephosphorylates Smad1 in a number of cell lines tested, including 293T, HepG2, HaCaT, Mv1Lu, and C2C12 cells. Because of its activity toward the dephosphorylation of Smad1, overexpression of PPM1A inhibits BMP-induced transcriptional responses, whereas knockdown of its expression promotes BMP responses. In addition to our finding that Smad1 is dephosphorylated by PPM1A, we have found that PPM1A effectively dephosphorylates the phospho-SXS motif in Smad5 and Smad8.

Our finding that PPM1A is a phosphatase for R-Smads does not preclude the existence of other Smad phosphatases. We expect there is redundancy on Smad dephosphorylation. Recently, Chen et al. (15) has reported that PDP is a Mad phosphatase in Drosophila (15). Interestingly, mammalian PDPs (e.g. PDP1/PPM2C and PDP2) have no effect on Smad2 dephosphorylation (15, 16), whereas PPM1A dephosphorylates all R-Smads (Ref. 16, this study, and data not shown). Unexpectedly, we failed to observe any effects of PDP1 or PDP2 overexpression on dephosphorylation of P-Smad1 (supplemental Fig. S2). Although our manuscript is in revision, Knockaert et al. (27) report dephosphorylation of Smad1 by small C-terminal domain phosphatases (SCPs) (27). Similar to the PDPs, overexpression of SCP1–3 has no effect on Smad1 dephosphorylation in mammalian cells, although SCP1–3 clearly dephosphorylates the linker region of Smad2/3 (28). These discrepancies may be explained by the possibility that PDPs and/or SCPs may require additional cofactors or downstream effectors (which is limiting in mammalian cells) to have their full phosphatase activity toward dephosphorylation of the phospho-SXS motif of Smad1. Thus, in the absence of increased expression of these cofactors, increased expression of PDPs or SCPs does not suffice to achieve their effects. Further experiments are needed to clarify these issues, to identify new Smad phosphatases, and to characterize how these phosphatases specifically impact Smad-dependent responses.

Identification of PPM1A as a Smad1 phosphatase will aid in the understanding of the mechanisms underlying shut-off of BMP signaling. We anticipate that PPM1A might be involved in the regulation of many developmental processes such as skeletal and cardiovascular development as well as human diseases, partly through its role as a Smad phosphatase.

**Acknowledgments**—We thank Joan Massagué for Mv1Lu-tTA cells, Kohei Miyazono for GCGG-lux, Christof Niehrs for Xvent-lux, and Peter ten Dijke for Id1-lux.

**REFERENCES**

1. Urist, M. R. (1965) Science 150, 893–899
2. Chen, D., Zhao, M., Harris, S. E., and Mi, Z. (2004) Front. Biosci. 9, 349–358
3. Kishigami, S., and Mishina, Y. (2005) Cytokine Growth Factor Rev. 16, 265–278
4. ten Dijke, P., Korchnytskyi, O., Valdimarsdottir, G., and Goumans, M. J. (2003) Mol. Cell. Endocrinol. 211, 105–113
5. Wu, M., and Cao, X. (2005) Biochem. Biophys. Res. Commun. 328, 651–657
6. Chambers, I., and Smith, A. (2004) Oncogene 23, 7150–7160
7. Tres, L. L., Rosselot, C., and Kierszenbaum, A. L. (2004) Mol. Reprod. Dev. 68, 1–4
8. Zhang, J., and Li, L. (2005) Dev. Biol. 284, 1–11
9. Feng, X.-H., and Derynck, R. (2005) Ann. Rev. Cell Dev. Biol. 21, 659–693
10. Miyazono, K., Maeda, S., and Imamura, T. (2005) Cytokine Growth Factor Rev. 16, 251–263
11. Feng, X.-H., and Lin, X. (2006) in Smad Signal Transduction (ten Dijke, P., and Heldin, C.-H., eds) pp. 253–276, Springer, The Netherlands
12. Izzli, L., and Attisano, L. (2004) Oncogene 23, 2071–2078
13. Inman, G. J., Nicolas, F. J., and Hill, C. S. (2002) Mol. Cell 10, 283–294
14. Xu, L., Kang, Y., Col, S., and Massague, J. (2002) Mol. Cell 10, 271–282
15. Lin, X., Liang, P., and Feng, X.-H. (2000) J. Biol. Chem. 275, 36818–36822
16. Lin, X., Liang, Y.-Y., Sun, B., Liang, M., Bruniciardi, F. C., Shi, Y., Chen, Y.-G., Meng, A., and Feng, X.-H. (2006) Cell 125, 915–928
17. Lin, X., Liang, M., and Feng, X.-H. (2000) J. Biol. Chem. 275, 555–565
18. Miyazono, K., Inoue, H., Ishidou, Y., Mishima, H. K., Kawabata, M., and Miyazono, K. (2000) Mol. Biol. Cell 11, 555–565
19. Lin, X., Liang, Y.-Y., Sun, B., Liang, M., Bruniciardi, F. C., Shi, Y., Shi, Y., and Feng, X.-H. (2003) Mol. Cell Biol. 23, 9081–9093
20. Kornchenskyi, O., and ten Dijke, P. (2002) J. Biol. Chem. 277, 4883–4891
21. Onichtchouk, D., Glinka, A., and Niehrs, C. (1998) Development (Camb.)
Dephosphorylation of Activated Smad1 by PP2C

22. Reynisdóttir, I., Polyak, K., Iavarone, A., and Massagué, J. (1995) Genes Dev. 9, 1831–1845
23. Jackson, M. D., Fjeld, C. C., and Denu, J. M. (2003) Biochemistry 42, 8513–8521
24. Miyazono, K., and Miyazawa, K. (2002) Science’s STKE E40
25. Gilardelli, C. N., Pozzoli, O., Sordino, P., Matassi, G., and Cotelli, F. (2004) Dev. Dyn. 230, 494–508
26. Pardali, K., Kowanetz, M., Heldin, C. H., and Moustakas, A. (2005) J. Cell. Physiol. 204, 260–272
27. Knockaert, M., Sapkota, G., Alarcon, C., Massague, J., and Brivanlou, A.H. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 11940–11945
28. Wrighton, K., Willis, D., Long, J., Liu, F., Lin, X., and Feng, X.-H. (October 10, 2006) J. Biol. Chem. 10.1074/jbc.M607246200